Stability of Drugs of Forensic Interest in Post Mortem Blood

A thesis submitted in accordance with the requirements of the University of Glasgow for the degree of

Doctor of Philosophy

by

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SUMMARY

The stability study of drugs of forensic interest in human post-mortem blood is an important forensic study, because in some cases, a requirement for the laboratory to undertake a full drug screening is after a few months of storage due to a need for new evidence. Therefore, it is necessary and important to know if drugs are stable over a period of time under different conditions to enable a solid interpretation to be made from any results.

Some studies have been published on the stability of drugs at different temperatures but none had covered the whole set of drugs that has been studied in this thesis. The periods of study that have been covered by other studies varied from a few days to a maximum of 70 weeks, but again not all drugs have been covered.

The drugs studies in this thesis are two sets of drugs, benzodiazepines and tricyclic antidepressants, their stability being determined over twelve months and at three different temperatures 25, 5 and -20°C.

In this thesis, blood was 'spiked' with eight drugs, Diazepam, Temazepam, Triazolam, Desmethyldiazepam, Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine. The samples were stored with blanks at different temperatures for different storage times. Each month a number of samples were removed from storage and analysed to test the effect of storage time and temperature on drug concentration.

Different solid phase and liquid-liquid extraction methods were tested for the determination of benzodiazepines. Liquid-liquid extraction methods for

the determination of Diazepam, Temazepam, desmethyldiazepam and Triazolam proved after study to be tedious and time-consuming. A method based on solid phase extraction was used to determine the four benzodiazepine drugs. The extraction method gave good recoveries and was highly efficient. The method of analysis used for the determination of stability of benzodiazepine drugs was the high performance liquid chromatography (HPLC) method.

Tricyclic antidepressant drugs are the other drugs studied for their stability in blood. Different solid phase extraction methods were used for the determination of drugs in post-mortem blood but gave poor recoveries. The best method of extraction used was a liquid-liquid extraction method which yielded high recoveries and proved to be quick. The method of analysis used for the determination of tricyclic antidepressants for the purpose of stability of the study was gas chromatography (GC).

At a recognised toxic level for each drug under study a reasonable amount of the drug was found to be detectable after one year at storage regardless of the storage temperature or media. The decrease rate of each drug concentration with time at the three storage conditions (25, 5 and -20°C) was obtained.

CHAPTER 1

1.1 INTRODUCTION

Depression is not only a common disorder but it is one that is readily treatable. Tricyclic antidepressants drugs play a major role in this treatment. They have been most beneficial in patients with endogenous depression. Besides being useful for treating depression they may be used for treating enuresis.

The depression problem is universal. Approximately 2 - 15% of adults are believed to suffer depressive symptoms in any given year. The major complication of depression is that it is the tenth greatest cause of death for all ages. Because depression is a common problem in medical practice and as tricyclic antidepressants are the most often prescribed, this may lead to drug overdose in patients and may lead to death.

Drug concentrations in blood and other biological fluids and tissues do not remain constant in a corpse in the interval between death and the collection of samples. Blood drug concentrations can change significantly during the post-

mortem period. Since the early 1980s it has been clear that this problem particularly affects the antidepressants.

One of the principal tasks in toxicological analysis is to detect whether a potentially harmful substance is present and to identify the substance(s) correctly, regardless of whether this is for clinical, forensic, environmental, workplace, drug abuse or doping control purposes. The analyst must be able to detect such compounds, identify them, differentiate between closely resembled ones and quantitate them within a reasonable amount of time. Establishing the presence of a harmful substance is not the only task in toxicological analysis. At the same time it is equally important to establish the absence of other toxicologically relevant substances within reasonable or significant limits.

The main task of a toxicologist is to measure and interpret levels resulting from drug overdose. In this study on antidepressants, their stability in post-mortem blood over a period of time was investigated.

1.2 BENZODIAZEPINES

When benzodiazepine was first synthesised in 1955, unlike other psychotropics they did not attract much attention. It was only 5 years later that several clinical investigators reported their anxiolytic properties.[1,2] The first report of the potential of benzodiazepines to cause a dependency came in 1961[3,4] only one year after the introduction of chlordiazepoxide. Since then there has been a steadily increasing number of papers concerned with benzodiazepines.[5,6]

In the UK benzodiazepine prescription figures produced by the Department of Health and Social Security (DHSS) indicate a fall from 30 million to 26 million between 1981 and 1985. Intravenous misuse of injected diazepines began in Britain in the mid 1980s. At first the drugs commonly used were Flurazepam and Diazepam but by the late 1980s, when the use of injected benzodiazepines had become widespread in several British cities, users were reported to be injecting mainly Temazepam from capsules.[7,8]

In 1989 the manufacturers replaced liquid filled Temazepam capsules with semi-solid gel filled capsules to prevent the drug from being injected.[9,10] These capsules, however, are also being injected and seem to lead to a greater rate of death in individual users.

Although benzodiazepine prescribing in the United Kingdom has been falling steadily, the number of prescriptions issued is still large. In England in 1989, 21 million prescriptions for hypnotics, sedatives and tranquillisers were issued, the vast majority of these being benzodiazepines.[11,12]

Despite the fact that the benzodiazepines are extensively used, some doctors feel that benzodiazepines no longer have a place in clinical practice[13,14] Whereas others have gone the other way and suggest that benzodiazepine hypnotics should be available without a prescription as they are so much safer than the alternatives. It is therefore difficult for the average psychiatrist to know when to prescribe benzodiazepines. It is also difficult to know which benzodiazepines to prescribe because of conflicting accounts of their advantages and disadvantages.

Nowadays, all practitioners prescribe benzodiazepines according to the circumstances. However, in anxiety occurring at a time of major stress, now normally diagnosed as acute stress, it may be appropriate to prescribe a benzodiazepine if the distress is severe and the prescription is likely to be short term. The other major use of benzodiazepines is in the treatment of alcohol withdrawal reactions. So, it was concluded that short treatment with benzodiazepines for a few weeks was safe from the risk of dependence.[15,16]

1.2.1 CHEMISTRY

The basic structure for most benzodiazepines is shown in Figure 1.

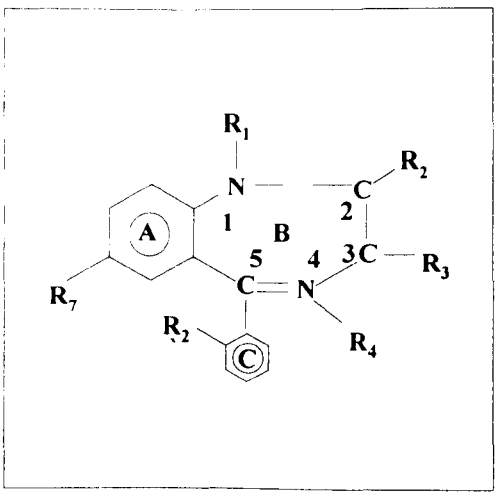


Figure 1: Basic Structure of Benzodiazepines

l, 4 benzodiazepine structure is only the fused ring portion. However, all the important central nervous system (CNS) depressant benzodiazepines contain a 5-aryl or cyclohexenyl substituent, so that the term benzodiazepines has come to mean the 5-aryl-l, 4- benzodiazepine.

The effects of the benzodiazepines result from the actions of these drugs on the CNS. The most prominent of these effects are sedation, hypnosis, decreased anxiety, muscle relaxation and anticonvulsant activity. It has minor cardiovascular effects except in severe intoxication.

1.2.1.1 Diazepam

Diazepam is a benzodiazepine with anticonvulsant, anxiolytic, sedative, muscle relaxant and amnesiac properties. Diazepam is used in the management of severe disabling anxiety disorders, insomnia, convulsions, particularly status epilepticus and febrile convulsions, and in alcohol withdrawal. It is also used as a sedative for surgical and other procedures and for the relief of muscle spasm.

Dependence may develop with regular use. Drowsiness, sedation and ataxia are the most frequent adverse effects.

Diazepam is Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one. The structure is shown in Figure 2.

$C_{16}H_{13}C1N_2O$

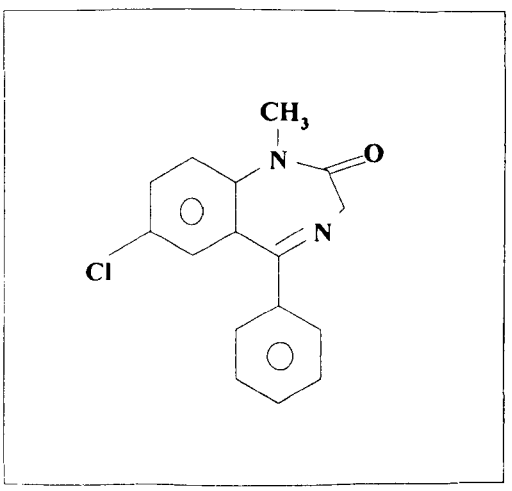


Figure 2: Diazepam

Pharmacokinetics

Diazepam is readily and completely absorbed from the gastro-intestinal tract, peak plasma concentration occurring within 0.5 - 2 hours of oral administration.

Diazepam has a biphasic half-life with an initial distribution phase followed by a prolonged elimination phase of one or two days.

Diazepam and desmethyldiazepam accumulate in the body on repeated administration and the proportion of desmethyldiazepam in the body increases on long term administration.

Diazepam is extensively metabolised in the liver and in addition to desmethyldiazepam its active metabolites include Oxazepam and Temazepam. It is excreted in the urine, mainly in the form of its metabolites either free or in conjugated form.

1.2.1.2 N-Desmethyldiazepam:

N-Desmethyldiazepam is 7-Chloro-1,3-dihydro-5-phenyl-2H-l, 4-benzo diazepin -2-one.

$C_{15}H_{11}ClN_2O$

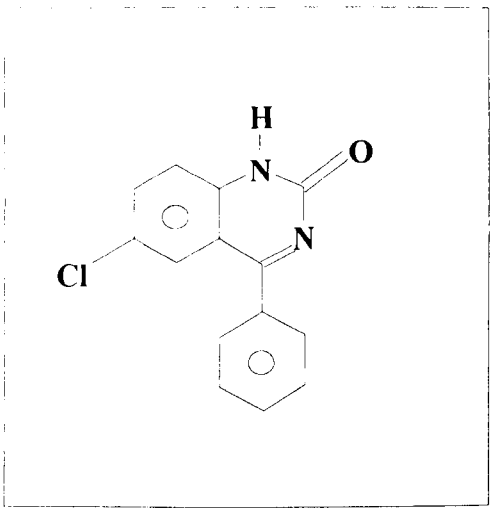


Figure 3: N-Desmethyldiazepam

Desmethyldiazepam is a long-acting benzodiazepine with the general properties of Diazepam. It is the principal active metabolite of several benzodiazepine drugs and has a half life of two to five days. It is used for the treatment of anxiety disorders and insomnia.

1.2.1.3 Temazepam

Temazepam is 7-Chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one. The structure is shown in Figure 4.

Temazepam is a relatively short acting benzodiazepine. It is used as a hypnotic in the short term management of insomnia. It is also given for premedication before surgery. Temazepam may be given to children as a premedication.

$C_{16}H_{13}C1N_2O_2$

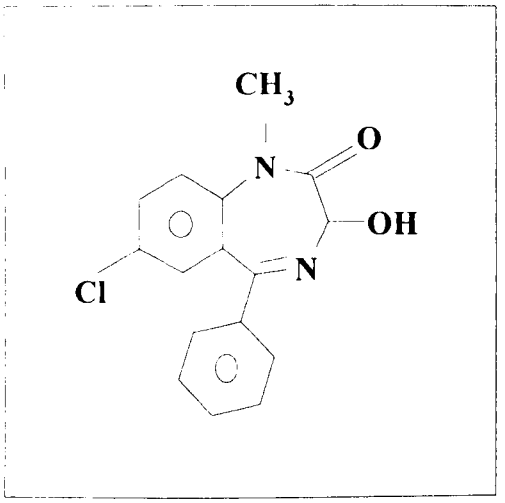


Figure 4: **Temazepam**

Pharmacokinetics

Temazepam is absorbed from the gastro-intestinal tract. It is 96% bound to plasma protein. Some studies show a half life of eight hours. Recent studies have shown a longer half-life of 15 hours. It is excreted in urine in the form of inactive glucuronide with small amounts of the desmethyl derivative, Oxazepam.

1.2.1.4 Triazolam

Triazolam is a short acting drug used in the management of insomnia. Dependence may develop after its regular use and its withdrawal symptoms are severe. Symptoms of anxiety, panic, paranoia and hallucinations have been

attributed to its continued use. As with all benzodiazepines, adverse effects may include drowsiness, sedation and ataxia.

Triazolam is 8-Chloro-6-(2-Chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4,3-a] [1,4]benzodiazepine. Its structure is shown in Figure 5.

$C_{17}H_{12}Cl_2N_4$

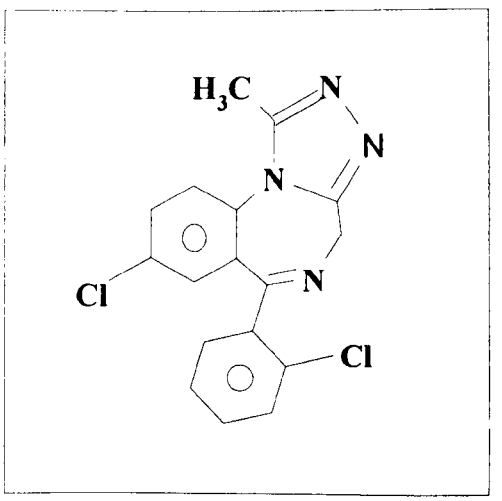


Figure 5: Triazolam

Pharmacokinetics

Triazolam is rapidly absorbed from the gastrointestinal tract, peak plasma concentrations being achieved within two hours of administration by mouth.

It is reported to be about 89% bound to plasma protein. Triazolam undergoes hydroxylation in the liver and is excreted in the urine in the form of its conjugated metabolites.

1.3 TRICYCLIC ANTIDEPRESSANTS

1.3.1 INTRODUCTION

Tricyclic antidepressant drugs were introduced in the 1950s and have been used clinically for more than 2 decades; these include Amitriptyline which is the most widely prescribed drug, Doxepin, Nortriptyline, Imipramine and Desipramine.

Amitriptyline has been implicated in roughly half of all deaths attributed to tricyclic antidepressants both in the United Kingdom and in the United States.[17,18] Since the introduction of tricyclic antidepressants into clinical practice, antidepressant drugs have become the primary method of treating depressive orders.[19,20] Tricyclic antidepressants are commonly encountered in autopsy toxicology. They probably present the most common life-threatening therapeutic drug ingestion world-wide. It is not surprising that they should be taken frequently in overdose which accounts for at least 5% of all positive toxicology screens.[21,22] Death from tricyclic overdose usually results from the well-documented cardiotoxicity of the compounds.[23,24]

A study was done on fatal toxicity of antidepressant drugs. They calculated a fatal toxicity index for antidepressant drugs on sale during the

years 1975-84 in England, Wales and Scotland, which showed that the tricyclic drugs introduced before 1970 had a higher toxicity index than the other drugs. The toxicity of Amitriptyline, Dibenzepin, Desipramine and Dothiepin was significantly higher, while that of Clomipramine, Imipramine, Protriptyline and Trimipramine was lower. The monoamine oxidase inhibitors had intermediate toxicity and the antidepressants, introduced in 1978, had significantly lower toxicity.[19,25]

Another study showed that twenty-nine coroners' cases were reported involving tricyclic antidepressants which included concentrations in the myocardium as well as in the liver and the blood.[26]

Amitriptyline is the most frequently involved drug in tricyclic overdose, accounting for half or more of the poisonings. So, whenever Amitriptyline was found by this screening procedure the drug, and its pharmacologically active metabolite Nortriptyline, were quantified in the liver, myocardium and blood.[27]

There are several groups of antidepressants: the tricyclic and related antidepressants; the monoamine oxidase inhibitors; and the serotonin re-uptake inhibitors.

Antidepressant therapy has mainly been with tricyclic antidepressants (TCA).

Both the dose and the duration of treatment are important when a patient is being treated for depression. An antidepressant must be given in and adequate dose to be effective.

1.3.2 CHEMISTRY

The 3-ring nucleus characteristic of these drugs has given them the name tricyclics. They closely resemble the phenothiazines chemically and, to a lesser extent, pharmacologically.

The general structure of antidepressants is shown in Figure 6.

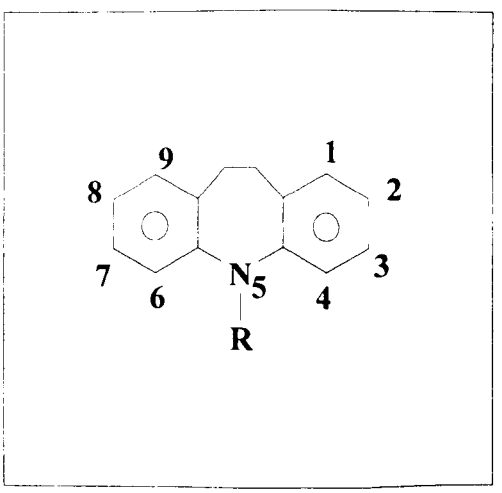


Figure 6: General Structure of Tricyclic Antidepressants

1.3.2.1 Amitriptyline

Death from Amitriptyline overdose is a significant problem in the Lothian and Borders region of Scotland, accounting for 21% of all fatal self-ingested overdoses presenting to the Forensic Medicine Unit over a five year period.

Twenty-four cases were reported in this study involving Amitriptyline as the major drug concerned.[28]

In the University of California study of fatal self-ingested overdose involving Amitriptyline, the average age was found to be 44 years. Only five of the twenty-four cases in this series reached hospital before death.[29]

Current evidence does not show any clear clinical superiority of one antidepressant over another, though antidepressants have been found to differ in their side-effect profiles and in their toxicity. Amitriptyline poisoning remains a major clinical problem. However, it remains a widely prescribed antidepressant.

Amitriptyline in general, typifies the group of drugs known as tricyclic antidepressants. It is used widely in melancholic depression and may also be

effective in some cases of atypical depression. It has marked antimuscarinic and sedative properties and prevents the re-uptake of Noradrenaline and Serotonin at nerve terminals.

Amitriptyline is usually given as a hydrochloride by mouth or in divided dose of 75 mg initially, gradually increasing if necessary to 150 mg daily.

Pharmacokinetics

Amitriptyline is readily absorbed from the gastro-intestinal tract, peak plasma concentration occurring within a few hours of oral administration. Since Amitriptyline slows gastro-intestinal transit time, absorption can be delayed, particularly in overdosage.

Amitriptyline undergoes extensive first-pass metabolism and is demethylated in the liver to its primary active metabolite, Nortriptyline. Other paths of metabolism of Amitriptyline include hydroxylation and N-oxidation; Nortriptyline follows similar paths. Amitriptyline is excreted in the urine, mostly in the form of its metabolites, either free or in conjugated form.

Amitriptyline and Nortriptyline are widely distributed throughout the body and are extensively bound to plasma and tissue protein. Amitriptyline has been estimated to have an elimination half-life ranging from about 9 – 36

hours, which may be considerably extended in overdosage. Plasma concentrations of Amitriptyline and Nortriptyline vary widely between individuals and no simple correlation with therapeutic response has been established.[30]

Chemistry

Amitriptyline is 3-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ylidene)-NN- dimethylpropylamine. The structure is shown in Figure 7.

$C_{20}H_{23}on$

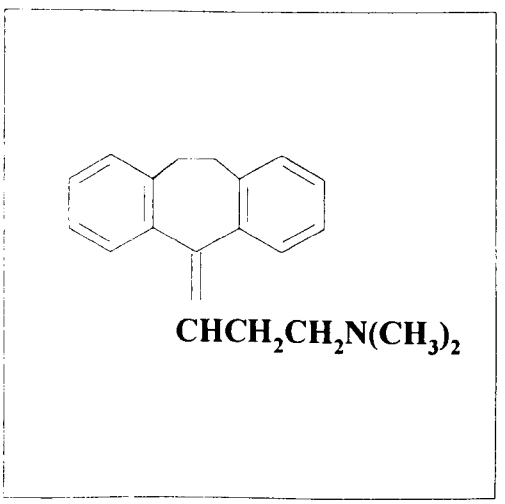


Figure 7: Amitriptyline

1.3.2.2 Imipramine

Imipramine is 3-(10, 11-dihydro-5H-dibenz[b,f]azepin-5-y1)-NN-dimethyl- propylamine. The structure is shown in Figure 8.

$C_{19}H_{24}N_2$

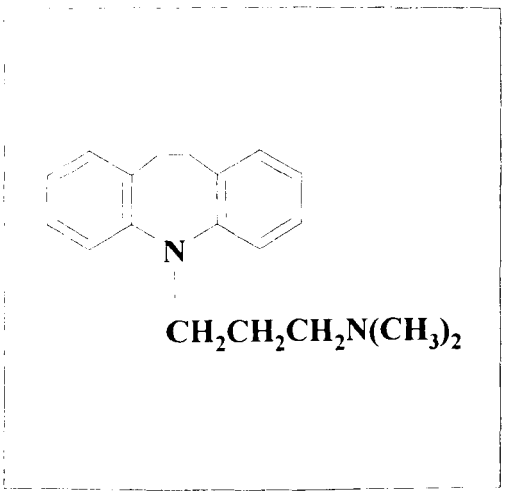


Figure 8: Imipramine

The structure is similar to that of Amitriptyline. It has properties typical of the group of drugs known as tricyclic antidepressants. It is used widely to treat melancholic depression and may also be effective in some cases of atypical depression.

This is given by mouth as the hydrochloride or the embonate. The usual dose is 25 mg three times daily initially, gradually increasing to 50 mg three times daily.

Pharmacokinetics

Imipramine is readily absorbed from the gastro-intestinal tract, and extensively demethylated by first-pass metabolism in the liver to its primary active metabolite Desipramine.

Paths of metabolism of both Imipramine and Desipramine include hydroxylation and N-oxidation. Imipramine is excreted in the urine, mainly in the form of its metabolites, either free or in conjugated form. Imipramine elimination half-life ranges between 9 - 28 hours.

1.3.2.3 Nortriptyline

Nortriptyline is a tricyclic antidepressant with actions similar to those of Amitriptyline and like other members of the tricyclic group it provides effective treatment for depression. In the treatment of depression it is given by mouth as a hydrochloride, starting with low dose and gradually increasing to the equivalent of 75 to 100 mg daily in divided dose.

The structure is 3-(10, 11-dihyro-5H-dibenzo[a,d]cyclohepten-5-ylidene) –N- methylpropylamine

The structure is shown in Figure 9.

$C_{19}H_{21}N$

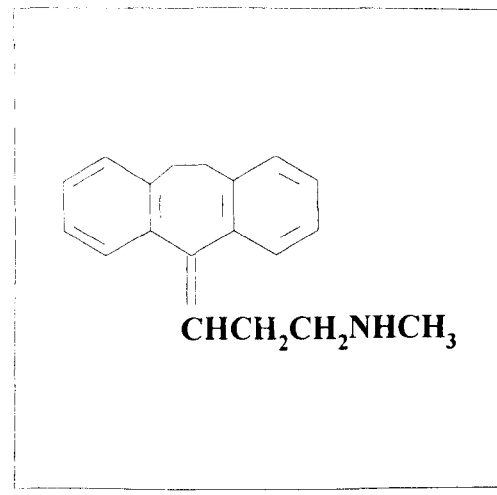


Figure 9: Nortriptyline

Pharmacokinetics

Nortriptyline is the principal active metabolite of Amitriptyline. It has been reported to have a longer plasma life than that of Amitriptyline. It is subject to extensive first-pass metabolism to 10-hydroxynortriptyline which is active.

1.3.2.4 Chlorpromazine

After more than thirty years of clinical use[31] its metabolism is still of interest. Chlorpromazine and its metabolites play an important role in clinical response.[32] Typical problems include instability, a tendency for adsorption loss and low analyte concentrations in clinical samples.[33] Chlorpromazine is a phenothiazine used for the control of psychosis, including schizophrenia and

mania, and severely disturbed or agitated behaviour. It is also used for the relief of nausea and vomiting, pre-operative anxiety and intractable hiccup.

Chlorpromazine is 3-(2-Chlorophenothiazin-10-yl)-NN-dimethylpropyl-amine. Its structure is shown in Figure 10.

$C_{17}H_{19}ClN_2S$

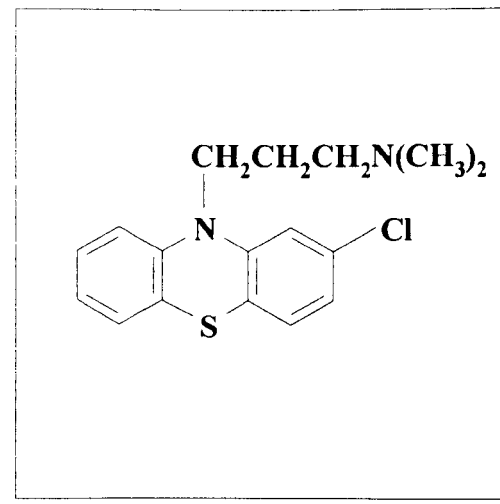


Figure 10: Chlorpromazine

Chlorpromazine and other phenothiazines produce in general a lesser degree of central depression than barbiturates or benzodiazepines. Phenothiazines have antimuscarinic properties, hypersensitivity reactions and various haematological disorders may occur.

The effect of Chlorpromazine on blood was provided in data from the UK Committee on Safety of Medicines. It provided data on reports it had received between 1963 and 1993 on agranulocytosis and neutropenia.[32] The study showed that the most frequently implicated phenothiazines were

Chlorpromazine with 51 reports of agranulocytosis (26 fatal) and 12 of neutropenia (2 fatal).

Pharmacokinetics

Chlorpromazine is readily absorbed from the gastro-intestinal tract; peak plasma concentration was attained 2 to 4 hours after ingestion. It is subject to considerable first-pass metabolism in the gut wall and is also extensively metabolised in the liver and is excreted in the urine and bile in the form of numerous active and inactive metabolites.

The paths of metabolism of Chlorpromazine include hydroxylation and conjugation with glucuronic acid, N-oxidation, oxidation of a sulphur atom and dealkylation.

CHAPTER 2

2.1 STABILITY OF DRUGS

The stability of drugs in biological specimens is of interest to forensic toxicology laboratories. The validity of toxicological test results for a given specimen may be disputed in the medicolegal community. This dispute may involve challenge by a defence attorney to have a specimen reanalysed by another laboratory. In this case, knowledge of the stability of a drug is also of importance for situations in which the time period between an initial presumptive screen and the subsequent confirmation is significant. Several studies on drug stability have been reported. A study was reported on post-mortem stability of benzodiazepines in blood and tissues; Diazepam was found to be very stable over a five month period when stored at room or refrigerated temperatures while decreases in Nordiazepam concentration were observed at both temperatures, with the greater and more rapid decreases in blood at room temperature. The presence of Chlordiazepoxide and Norchlordiazepoxide was not detected after 18 days from the time of addition to the blood. Diazepam showed no significant change in concentration after three months of storage in both liver and brain. Nordiazepam at both temperatures after two months of storage showed a decrease, with the greatest decrease occurring in the liver stored at room temperature.[34] Another study reported the stability of drugs

and poisons in putrefying human liver tissues, where Chlorpromazine showed a total loss from liver tissue containing 100 µg/g of drug when stored in closed vessels at 15°C and 29°C over a period of 53 days. However, the concurrent storage of tissues containing Amitriptyline and Imipramine resulted in no loss of these drugs from the tissues.[35] Formalin blood solutions were monitored daily for thirty days. The formalin blood solution losses were found for Diazepam and Phenytoin over the 30-day period of at least 41% and 33%, respectively. The study revealed that the drugs examined at toxic concentrations can be detected, with variable recoveries, for up to 30 days after fixation with formalin.[36] Stability of drugs of abuse in urine samples stored at -20°C was determined for Amphetamine, Methamphetamine, Morphine, Codeine, Cocaine, Benzoylecognine and Phencyclidine in 236 physiological urine samples for a period of twelve months. The average change in the concentrations of these drugs was not extensive except for an average change of -37% in cocaine concentrations.[37] The storage effect at room, refrigerator and freezer temperatures, on the stability of Morphine and Codeine in urine was reported; total Morphine, total Codeine, free Morphine and free Codeine in samples were analysed at thirty day intervals for an eleven month period. Total Morphine and Codeine concentration decrease was observed for all specimens in all storage conditions. For samples stored in the refrigerator and freezer, decrease was in the range from 10 – 40%. For samples stored at room temperature, the decrease was larger for total Morphine, total Codeine. Total Morphine concentrations show a decrease pattern similar to that observed for

the freezer and refrigerator storage conditions.[38] A five year stability of common illicit drugs in blood was reported. The drugs of interest in this study were cocaine and Benzoylecgonine, Methamphetamine and amphetamine, nonconjugated Morphine and Codeine, and phencyclidine (PCP). The specimens were collected in Vacutainer tubes containing sodium fluoride and potassium oxalate. The tubes were stored at room temperature. Cocaine and Benzoylecognine have poor stability. Methamphetamine and PCP were both fairly stable and had a high probability of confirmation upon reanalysis. While Morphine initially decreased then increased at the 3 year interval, and finally decreased at 7 and 5 year intervals.[39]

The stability of Morphine and Buprenorphine were also reported in whole blood. Both drugs were found to be very stable for up to six months under the storage conditions (4°C, 25°C, -20°C). At least 85% of Morphine and 77% of Buprenorphine were recovered and not less than 70% was still detectable after one year of storage regardless of the temperature, especially when blood samples were stored in silanized glass vessels.[40] Another stability study was done on a wide range of drugs including Temazepam, where Temazepam stability was studied at 5°C, 25°C, -20°C, in aqueous and blood samples for a period of one year. Blood Temazepam samples showed a significant decrease on their concentration after one month of storage with a recovery of 92% from the day zero. The decline in Temazepam concentration continued and 80% of the drug was recovered after six and a half months. The lowest recovery

reached 50% after one year of storage. While Temazepam aqueous samples showed no significant change in concentration for day zero up to two and a half months. A significant decrease in concentration was noticed after six months of storage with a recovery of 88% from day zero. 50% of the drug was still detected after one year of storage in aqueous samples.[41]

2.1.1 BENZODIAZEPINES

2.1.1.1 Introduction

Although many techniques have been developed for the extraction of benzodiazepines and their metabolites from body fluids, difficulties remain in the detection and quantification of these compounds in the blood samples routinely encountered in forensic casework.[42] Such samples are varyingly and usually extensively haemolysed and are often putrified. Large amounts of solidified and coagulated materials may be present and there may be serious contamination by plasticizers and by components of the elastomers e.g. in rubber septa, with which the samples may come into contact during their collection and storage. The amounts of blood available for examination may be small and further restricted by the requirements of other analyses. Sometimes the concentration of benzodiazepines might be very low. Therefore, a good

extraction technique with high recoveries is needed to detect benzodiazepines and metabolites in biological material presented for toxicological analysis.

Several extraction techniques based on either liquid extraction or solid phase extraction have been developed for the determination of benzodiazepines in urine, plasma and whole blood.

2.1.1.2 Solid Phase Extraction

Solid phase extraction techniques have gained popularity for many biochemical, clinical, industrial and environmental applications because of their efficiency and ability to provide a clean extract in a single step.

High recoveries and selective extraction are especially important in drug screening in forensic toxicology, where specimens are complex and sometimes contain drugs at low concentrations.

The principle of liquid/solid phase extraction is closely related to conventional liquid-liquid extraction. Described in 1976[43] it involves the absorption of the aqueous phase onto diatomaceous earth, a porous material which acts as a support for the aqueous phase. This provides a large surface area for partition into an eluting solvent, which flows through the immobilised specimen under gravity, eluting the analytes of interest. The next major

development was the partition chromatography where a polar solvent (water) stationary phase was held by adsorbing it on silica gel and moving a second solvent (chloroform modified with ethanol) through the column as the mobile phase to isolate and separate acetyl derivatives of aminoacids. In this system, chromatographic separation was achieved, not by adsorption of the compounds onto a solid phase but by partitioning of the solute between two liquid phases according to distribution isotherm.[44]

Over the past decade, solid phase extraction (SPE) has emerged as a powerful technique for the pre-treatment of biological samples for clinical and toxicological analysis.[43-47]

Solid phase extraction has given more reproducible results since it is based on specific molecular interactions. The easy use of the column allows a large number of samples to be processed and the procedure can be automated.[48]

The majority of publications have been geared towards the extraction of the individual drug or groups of related drugs, for example, extraction of Temazepam on its own or extraction of weak and strong basic drugs using the same extraction column.[49-53]

The use of solid phase extraction columns as an alternative to liquid-liquid extraction for the isolation of drugs from biological samples has gained popularity over recent years because of the reported excellent recoveries and ease of use.[54-57]

The majority of reported solid phase methods for the extraction of benzodiazepine drugs from body fluids involve the use of reversed bonded phase columns (C_2, C_8 and C_{18})[57-62] and since benzodiazepines are considered as weak basic compounds and are better extracted on non-polar sorbents, other methods were reported where ion exchange/ion pairing extraction and mixed mode extraction methods have been used and were reported to be highly efficient in the recovery of various drugs from biological fluids[53,61,62]. One of the extraction procedures reported was for the quantitation of diazepam and N-desmethyldiazepam from blood using an inexpensive source of diatomaceous earth (Celite 560) which gave good recoveries.[60] Another method was reported where non-ionic Amberlite XAD-2 (polymeric sorbent consisting of styrene-divenylbenzene and polyacrylate structure) was used for confirming the presence of benzodiazepines in urine by hydrolysing benzodiazepines to benzophenones in an acid medium. It was described as a simple and reliable method but has no differences in hydrolysis efficiency among benzodiazepines which preclude the use of a single calibration graph.[61] C2-AAP cartridges were used for screening of benzodiazepines in biological samples. The extraction time was five minutes and recovery ranged from 92 – 104% and was

independent of concentration.[59] A rapid method of isolation of benzodiazepines from human samples was with Sep-Pak C_{18} cartridges where the drugs were dissolved in alkaline samples, directly applied to the cartridges and eluted with hexane : isopropanol (9 : 1). The recoveries were excellent for all drugs in urine samples but were lower in plasma samples.[50] An extraction method for the determination of Triazolam and its metabolites in human urine has been reported where a Sep-Pak C_{18} cartridge was used and further purified by Sep-Pak Silica cartridge. The reported recoveries of Triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam were 88, 75 and 75%.[49]

An automated sample preparation module (ASPEC) was reported for the determination of four benzodiazepines in plasma. Extraction was carried out on disposable C_{18} cartridges and involved several washing steps. The drugs were desorbed with ethylacetate. The limits of detection in plasma were 0.5 – 2 ng/ml.[61] Another automated on-line solid phase extraction method was reported where the extraction was performed on LC-18 cartridges and eluted with ethylacetate. The method gave satisfactory recoveries when using a washing mixture of water : methanol (95 : 5 v/v) prior to elution.[62]

The mixed mode solid phase extraction reported was Bond Elut Certify columns where the columns were used for isolation of a broad range of drugs including benzodiazepines and where urine or serum samples were mixed with phosphate buffer before applying to the pre-conditioned column. The drugs

were eluted with methanol : 10% ammonia (5 : 1). The reported recoveries for drugs ranged between 71 – 99% at a concentration of 10 μmol/L.[63] The other Bond Elut Certify extraction reported was for benzodiazepines in urine samples where the samples were incubated with β-glucuronidase enzyme to convert benzodiazepines to aminobenzophenones before applying them to the sorbents. The recoveries were reported as excellent recoveries.[64]

2.1.1.3 Liquid-Liquid Extraction

Several liquid-liquid extraction methods have been developed for the quantification of benzodiazepines from liver, serum, plasma and whole blood. A single step liquid-liquid extraction method was used to extract benzodiazepines from liver where 1-chlorobutane was used for the extraction and the assay allowed detection of 5 to 20 μg of drug/kg of tissue.[65] Another method of liquid-liquid extraction was used to extract twelve benzodiazepines from serum. The procedure required only 1ml of serum. The samples were extracted using potassium carbonate and chloroform and it gave recoveries of more than 95%.[66]

An extraction method that had definite forensic application to post-mortem blood, where only one millilitre of blood sample is required and n-butylchloride is used for extraction, the sensitivity of the procedure was Diazepam (1-5 μg/dL) and NorDiazepam (5-10 μg/dL).[67]

A method was reported which used a one step extraction of whole blood. It required 1.0 ml sample, 1.0 ml buffer, a few hundred µl of solvent and less than 2 min. of technologist time and had recoveries of 80 - 82%.[68] A liquid-liquid extraction method was reported for benzodiazepines where the drugs were extracted from 1ml of buffered blood with n-hexane-dichloromethane (70 : 30) at pH9 and where no derivatization step was needed, prior to gas chromatography analysis. The recoveries were between 70 - 97%.[69] Ether extraction of sixteen benzodiazepines in blood samples treated with aqueous ammonia has been reported and that method yielded recoveries of 70 - 75%.[70]

A sensitive method for the determination of Temazepam in plasma was reported where a liquid-liquid extraction method was used, with dichloromethane : pentane (1 : 1) as the extracting solvent. The detection limit was 3.5 ng/ml.[71] Toxi-tube A was reported to be used for liquid-liquid extraction of 600-compounds including several benzodiazepine drugs of interest, where 1 ml of urine or whole blood was mixed with distilled water after extraction and centrifugation. The sample was reconstituted with acetonitrile : water (50 : 50 v/v) before analysis on the HPLC system. Clear extracts were reported and this gave low interference chromatographic analysis which enhanced the drug detection.[72] A method was reported where parent benzodiazepines can be identified by converting them to aminobenzophenone using β-glucuronidase enzyme. The extracting solvent used was a mixture of

isobutyl alcohol : methyl-chloride (1 : 9). The limit of detection was reported as 200 ng/ml.[64]

2.1.2 TRICYCLIC ANTIDEPRESSANTS

2.1.2.1 Introduction

Antidepressant drugs are highly protein bound and the therapeutic concentrations of the free fraction of these drugs are quite low.[73] Routine determination of the free fraction of antidepressant drugs is an analytical challenge.

Various extraction procedures are available, varying from triple liquid-liquid extractions to solid phase extractions. Colour tests, such as Forrest's reagent, are also used by some laboratories to detect the presence of some of the tricyclics in toxicological specimens.[74]

2.1.2.2 Liquid-Liquid Extraction

Until recently, extraction of antidepressants from biological samples with water immiscible organic solvents has been the most popular technique. A number of different approaches have been used.

Liquid-liquid extraction equipment is mainly glass culture tubes with PTFE-lined screw caps with different dimensions. Some procedures require silanisation of the tubes to minimise adsorption of drugs onto the glass. Different methods have been used to silanise the tube, for example, with dichloromethylsilane. In some cases it was soaked overnight,[75] while in others for as little as 5 minutes.[76] Another approach to avoid adsorption of drugs, is to extract the drugs in plastic polypropylene tubes. There is a wide range of time suggested for vortex mixing the sample with the organic solvent ranging from 30[77] to 15 minutes.[78] Mixing, particularly high speed vortex mixing of plasma with organic solvents, can lead to the formation of an emulsion. Use of rotary mixers at slow speed allows simultaneous mixing of a large number of samples uniformly with reduced emulsion formation.

N-hexane is an example of one of the solvents used for the extraction of antidepressant drugs where the recoveries reported were 92 - 110%.[79,80]

The liquid-liquid extraction procedure, to extract tricyclic antidepressants from liver, was published where chloroform was the solvent used for extraction and where it gave good recoveries. The purpose of this study was to further examine the experiences with liver TCA concentrations as causes of death.[81] N-chlorobutane was used as the extraction solvent in another study where a 2 ml blood sample was used. After performing the extraction and evaporation steps, the drugs were reconstituted with 50% hexane

in ethanol. The study gave good recoveries for the Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine as follows:

69.2%, 59.8%, 79.8% and 56.9%, respectively.[82,83]

Amitriptyline and Nortriptyline were liquid-liquid extracted from plasma by multi centrifugation steps and one washing step with normal saline, which gave high recoveries for plasma Amitriptyline and low recoveries for plasma Nortriptyline due to the difference in distribution in blood compartment. Amitriptyline is found predominantly in plasma whereas Nortriptyline is more concentrated into the cellular fraction.[84]

N-butylacetate, in another study, was the solvent of choice for the extraction of tricyclic antidepressants from blood due to its lack of volatility and its polarity which is essential in a single-step extraction without evaporation, and gave good recoveries at the same time.[68]

Another simple and rapid method was reported. 1 ml of plasma was used where it was extracted with Butan-1-ol in hexane. The recoveries of Amitriptyline and Nortriptyline were 90 and 87%, respectively.[85]

A simultaneous method of extraction was reported for Imipramine, Desipramine and their 2- and 10-hydroxy metabolites. The drugs were

extracted from plasma or urine at pH 8.5 with diethylether and back extracted in 0.1M orthophosphoric acid. The recovery of the compound ranged from 78.6% for Imipramine to 94.3% for 2-hydroxydesipramine.[86]

A forensic determination of eleven cyclic antidepressants using liquid-liquid extraction was reported. The samples were extracted by 20% Na_2CO_3, water and n-hexane-isoamylalcohol (98.5 : 1.5 v/v). The relative recoveries in human serum ranged from 94-103% for all drugs.[87]

2.1.2.3 Solid Phase Extraction

In the last few years a number of solid phase extraction procedures have been described for the determination of antidepressants.

Bonded phase silica, e.g. Octadecyl, octyl, phenyl and cyanopropyl have been the commonly used supports for the extraction of antidepressant drugs. Cation-exchange and mixed phase bonded silica are also being used for the antidepressants.

In some procedures, cartridges packed with diatomaceous earth, e.g. ClinElut or Extrelut, have been used for the extraction of antidepressants.[88] Other procedures used Sep-Pak C_{18},[89,90] Baker Bond C_{18}[91], Bio-Rad C_{18}[92], Bond Elut CN[93], Baker Bond CN[55] and Bond Elut C2 Extraction Cartridges. [94]

All of these solid phase extraction procedures claim to give clean extracts with excellent recoveries of the desired drugs.

Antidepressant drugs are high protein-bound. Recovery of these drugs by solid phase extraction can be variable and low due to incomplete liberation of drugs bound with proteins. The simplest approach to minimise the effect of protein binding is to pass the sample through the sorbent at a slow rate.[91] In another study, the sample was diluted with an equal volume of water,[55] and in another it was diluted with an equal volume of 0.1M ammonium hydroxide.[92] A study described the addition of methanol to precipitate proteins. The resultant supernatant was applied to the extraction cartridge.[90]

Another study used a method to precipitate proteins by adding two volume of acetonitrile and then processing the supernatant by solid phase extraction which recovered Imipramine with a yield of 97%.[55]

Several solid phase extraction methods has been described for the extraction of antidepressants from plasma, whole blood and urine samples.

Some studies were done on the extraction of tricyclic antidepressants from urine. One was carried out on 1 ml Bond Elut strong cation exchange (SCX) columns. These contain 40 µm silica particles with an alkyl bonded

benzene sulfonyl propyl moiety which give the sorbent the ability to participate in both cation exchange and nonpolar interactions.[53]

Some other methods were reported for the extraction of tricyclic antidepressant from serum. One was done using two solid phase cartridges of a graphitized carbon black (Carbopack B) cartridge and a silica based strong acid exchanger. After applying serum samples on Carbopack cartridge, the tricyclics were removed from the Carbopack surface and selectively readsorbed onto the cation exchange surface by passing methylene chloride : methanol (60 : 40 v/v) through the two cartridges. The drugs were eluted from the cation exchange surface with acetonitrile : methanol : water (72 : 18 : 10 v/v/v). The recoveries exceeded 90%.[95] Another on-line solid phase extraction method was reported. It was performed on a bonded phase CN column to extract Amitriptyline and Nortriptyline from serum. The relative standard deviation ranged from 2.8 - 8.0% for concentrations of 200-40 ng/ml.[96]

Other studies were reported for the extraction of tricyclic antidepressants from plasma. One was performed on Cyano bonded silica cartridges. The samples were acidified then applied to the preconditioned column. The drugs were eluted with acetonitrile : triethylamine (100 : 0.15 v/v). The recoveries ranged between 93 - 102%.[97] Another was described as a rapid and reliable procedure for the simultaneous determination of Chlorpromazine and thirteen of its common metabolites in human plasma using C_8 bonded phase columns.[98]

Some reported studies show the extraction of tricyclic antidepressant from blood. One was done on C_8 Empore solid phase extraction membrane (SPE14) where drugs were eluted from the column with a mixture of water-acetonitrile-aceticacid-n-butylamine (600: 400 : 2.5 : 1.5 v/v/v), which was the mobile phase, thereby avoiding evaporation steps that can affect drug stability. Recovery for all drugs exceeded 90%.[99] Another method was used where aqueous blood sample (blood diluted with water) was centrifuged, then supernatant was applied to a carbon sorbent (Supelclean – ENVIcarb) then eluted with TFA methanol (15% trifluroacetic acid) followed by xylene-methanol. The eluent was then applied to the second sorbent, a cation exchange sorbent (Bond Elut PRS cartridges), and eluted with 2% ammonia-methanol-ethylacetate. The absolute recoveries reported were 96.7%, 100% and 88.1% for Nortriptyline, Imipramine and Chlorpromazine, respectively.[100] A method was described for the extraction of tricyclic antidepressant from plasma, whole blood and urine on Bond Elut Certify Columns. According to this study the tricyclic antidepressants were detected at concentration of 100 - 200 ng/ml.[101] While the recoveries were described as reproducible in another Bond Elut Certify solid phase extraction method, the blood was pretreated by mixing it with phosphate buffer (pH 6.0) then the supernatant was applied to the preconditioned column and, finally, eluted with ethylacetate containing 2% (v/v) of 25% ammonia solution.[102]

For the extraction of a large number of samples, manual solid phase extraction is tedious and somewhat slow. A number of manufacturers have marketed instruments which allow automatic performance of some or all of the steps of the solid phase extraction procedure using different extraction sorbents.[103,104]

2.2 DRUG ANALYSIS

2.2.1 BENZODIAZEPINES

2.2.1.1

Because the world-wide market for benzodiazepines anxiolytics and hypnotics is extremely large, benzodiazepines continue to be developed, evaluated and introduced for clinical use. Demonstrating the presence or absence of a benzodiazepine in a biological sample can have important legal and medical consequences. Identification of a benzodiazepine in an impaired driver's blood can help corroborate an arresting officer's statement, while, in a hospital setting, detection of benzodiazepine is necessary to ensure compliance with prescription orders. The assay methods used to determine their concentrations are important to evaluate their pharmacokinetics, bioavailability and clinical pharmacology, and to detect and identify them in toxicological and forensic samples. The assays methods include mainly gas chromatography, gas chromatography-mass spectrometry,[105,106] radio-immunoassay and radio-receptor assay.[107] Colorimetric and spectroscopic techniques[108] lack sensitivity and specificity.

2.2.1.2 Thin Layer Chromatography (TLC)

Thin layer chromatography R_F x 100 values of parent benzodiazepines have been measured on three TLC systems.[109] Two dimensional TLC identification of twelve 1,4-benzodiazepines or their hydrolysis products (the corresponding aminobenzophenones) has been reported.[110]

Methods for the rapid, reliable and sensitive detection and identification of eighteen 1,4-benzodiazepines drugs (including Chlordiazepoxide, Diazepam, Nitrazepam, Temazepam, Prazepam and others), have been presented.[111] The methods were based on the hydrolysis of the 1,4-benzodiazepines in acidic media to the corresponding aminobenzophenones and their thin layer chromatography separation using two mobile phases and detection with Bratton-Marshall reagent directly or after photochemical dealkylation. A combination of TLC and other chromatographic systems were reported. A method of drugs screening was reported where solid phase extraction and combined TLC and GC/MS were used to identify different drugs including benzodiazepines.[112]

2.2.1.3 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatographic methods have been described for the analysis of benzodiazepines. These techniques have offered greater

specificity and sensitivity over immunological procedures and colorimetric and spectrophotometric methods, are also more suitable than CG for thermally labile compounds, and do not require derivatization or hydrolysis prior to analysis.

Retention data using both silica and ODS-silica in HPLC have been given. HPLC retention characteristics of 21 benzodiazepines and some of their metabolites have been examined on both silica and ODS-silica packing materials.[113] Four HPLC systems have been considered and retention data were presented for the compounds on these four HPLC systems. The correlation of retention data on the system was considered with reference to the problem of identifying unknown benzodiazepines. An HPLC support was prepared, based on silica beads coated with β-cyclodextrin containing polymer, which allows the elution of solutes in order of their affinity to β-cyclodextrin.[114]

A complete reversed-phase HPLC separation of benzodiazepines was achieved in 50 minutes with detection limits of less than 3 ng/ml in urine and 5 ng/ml in other biological fluids when using a suitable gradient elution.[115]

A molecular rearrangement of aminobenzophenones (hydrolysis products of 1,4-benzodiazepines) to 9 acridones has been studied. Its chemical structure is shown in Figure 11.[116]

$C_{16}H_{13}ClN_2O$

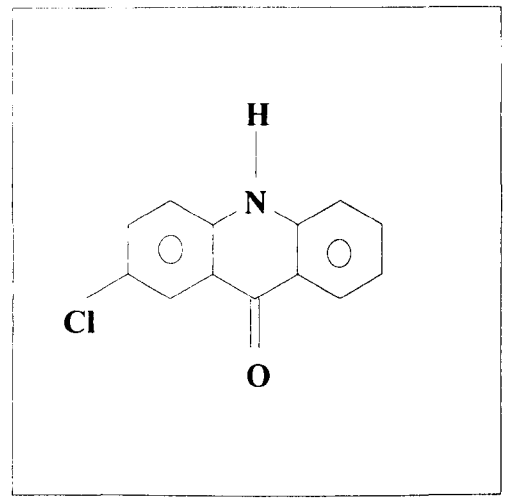


Figure 11: 9-Acridones

The compounds synthesised were analysed by HPLC with fluorescence detection, because of their high luminescence. The method can detect sixteen benzodiazepines simultaneously, but ten different aminobenzophenones are formed from the sixteen benzodiazepines considered. It is reliable for the analysis of these compounds in biological samples at therapeutic concentrations.[116] The separation, isolation and identification of 1,4-benzodiazepine glucuronide (oxazepam, cinolazapam) from biological fluids by reversed-phase HPLC has been reported.[117] A number of HPLC methods have been described for the determination of Diazepam and its major metabolites in urine samples.[118] Other methods are available for their analysis in blood, serum and saliva samples by HPLC.[119] HPLC methods for the separation of benzodiazepines in plasma has been studied.[120] Another method for the analysis of benzodiazepines in serum where a 100 x 2.1 mm I.D, 3 µm particle size C_8 analytical column was used and the assay for major benzodiazepines was linear in the range of 50-2000 ng/ml. Regression analysis showed a

correlation coefficient of greater than 0.999.[52] Separation of parent benzodiazepines and their major metabolites by reverse-phase ion-pair chromatography from biological specimens was studied, where the separation used a reversed-phase C_8 column with a variable ultraviolet (UV) detector.[121,123] A combined GLC-ECD and HPLC procedure reported in 1978 for the analysis of the most commonly encountered benzodiazepine drugs has been applied to both plasma and post-mortem blood samples.[122] Two HPLC systems, one based on silica and the other on a reversed-phase ODS-silica, were used to detect the efficiency and cleanliness of ether extraction for benzodiazepines in blood samples treated with aqueous ammonia.[70] HPLC determination of Triazolam and its metabolites in human urine where the extract was chromatographed on a reversed-phase column with a UV detection at 220 nm was used. The overall recovery for Triazolam was 88%, and its detection limit was 5 ng/ml, using a 10 µl specimen.[123] Another reported method used HPLC with electrochemical reductive detection. This enabled the determination of benzodiazepines and their metabolites in small samples of degraded and contaminated blood, which are typical in forensic science work, and the recovery values were 75 - 95%.[124] A rapid isolation of benzodiazepines with Sep-Pak C_{18} cartridges from urine samples were reported. The recoveries recording to the study were excellent.[58,72] Simultaneous determination of twelve benzodiazepines in human serum was performed on a C_{18} reversed-phase column (ODS-C_{18}). The method was sensitive for the twelve drugs.[66] Six hundred compounds including benzodiazepines were analysed with a

multi-step gradient on a symmetry C_8 5 µm column, operated at 30°C. The method gave less peak tailing, lower identification limits and more stable retention times.[72] HPLC with photodiode-array detection for basic drugs in serum was reported using Hypersil 5 µm (particle size) octadecylsilane C_{18} column.[63]

2.2.1.4 Gas Chromatography (GC)

The analysis of benzodiazepines and their metabolites by gas chromatography (GC) has been the subject of many investigations. The analytical problem is compounded as these drugs undergo extensive biotransformation to products that are widely distributed and are present in low concentrations after therapeutic doses.

Several methods have been reported for the analysis of benzodiazepines in biological samples. A general method for the trace analysis of benzodiazepines and their major metabolites in 0.2ml blood samples has been described. The method involves solvent extraction of blood with toluene, and isolation of the analyte using deactivated Amberlite XAD-7 porous polymer beads. The subsequent analysis of the cleaned up extracts used capillary column GC with ECD. The clean-up technique eliminates lipids and other interfering material, enabling routine analysis of blood extracts to be carried out with no significant deterioration in column or detector performance over a

period of many months. The use of fused-silica capillary columns coated with SE-52 and the correct choice of chromatographic conditions permits benzodiazepines of widely differing volatilities and polarities to be analysed without derivative formation. Data for 26 benzodiazepines and metabolites are presented.[126]

Another method for the analysis of benzodiazepines in post-mortem blood has been reported. The method reported used optimum characteristics from published methodology using only one millilitre of blood sample. The data obtained for qualitative and quantitative analysis is presented to show the scope and reliability of the method. The method provides an effective screen for the common benzodiazepines in a total of 30 minutes from extraction to the GC. The approximate sensitivity of the procedure was 1 - 5 μg/dl for Diazepam and Clonazepam and 5 – 10 μg/dl for Nor Diazepam.[127]

A solvent modified solid phase micro-extraction for the determination of Diazepam in human plasma sample by capillary gas chromatography was reported. The method was shown to be reproducible with a detection limit of 0.10 nmol/ml in human plasma.[128] An automated on-line solid phase extraction using gas chromatography with nitrogen-phosphorous detection for the determination of benzodiazepines in human plasma was reported in 1997.[62] With a sample volume of 1 ml the procedure showed good linearity and repeatability in the range 5 – 50 ng/ml. The limits of detection in plasma were

0.5 – 2 ng/ml. This study showed that capillary gas chromatography (GC) is the separation method of choice for the trace analysis of complex mixtures because of its good separation efficiency and the availability of a wide range of sensitive and selective detectors.

A rapid twin-column GC method for the simultaneous screening and determination of commonly prescribed benzodiazepines from plasma and whole blood is presented.[69] Identical fused silica SE-54 columns were commited to a common split-splitless injector. Nitrogen-phosphorous and electron capture detectors were used to monitor the effect for such columns. By combining these specific and sensitive detectors considerable and accurate chromatographic information was obtained in a single run. The drugs were extracted from 1 ml of buffered plasma or blood with n-hexane-dichloromethane (10 : 20) and analysed without derivatization. The method was sensitive enough to permit reliable quantification of plasma Diazepam, Nordiazepam, Temazepam and Triazolam within twelve minutes. Toxicological analysis of whole blood samples by means of GC with nitrogen-phosphorous detection was studied for a wide range of drugs including benzodiazepines.[102] An automated sample preparation module was interfaced with a capillary gas chromatograph (GC) by means of a loop-type interface. The system was optimised for the determination of four benzodiazepines in plasma. Only 110 µl aliquot of the eluate was injected into the GC via the loop-type interface using fully concurrent solvent evaporation conditions. Detection was

by means of a nitrogen phosphorous detector (NPD). The procedure was reported to show good linearity and repeatability in the range 5 – 50 ng/ml and limits of detection in plasma were 0.5 – 2 ng/ml.[62] Determination of Triazolam in a drug tablet by thermally desorping the drug and then applying it to the GC system with flame ionisation detection (FID), Triazolam in the tablet sample (about 3 µg/mg) could be determined using the calibration curve.[129]

2.2.1.5 Gas Chromatography-Mass Spectrometry (GC-MS)

A GC-MS method for identification of parent Benzodiazepines from urinary extracts treated with βglucuronidase was reported, excellent quantitation was achieved within eight minutes run time and the cutoff concentration for a positive result was set at 200ng/ml.[64] Another method for the identification and differentiation of 30 benzodiazepines and their metabolites in urine after acid hydrolysis and acetylation has been published.[130] The acetylated extract was analysed by computerised GC-MS. An on-line computer allowed rapid data processing of ions using ion chromatography with mass to charge ratio of its ions as follows:

205, 211, 230, 241, 249, 312 and 333.

The identity of positive signals to the reconstructed ion chromatograph was confirmed by a comparison of the stored full mass spectra with reference

spectra. The ion chromatogram relevance mass spectra on GC retention indices on OV-101 are documented.

A specific and sensitive assay has been developed to quantitate Triazolam in post-mortem blood using $^{2}H_{6}$.Triazolam as an internal standard. Triazolam was isolated from whole blood by adsorption on an Amberlite XAD-2 resin and subsequentlty eluted with an organic solvent. The extract was analysed by GC-MS using selected ion monitoring (GC/MS/SIM) in the negative chemical ionisation mode (CI^{-}).[131]

A simple confirmation procedure for the presence of benzodiazepines in previously screened positive urine samples was reported where samples are manually hydrolysed to the corresponding benzophenones in an acid medium. The hydrolysis products are aspirated into a photometric flow system for screening; positive samples are then confirmed and benzodiazepines identified by using a different flow system that conditions the analytes for GC separation and unequivocal mass spectrometric confirmation. Detection limits for benzodiazepines in the nanogram/millilitre range are achieved by using 0.5 ml of hydrolysed urine. The high specificity and sensitivity of the method enables the confirmation of different benzodiazepines in urine samples.[57]

2.2.2 TRICYCLIC ANTIDEPRESSANTS

2.2.2.1 Introduction

Methods used for the analysis of tricyclic antidepressants in a forensic laboratory must be able to detect therapeutic levels and be applicable to small volumes of blood samples of poor quality. Many analytical methods have been applied to the separation of tricyclic antidepressants, and their metabolites from serum and plasma, but few satisfactory methods for the analysis of whole blood samples, such as those encountered in drugs/driving, murder, or rape cases, have been published. A successful method should facilitate the analysis of tricyclic antidepressants and their active metabolites without interference from coextracted endogenous materials present in blood.

Many analytical methods have been applied to the analysis of tricyclic antidepressants in biological fluids. The assays generally preferred are by either enzyme-immunoassay[132,133] or liquid chromatographic techniques.[134] Enzyme multiplied immunoassay techniques (EMIT) with monoclonal antibodies is simple and easily adaptable to different automated analysers. However, at present, this technique is usable only for assays of first generation tricyclic antidepressant. Quantitative thin layer chromatography is another method used.[135] GC has been used with flame ionisation detection, nitrogen phosphorous detection,[104] electron capture detection,[102] and mass

spectrometry.[135,136] Liquid chromatographic procedures have been published for both normal phase and reversed-phase columns.[147-139]

2.2.2.2 Thin Layer Chromatography (TLC)

Analysing for tricyclic antidepressants by thin layer chromatography (TLC) is either by quantitative or qualitative TLC.

Quantitative TLC is relatively faster than other chromatographic techniques where more than eight samples are processed. Furthermore, the compounds separated on a TLC plate can be chemically converted to coloured or fluorescent products, a feature not readily and efficiently possible with liquid chromatography. Antidepressant drugs do not have strong UV absorption and, with the exception of Protriptyline, do not have strong native fluorescence.

TLC has been applied in the 1980s for the determination of Imipramine and Desipramine.[140] In this procedure the specificity and sensitivity of detection has been increased by exposing the plate to nitrous acid fumes The resulting intense yellow spots are measured at 405 nm. While it appears that TLC is the most viable technique for the detection of antidepressant overdose in a majority of clinical laboratories, generally speaking, TLC does not have the required sensitivity to determine therapeutic concentrations of these drugs in plasma or serum. However, TLC has been quite useful for the screening of

antidepressants. Rf values, colours produced by these drugs with different reagents, e.g. Mandelin's reagent (perchloric acid),[141,142] help in the identification of the drug.

2.2.2.4 High Performance Liquid Chromatography (HPLC)

Different types of reversed-phase and adsorption columns with different chemically bonded phases have been tested to separate many of the tricyclic antidepressants.[143] Following the introduction of chemically bonded phases for HPLC,[144,145] much attention has been focused on the analysis of drugs by reversed phase chromatography using a primarily aqueous mobile phase. Columns packed with a polar stationary phase such as unbonded silica or silica bonded with cyanopropyl groups (CN) have been used in the normal phase mode. The mobile phase in these procedures is polar and based upon water-miscible solvents. Columns packed with a non-polar stationary phase, C_8 or C_{18} silica, are preferred because of their long life unless a better separation can be achieved using an alternative stationary phase. With a given mobile phase retention of antidepressants on a C_{18} column is greater than on a C_8 column of the same brand. Therefore, a C_8 column may be better than a C_{18} column for faster analysis.

A major failure offered by bonded-phase systems using aqueous eluents is the ability to analyse biological fluids either directly or following removal of colloidal material such as plasma protein.[146]

An isocratic reversed-phase HPLC procedure was reported[87] for the simultaneous detection of Amitriptyline, Nortriptyline, Imipramine, Clomipramine and Desipramine in serum. Detection is achieved at 254 nm.

The recovery of tricyclic antidepressants was 92 - 110% with a relative standard deviation of less than 5%. Another column switching system for the direct injection of plasma or serum samples, followed by isocratic HPLC and ultraviolet detection, is described for the simultaneous quantitation of the tricyclic antidepressant Amitriptyline and Nortriptyline. The method included adsorption of Amitriptyline and its metabolite as a reversed-phase C_8 clean-up column. In plasma samples 'spiked' with 25 - 300 ng/ml, the recoveries were between 84 and 112%. There were linear correlations (> 0.99) between drug concentrations of 5 – 500 ng/ml.

An analytical procedure was described in which a small volume of blood was taken (100 - 500 µl) and analysed using an ODS-Hypersil reversed-phase HPLC column.[79]

A method for the analysis of serum was reported.[146] The extract was chromatographed on a cyano column and the absorbance was measured at 215 nm. The mean analytical recoveries of tricyclic antidepressants added to the serum within the range 10 – 200 µg/L exceeded 90%. Antidepressants and other neuroleptics were measured in serum or plasma were detected by high performance liquid chromatography using octodecyclsilica column with a photodiode array detection.[147] A method for the analysis of Amitriptyline and its metabolite Nortriptyline in a diasylate from blood plasma by HPLC without prior extraction with organic solvents has been reported.[148] The method is highly precise, easy to perform and seems suitable for determining the free fraction of drug in plasma.[149] The analysis was performed on a reversed-phase spherisorb ODS Superpak cartridge 3 µm (100 mm x 4.0 mm I.D].

A method for the analysis of both benzodiazepines and tricyclic antidepressants in serum has been described.[52] A 30% solution of acetonitrile in phosphate buffer containing dimethyloctylamine was used as a common isocratic mobile phase for the analysis of both benzodiazepines and TCA on a reversed-phase column and detection was 242 nm. The sensitivity limit of the assay was 25 mg/ml in serum with recoveries of 76 - 95% for tricyclic antidepressants.

The analysis of tricyclic antidepressants was not only in plasma, serum and blood but also in mice brain,[150] where a method was developed for the

assay of Amitriptyline, Amitriptyline-N-oxide, Nortriptyline, Desmethyl-nortriptyline and its cis and trans-isomers in the plasma and the brain of mice using HPLC with ultraviolet detection (254 nm). The method was used to determine levels of Amitriptyline and its major metabolites in mice 30 minutes after a single administration of Amitriptyline (20 mg/kg). Other method were reported for the analysis of Amitriptyline, Nortriptyline, Imipramine in plasma or serum by reversed-phase liquid chromatography.[151,152] Other methods reported the use of C_{18} columns. One method reported the use of reversed-phase C_{18} columns and the use of mobile phase containing 1,8-diamino-octane (DAO) as amine modifier and heptanesulfonate as counter ion. This study demonstrated the high resolution and selectivity of the chromatographic system compared to the mobile phases prepared with primary amines.[153] Some other methods reported the use of HPLC using reversed-phase C_{18} with ultraviolet detection for the determination of tricyclic antidepressants from biological samples.[80,85,154]

Another method was reported where the analysis was performed on two different C_8 reversed-phase columns. One was TSK gel super-octyl, particle size 2 µm and the second was Hypersil MOS-C_8 with a particle size of 5 µm. The results show that the new ODS column packing with a particle size of 2 µm gives higher sensitivity and a shorter analysis than the conventional ODS column packing when applied to the analysis of biological samples.[87]

2.2.2.4 Gas Chromatography (GC)

Gas chromatography (GC) remains a highly sensitive and specific technique. For some applications, such as the determination of the free concentration of antidepressants, GC may be the only feasible technique. For chromatographic analysis, particularly for GC procedures, it is common practice to add one or more compounds as an internal standard to the sample to avoid precise measurement of volumes during extraction and analysis. There are exceptions, however. There is a report on the determination of Amitriptyline and Nortriptyline in rat brain by gas chromatography without the addition of any internal standard.[155] Drugs usually need derivatization before their analysis on GC. In general, there is a tendency for the absorption of primary and secondary amines on packed columns and to a lesser extent on capillary columns. This adsorption can be minimised by preparing suitable derivatives. However, there is a trend to avoid the preparation of derivatives even if it merely involves the addition of a few microlitres of a volatile reagent to the final extract prior to its evaporation. A majority of GC procedures for the determination of antidepressants published in the 1980s have used 1-2 m long packed glass columns. The liquid phase most commonly used for the GC separation of antidepressants remains OV-17 or an equivalent packing, SP-2250.[156] Some reports on the use of quartz capillary columns with bonded liquid phases for GC determination of drugs has been reported.[157,158]

Helium is the recommended carrier gas used to exploit the high efficiency of the capillary columns. Use of hydrogen, which can provide even higher efficiency than helium, cannot be used with the commonly used detectors, nitrogen-phosphorous detector (NPD) and mass spectrometer, for the determination of antidepressants. Splitless injection provides high sensitivity and good reproducibility for the determination of antidepressants.

Use of a flame ionisation detector (FID) for the determination of antidepressants was often used in the past. Methods have described a clean chromatogram for the determination of considerably low concentrations of Amitriptyline and its metabolites with the use of Flame Ionisation detectors.

It has been shown that Nitrogen Phosphorous detection is the most suitable detector for GC detection of psychoactive drugs which are present in plasma in low concentrations.

A method was described for the comprehensive screening and analysis of more than 80 basic compounds in blood and other biological specimens. This included Amitriptyline, Nortriptyline, Imipramine, Chlorpromazine and some other tricyclics.[73] The method was performed using 1-Chlorobutane as the extracting solvent with GC analysis which as been developed and modified so that identification and quantification of as little as 0.1 µg/ml of virtually all basic drugs in blood can be accomplished in approximately one and one half

hours. Amitriptyline, Nortriptyline and Imipramine were extracted from 1 ml of serum, urine or other biological fluids under alkaline conditions, the resulting extracted aliquot of chloroform: isoamyl alcohol was injected onto a temperature programmed GC equipped with two nitrogen phosphorous detectors and two fused capillary column.[159]

Other methods were reported where the extracted drugs were analysed on GC with nitrogen phosphorous detection and have proven to be simple, quick, sensitive and comprehensive, requiring only a small amount of sample.[69,160]

Another method is the detection of tricyclic antidepressants in body fluids by GC with a surface ionisation detector.[161] It is an extremely specific and sensitive method for organic compounds, such as secondary and tertiary amines that form their dissociative species at a low ionisation potential. The necessary equipment for surface ionisation detection is simply a modification of the standard thermoionic ionisation detector.[104]. This method is more suitable for plasma or blood analysis than for urine analysis. Detection of tricyclic antidepressants in whole blood was reported, where head space solid phase extraction was performed, the vial was exposed to the headspace to allow adsorption of the drug and analysis was performed on capillary GC with flame ionisation detection. The detection limit of each drug was 16–25 ng/0.5 mL.[162]

2.2.2.5 Gas Chromatography - Mass Spectrometry: (GC-MS)

The mass spectrometer is the most sensitive and specific detector for GC. Until recently, a mass spectrometer was available in only a few laboratories because of high initial and operating costs. Now a number of companies are marketing simplified bench top mass spectrometers which are adequate for drug analysis.

Several GC-MS methods have been reported for the analysis of tricyclic antidepressants in different biological materials.

A method was described for the analysis of TCA in urine and plasma, where a 10 ml sample of urine or a 1 - 3 ml plasma sample were extracted twice at pH3 and subsequently at pH10 with diethylether. The two extracts were combined and the organic layer solvent was evaporated with a dry stream of nitrogen. The residue was dissolved in 100 µl of methanol, 1 – 3 µl aliquot was used for GC-MS. Mass spectra were run on 4021 a GC-MS. Gas chromatography, with a fused-silica capillary column (25m x 0.32mm ID), was used with an injection port temperature of 290°C and an oven temperature programme at 75 - 300°C at 15°C/min. and the carrier gas was helium.[163]

Other GC-MS methods were reported. One method was that the required samples were liquid-liquid extracted and dissolved in 50 µl of tertbutyl

methylether. 1 µl aliquot was analysed by GC-MS in an electron impact mode.[100] Another GC/MS method for the determination of tricyclic ntidepressants from whole blood was reported where both high recoveries and clean extracts were achieved. GC/MS used was equipped with a fused silica capillary column. In the electron ionisation mode (70 eV) scanning was from 40 – 440 amu.[164]

2.3 CHROMATOGRAPHIC ANALYSIS

2.3.1 INTRODUCTION

Chromatography is a method used for the separation of the components of a sample in which the components are distributed between two phases, one of which is stationary while the other is a mobile phase.[165] It is a technique that enables samples of chemical mixtures to be separated by exploiting differences in their physical or chemical properties. These differences govern the rate of migration of the components of a mixture passing under the influence of a moving fluid through a bed of stationary phase. The individual components flow at different rates through the column, under the influence of the mobile phase. This action is known as the elution of the sample from the column. The differential rates of elution arise from interaction between the components of the sample and the material used to pack the column or a coating thereon.

The interaction of a sample with the column packing is referred to as retention. The degree of retention is a characteristic of that sample, since this depended on the solubility, absorption, size and ionisation characteristic of that compound in the specific environment of that chromatographic system employed.

There are many forms of chromatography available. Since HPLC (High Performance Liquid Chromatography) and GLC (Gas Liquid Chromatography) are the techniques used in this study, they will be discussed here.

Retention volume is a characteristic of a given sample and it is expressed as retention of a sample relative to the elution of a non-retained sample. This is referred to as the relative partition coefficient or capacity factor, K'.

$$K' = (V_R - V_0) / V_0 \qquad \textbf{Equation 1.}$$

where V_0 is the void volume, volume representing interstitial spaces between the particles packed in the column, V_R is the sum of the void volume and the volume of mobile phase necessary to overcome the interactions between the sample and the column packing.

When no change in the mobile phase occurs during the elution of the sample K' may be considered as:

$$K' = (t_R - t_0) / t_0 \qquad \textbf{Equation 2.}$$

where t_R and t_0 are the retention times of a retained and non-retained sample.

For any separation to be possible, it is essential that each component has a different value for the capacity factor; each component must be retained to a different extent. In these circumstances the system is said to be selective towards the components being analysed.

In chromatographic terms selectivity, α, is expressed as:

$$\alpha = \frac{K'b}{K'a} = (t_{Rb} - t_{R0}) / (t_{Ra} - t_{R0}) \qquad \textbf{Equation 3}$$

where K'a and K'b are the capacity factor for components a and b, tRa and tRb are retention times of peaks a and b, tR_0 retention time of non-retained sample and α is the selectivity factor.

The ability of a column to minimise peak spread is referred to as the efficiency of a column. The efficiency in all chromatographic techniques is expressed quantitatively as the number of theoretical plates, N, at the column.

$$N = 16 (tR/Wb)^2 \qquad \textbf{Equation 4}$$

where Wb is the base width of the peak.

The characteristics of a column may be defined using the term height equivalent to a theoretical plate (HETP) which is referred to as the peak height, H.

This is calculated by:

$$H(mm) = \text{length (mm)}/N \qquad \textbf{Equation 5}$$

Another characteristic of a column is defined by the resolving power, which related the width of eluted peaks to the distance between the peak maxima, the resolving power, or the resolution factor,

R_1 is calculated as follows:

$$R = 2\,(tRb - tRa) \,/\, (Wb + Wa) \qquad \textbf{Equation 6.}$$

where tRa, tRb, Wa and Wb are the retention times and base width, respectively, of peaks a and b showing in Figure 12A.

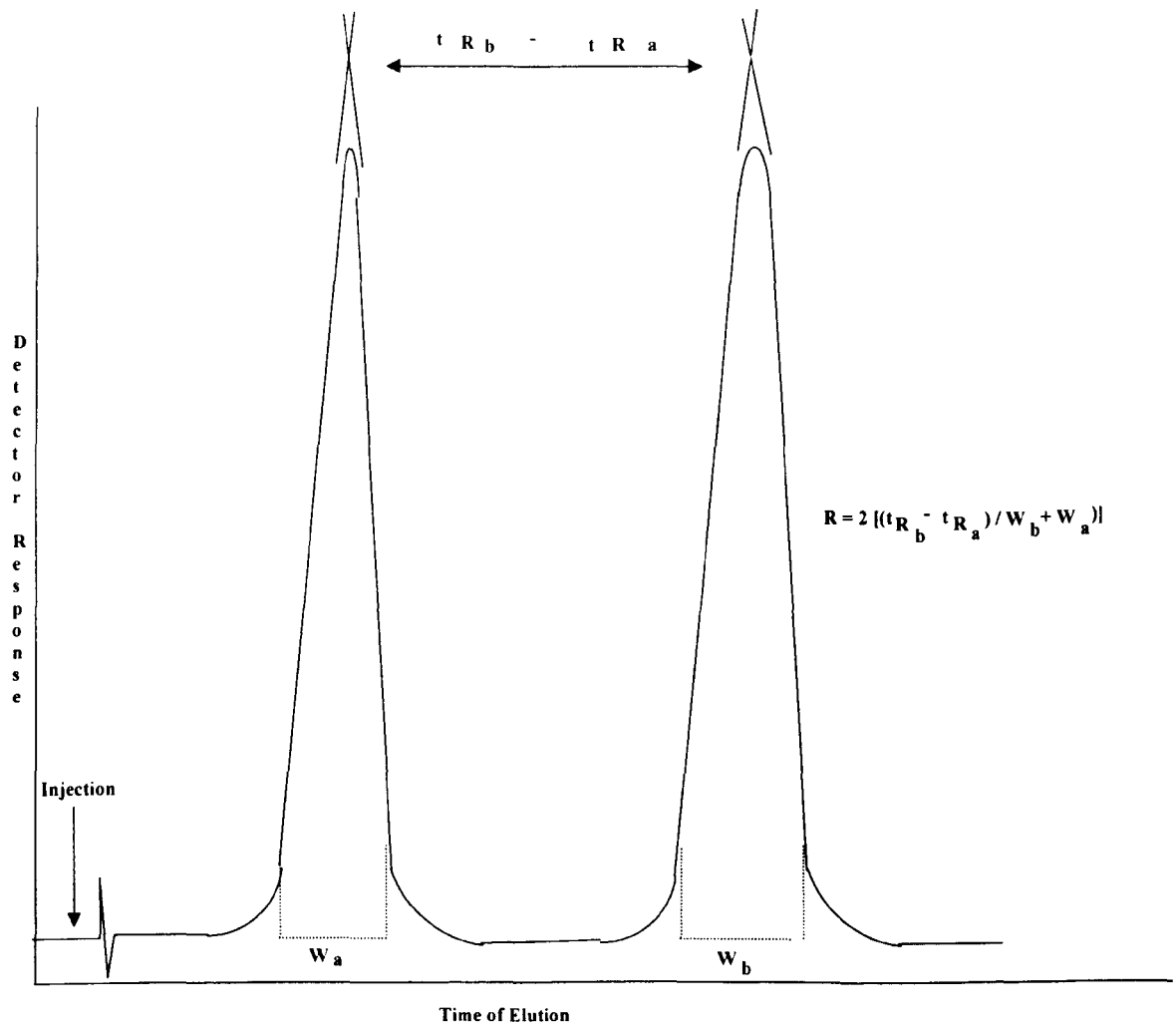


Figure 12A: Schematic Diagram of GC and HPLC Chromatogram showing the Measurable Parameter.

W_a and W_b = base widths of peak a and b

t_{Rb} and t_{Ra} = retention times of peak a and b

$t_{Rb} - t_{Ra}$ = sum of half widths of the bases at the constructed triangles (the adjacent triangles just touch at the base line).

R = resolution factor.

2.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.3.2.1 Reversed-Phase Chromatography

Reversed-phase (RP) chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase.

Chemically bonded octadecylsilane (ODS), an alkane with 18 carbon atoms, is the most frequently used stationary phase in RP chromatography. Non-aqueous eluents are frequently used for the RP chromatography of non-polar substances. The bonded phase columns are stable in the pH range (2 - 7) and at elevated temperatures. The classification of RP chromatography are discussed in reference 165.

2.3.2.2 Normal Phase Chromatography

Adsorption chromatography is referred to as normal phase chromatography.

Normal phase chromatography involves interaction between adsorbent, usually silica, and the solvent molecules in solution.

The mechanism of adsorption chromatography on the column packing is considered as a dynamic equilibrium, as shown in Figure 12B. The figure shows

that before a sample is introduced, a state of equilibrium exists whereby molecules of mobile phase are continually being adsorbed on the surface then subsequently desorbed; molecules of the sample and mobile phase compete for the adsorptive sites on the surface of the column packing.

For the sample to be able to elute from the column, the adsorption of the sample must be represented by an equilibrium distribution with a small proportion of the sample being in the mobile phase. Discussion of normal phase chromatography is discussed in reference [166].

2.3.3 GAS CHROMATOGRAPHY (GC)

2.3.3.1 Introduction

Like all chromatographic techniques, GC separates mixtures by taking advantage of their components' differential distribution between two phases, one stationary phase, which is maintained at a defined temperature in an oven and has a constant flow of carrier gas. A sample of the mixture to be separated is introduced into the gas stream just before it encounters the stationary phase; the components are separated by elution and detected as they emerge in the gas at the other end of the column. They are distinguished by the different times which they take to pass through the column, the retention times.

The retention time of a substance is dictated by the position at its distribution equilibrium between the two phases. Therefore, separation of a mixture depends on its components having significantly different distribution equlibria. In order to be able to communicate GC retention data between laboratories independent of the instrument used, the concept of Retention Index (RI) has been introduced to GC analysis. The Retention Index is a measure of retention times in relation to standards that are analysed under the same conditions.

The first RI system developed, and still widely used, is the Kovat's Retention Index ($RI_{\underline{K}}$). In this system, normal alkanes are assigned and $RI_{\underline{K}}$value at 100*X* carbon number. For Isothermal operation values are calculated according to the following expression:

$$RI_{\underline{K}} = 100 \left[\frac{\log ti' - \log t'n}{\log t'n + l - \log t'n} \right] + 100n \qquad \textbf{Equation 7}$$

where t'i = corrected retention time of sample peak [ti - t air], t'n = corrected retention time of normal alkane with carbon number N, which elutes before the samples peak, and t'n + 1 = corrected retention time of normal alkane with normal carbon number n + 1, which eluted after the sample peak.

Full discussion and the graphical representation of Kovat's Retention Index values is given in the literature.[166]

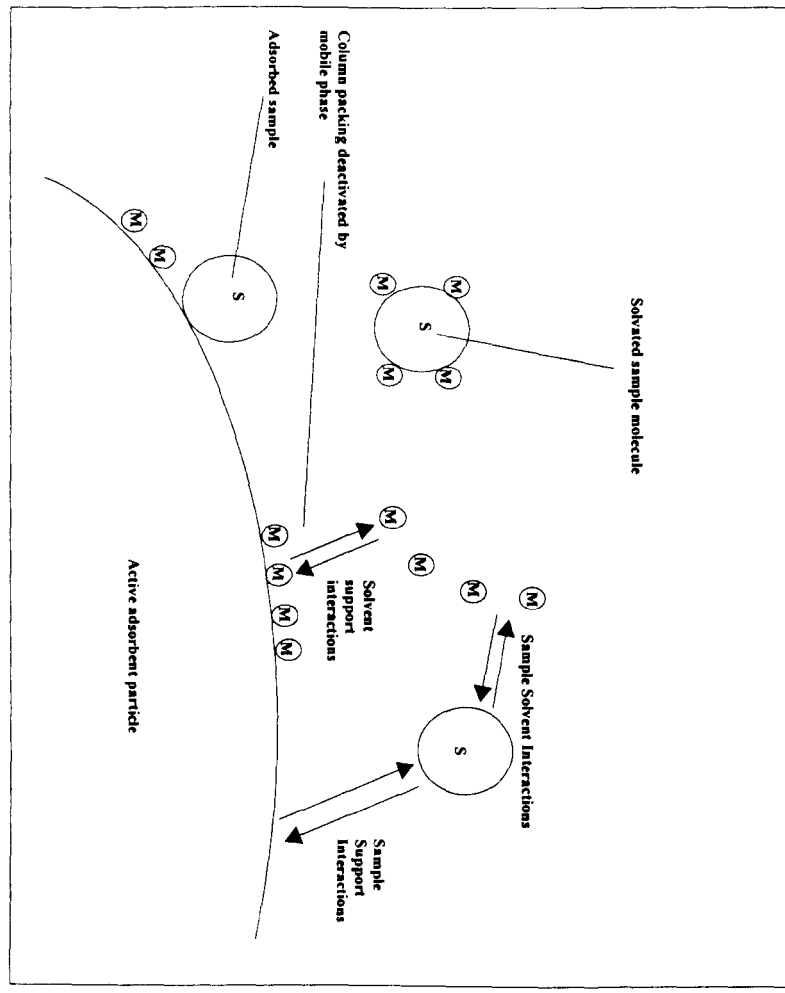


Figure 12B: Equilibria at a Liquid-Solid Adsorptive Surface [166]

2.3.3.2 Gas Chromatographic Columns

The GC column is the central item in a gas chromatograph. Two main column types are in common use - packed and capillary columns. The packed columns are used for 20% of GC analyses, and are used for simple preparations where high resolution is not required. Capillary columns of 0.25 and 0.53mm i.d. are the most popular, as are column lengths of 10 - 30m. The methyl silicones and polyethylene glycol stationary phases are the most preferred for capillary separations. The choice of phases for capillary columns is less than for packed columns, particularly for silica columns. However, the high efficiency available with a capillary column will often more than compensate for the lack of selectivity obtainable with a different phase. Bonded phases are divided into three:

(i) Non-polar phases: Apiezon L (a hydrocarbon grease used for the separation of barbiturates), SE-30, OV-1, OV-101 (and dimethyl silicone polymers separation on these phases are on the basis of molecular weight) and Apolone-87 (24, 24-diethyl-19,29-dioctadecyl-heptatetracontane), is a high temperature non-chiral hydrocarbon phase.

(ii) Polar phases: Carbowax 20M (is a polyethylene glycol which can be used for alkaloids and other basic drug separation), OV-17 (phenylmethylsilicone, moderate polar silicone phase), XE-60

(cyanoethylsilicone, favoured for steroid analysis), OV-225 (cyanopropyl phenylmethyl silicone), Polyesters (used for separation of fatty acids), Polyamides (Poly A103, used for separation of barbiturates and tricyclic antidepressants and Chirasil-Val (chiral phase used for the separation of enantiomers).

(iii) Mixed phases: mixture of phases used to tailor a column to solve a particular problem. As an example separation of 11 commonly used anticonvulsant drugs can be achieved using a mixture of SP-2110 and SP-2510-DA.

The coating of the stationary liquid phase inside the capillary column can be in several forms: wall coated tubular column (WCOT); support coated open tubular column (SCOT); and fused-silica open tubular column (FSOT]. The first column materials employed in the developmental stage of the technique were fabricated from plastic materials (Tygon and nylon) and metal (aluminium, nickel, copper, stainless steel and gold). Plastic capillaries, being thermoplastic in nature, had temperature limitations whereas metallic capillary columns had the disadvantage of catalytic activity. Today, the superior separations are achieved using fused-silica capillary column which, as an inert surface, yields better peak shapes; bands are sharper with less peak tailing, which facilitates trace analysis as well as provides more reliable quantitative and qualitative analyses. Full discussion on GC columns is discussed in the literature.[166]

2.3.4 GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC-MS)

2.3.4.1 Introduction

Gas chromatography-mass spectrometry (GC-MS) instruments have been used for the identification of hundreds of components that are present in natural and biological systems. In this instrument, the gas chromatography provides the separation of compounds and introduces them to mass spectrometry through the interface. Mass spectrometry produce ions from a molecule, separates these ions as a function of their mass to charge (M/Z) ratios and records and displays the relative abundance of these ions. Figure 13 shows the basic component of a typical GC-MS system.

More details of GC-MS are available in references 166-168

2.3.4.2 GC/MS Interface

The GC element leaves the column at atmospheric pressure whilst the MS ion source operates at 10^{-5} Torr. So for the direct connection of GC and MS the volume or pressure of the carrier gas must be reduced to avoid destroying

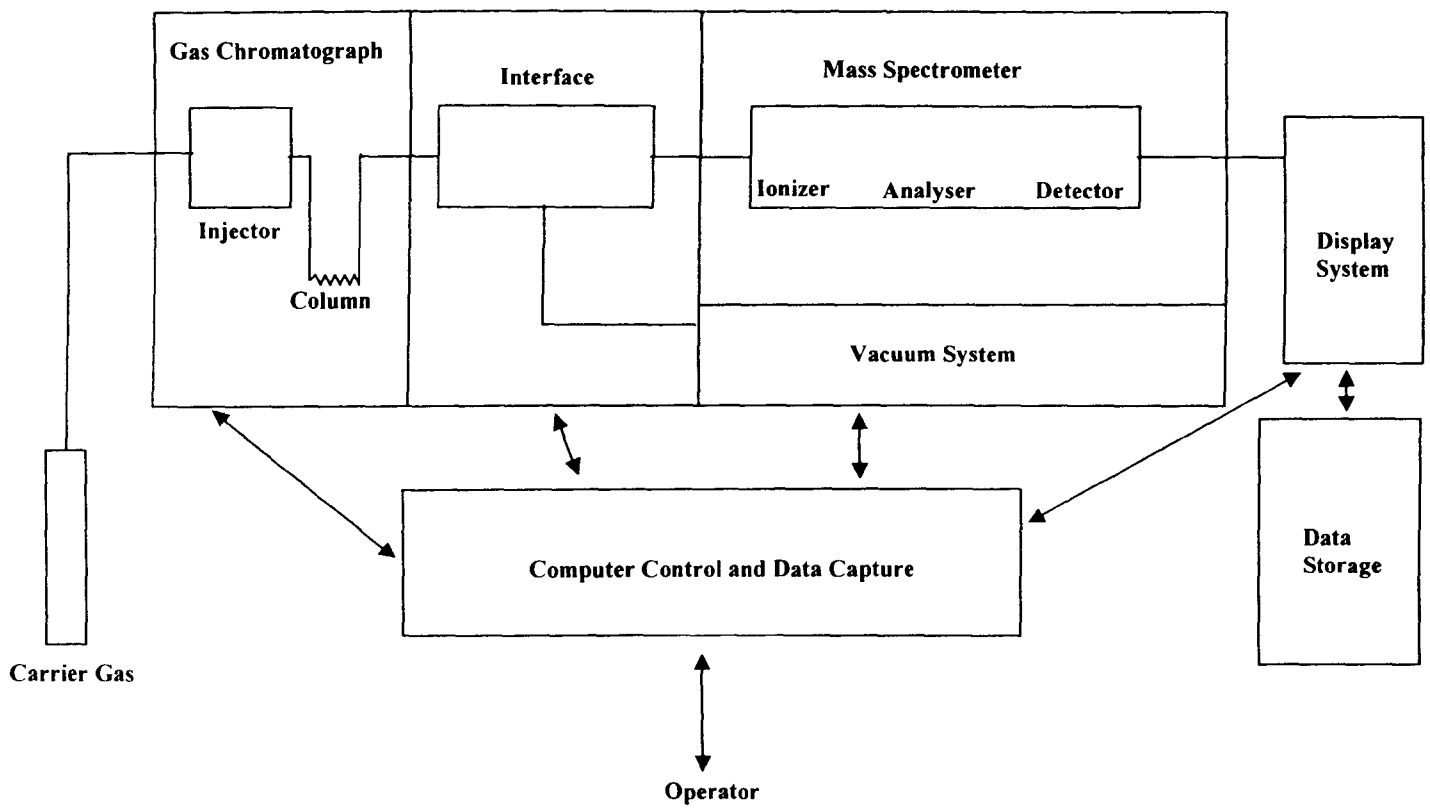


Figure 13: The Basic Components of Typical GC/MS System

the high vacuum condition. The ideal interface device would remove all the chromatographic mobile phase and transfer all the solute to the ion source without degrading chromatographic elution. The flow rate of the capillary column is in the range of 0.5 – 3 ml/min which can be accommodated by the vacuum system of the MS while still maintaining a suitable pressure in the ion source. So it is possible to introduce the fused silica column directly into the ion source. All of the sample eluted from the GC enters the MS ion source.

Mass spectrometry requires that molecules for analysis first be ionised. Two types of ionisation systems are available: Electron Impact (EI) and Chemical Ionisation (CI). Only one type will be discussed, electron impact.

Electron Impact (EI)

This is the most common method of ionisation in which electrons, obtained from a heated filament in vacuum, are accelerated through voltage (V) at 70V and directed across the ion chamber; the electrons thus have energy zV, where z is the electronic charge. A volatilized sample molecule (M) and an electron of energy greater than the ionisation energy of M will react if they pass close enough for the electron to impart sufficient of its energy to the neutral species. Not all of the energy of the electron is transferred.

$$M + e \rightarrow M^{+\cdot} + 2e$$ **Equation 8**

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

However, the reaction of an electron with a molecule is rather non-specific. Positive-ion formation is accompanied by other processes such as electron capture and electronic excitation and those are represented by equations 9 and 10, respectively:

$$M + e \rightarrow M^{-\cdot} + e$$ **Equation 9**

$$M + e \rightarrow M^{*} + e$$ **Equation 10**

Less than 1 percent of the sample molecules are converted into positively charged ions. Ions with a life time more than 10^{-5} sec will be detected intact. Those with very short life times, less than 10^{-6} sec, will decompose in the source of the mass spectrometer and will not be detected. To monitor negative ions, the repeller plate and acceleration potential must be reversed in sign.

2.3.4.3 Separation Of Ions

After leaving the ion chamber, the ions are accelerated with an acceleration potential (V) into the mass analyser, the separation of ions according to their mass to charge ratios (m/z) can be achieved with magnetic and electric fields alone or combined. Most of the basic differences between the various types of mass spectrometer lie in the manner in which such fields are used to effect separation. Those fields are magnetic-sector mass spectrometers,

quadrupole mass spectrometers, time-of-flight mass spectrometers and ion cyclotron resonance mass spectrometers.

2.3.4.4 Ion Detection And Data Analysis

There are four main ways of detecting ions: by photographic plate, Faraday cup or electron multiplier. They all work differently but in the simplest detector, ions impinge on a metal plate which is connected to earth through a resistor. The resulting neutralisation of the charge on the ions leads to a flow current through a resistor which can be detected and affords a measure of ion abundance. The commonest detector is the electron multiplier which consists of a series of about ten electrodes. When an ion impinges on the first electrode, it causes the release of a shower of electrons to the second electrode and each of these electrons causes a further shower of electrons at the third electrode. This cascading effect continues through the whole series of electrodes and provides gains of the order of 10^6. The amplified signals from the detector are passed to a recorder and the signals are used to deflect mirror galvanometers of different sensitivities. At a more sophisticated level, these electrical signals are passed indirectly via a magnetic tape recording, or directly to a computer which evaluates the incoming data and prints out the required information.

2.4 EXTRACTION TECHNIQUES

2.4.1 SOLID PHASE EXTRACTION

2.4.1.1 Introduction

Over the past decade, sorbent extraction has emerged as a powerful tool for chemical isolation and purification. It plays an important role in a broad range of applications: pharmaceutical, fine chemical, biomedical, food analyses, organic synthesis, environmental, and many others.[169-171] Sorbent extraction is a physical extraction process that involves a liquid and a solid phase. In sorbent extraction the solid phase has a greater attraction for the isolate than the solvent in which the isolate is dissolved. As the sample solution passes through the sorbent bed, the isolate concentrates on this surface, whilst the other sample components pass through the bed. Very selective extractions resulting in highly purified and concentrated isolates can be achieved by choosing sorbents with an attraction for the isolate but not for the sample components. Sorbents are formed by the reaction of organosilanes with activated silica. The product is a sorbent with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage.

The bonded silica sorbent product is stable within a pH range of approximately 2 - 7.5. Above pH 7.5 the silica substrate is susceptible to dissolution in aqueous solutions. Below pH 2.9 the silyl ether linkage is labile, and the functional groups on the surface will begin to cleave. In practice, bonded silica may be used for sorbent extractions in a pH range of 1 - 14, since degradation of the sorbent is a finite process and sorbents are typically exposed to solvent for only short periods of time.

Bonded silica sorbents are rigid materials that do not shrink or swell in different solvents, unlike many polystyrene based resins. For this reason, bonded silicas equilibrate rapidly to new solvent conditions. This allows complex extraction procedures involving many different solvent changes to be performed rapidly. The silicas most commonly used in making bonded silica sorbents have a particle size distribution of 15 - 100 microns. The particles are irregular rather than spherical.

The nominal porosity of most of the sorbents is 60 Angstroms, adequate for compounds with molecular weights up to approximately 15,000 to pass through the sorbent.

At the microscopic level, a silica sorbent resembles a forest of functional groups anchored through a root system of siloxane bridges. These bonds, and the silica beneath are responsible for two important properties of silica sorbents:

the need for solvation of the materials, and the potential for secondary interactions with isolate molecules.

Solvation of a sorbent is necessary before the sorbent will interact reproducibly with isolates. Solvation simply means that a solvent is passed through the sorbent to wet the packing material and to solvate the functional groups of the sorbent. Retention is accomplished by passing several bed volumes of a suitable solvent. The adsorptive or retentive properties of a bonded silica are due principally to the functional groups bonded to the silica substrate and any unbounded silanols remaining on the surface. Interactions between the substrate and isolate molecules are called secondary interactions. When there is an attraction between the sorbent and isolate molecules, causing the isolate to be immobilised on the sorbent surface as the sample solution passes through the sorbent bed. This phenomenon is called 'retention' whilst the process by which an isolate is removed from a sorbent bed on which it has been retained is called 'elution'.

Capacity of a given sorbent is defined as the total mass of a strongly retained isolate that can be retained by a given mass or sorbent under optimum conditions. Bonded silica ion exchange sorbents typically have capacities of 0.5 to 1.5 meq/g. For other sorbents, capacity values range from less than 1% to as high as 5%. That is, 100 mg of sorbent might retain as much as 5 mg of a strongly retained isolate.

Selectivity is the ability of the sorbent to discriminate between the isolate and all other sample matrix components. It is a function of three parameters: the chemical structure of the isolate, the properties of the sorbent, and the composition of the sample matrix. Maximum selectivity is achieved when a sorbent is chosen that interacts through isolate functional groups that are not common to other matrix components.

2.4.1.2 Non-Polar Interactions

Non-polar interactions are those that occur between the carbon-hydrogen bonds of the sorbent functional groups and the carbon-hydrogen bonds of the isolate. For many sorbents non-polar interactions are of lesser importance because a predominance of other functional groups mask the non-polar aspects of the sorbent. All isolate species have a potential for non-polar interactions.

Exceptions include inorganic ions and compounds, e.g. carbohydrates whose structure contains so many polar or ionic groups that the carbon structure of the molecule is masked.

Non-polar interactions are very effective for isolating groups of compounds that are not similar in structure. Retention of isolates by non-polar interaction is facilitated by polar solvents.

On the other hand, non-polar interactions between the isolate and the sorbent are best disrupted by solvents having some degree of non-polar character.

Octadecyl Silane C_{18}

The most widely used sorbent for non-polar interactions is octadecyl silane bonded to the silica substrate. Many isolate molecules can be retained by it. C_{18} is a very non-selective sorbent.

2.4.1.3 Polar Interactions

Polar interactions are exhibited by many different sorbents and functional groups on isolates. Polar interactions include hydrogen bonding, dipole/dipole, induces dipole/dipole, pi-pi, and a variety of other interactions in which the distribution of electrons between individual atoms in the functional groups is unequal, causing positive and negative polarity. Groups that exhibit these types of interactions typically include hydroxyls, amines, carbonyls, aromatic rings, sulfhydryl, double bonds and groups containing hetero-atoms such as oxygen, nitrogen, sulphur and phosphorus. Retention of isolates by polar interactions is facilitated by non-polar solvents.

Elution of isolates from polar sorbents is facilitated by polar solvents, and high ionic strength.

Activated Silica (SI)

Silica is generally regarded as the most polar sorbent available. Unbounded, activated silica is also fairly acidic. The extreme polarity of silica accounts for its ready adsorption of water and a very polar solvent such as methanol SI is one of the best sorbents available for selectively separating isolates at a very similar structure.

Aminopropyl (NH_2)

Like many of the ion-exchange sorbents, NH_2 is capable of exhibiting all possible interactions. NH_2 is a very polar sorbent, and a strong hydrogen bonder that also can function as an anion-exchanger. Thus, analytes have the opportunity to interact not only with the bonded phase but also with the underlying silica sorbent. Furthermore, because the hydrocarbon chain is short, only three carbons in length, the bonded phase may interact with the underlying silica through hydrogen bonding. Since the pKa of the NH_2 sorbent is 9.8, at any pH below 9.8 NH_2 is positively charged.

Although NH_2 has been used for non-polar isolations from polar solvents, its extreme polarity makes its non-polar character less significant than its other properties. Normal phase sorbents are good sorbents for the extraction of tricyclic antidepressants since they have amines in their structure which can interact with the functional groups of the sorbents, but it is not the sorbent of choice for the extraction of benzodiazepines which are considered as weak basic compounds that interact weakly with the functional groups at the polar sorbents.

2.4.1.4 Ionic Interactions

The interaction occurs between an isolate molecule carrying a charge (either positive or negative) and a sorbent carrying a charge opposite to that of the isolate. Groups of isolates and sorbents that can exhibit ionic properties allowing ion-exchange interactions can be divided into two classes: groups that can be cationic (positively charged) including primary, secondary, tertiary and quaternary amines; and inorganic cations such as calcium, sodium and magnesium.

Anionics (negatively charged) include carboylic and sulfonic acids, phosphates and other similar groups.

Retention of isolates by ion-exchange interaction is facilitated by solvent/matrix pH between the pKa of isolate and sorbent, while elution of isolates from ion-exchange sorbents is facilitated by solvent/matrix pH above pKa of cation or below pKa of anion.

Carboxymethyl (CBA)

CBA is a medium polarity sorbent, exhibiting either polar or non-polar interactions depending on the solvent environment.

One of the most useful characteristics of CBA is its weak cation-exchange properties. The most commonly encountered cations in organic isolates are amines.

CBA has a pKa of 4.8, above a pH of 4.8. CBA carries a negative charge that can be used for retaining cationic isolates. Dropping the pH below 4.8 neutralises the CBA, permitting the retained isolates to elute from the sorbent.

For this reason, CBA is often the best choice for cation exchange especially when dealing with very strong cations (i.e. high pKa cations).

Propylbenzenesulfonyl (SCX)

SCX is a strong cation-exchange with a very low pKa, due to the presence of the benzene ring on its surface. SCX has a high potential for non-polar interactions. This dual nature is useful with isolates that exhibit both cationic and non-polar character. After retaining the isolate, the sorbent can be washed with non-polar solvents and with high ionic strength solvents without displacing the isolate from the sorbent. The isolate can be eluted from the sorbent with a solvent that disrupts both ionic and non-polar interactions simultaneously, such as methanolic hydrochloric acid.

CN Sorbent (Cyanopropyl):

A medium polarity sorbent, CN can be used as a polar sorbent for analytes that might be retained irreversibly on the more polar sorbents, such as silica or diol. The cyano group provides a unique selectivity which can be moderated by intelligent use at eluting solvents.

CHAPTER 3 – EXPERIMENT

3.1 DRUGS SPIKING

Blank blood, supplied by the Scottish Blood Transfusion Service, was tested for the presence of drugs under investigation and was found to be negative. Portions of this blood were transferred into clean vials, sealed and designated as blank blood. Other portions were 'spiked' with the drugs under investigation, transferred into clean vials and sealed. Both lots were then stored under the same conditions. The concentration of drug in the blood was set to levels of each of the drugs under investigation where toxic effects had been found. At the day of spiking, a sample of 'spiked' blood was analysed to measure the concentration of the drug on that day. This was designated as the day zero concentration.

3.1.1 BENZODIAZEPINES

A stock of standard solution of Diazepam, Desmethyldiazepam, Temazepam and Triazolam was made up in methanol at a concentration of 50 µg/ml, 30 µg/ml, 50 µg/ml and 300 µg/ml, respectively. 36 ml of each of the above solutions were transferred into clean conical flasks and evaporated to

dryness under a stream of nitrogen gas on a hot block 60°C. Each solution of Diazepam, Desmethyldiazepam, Temazepam and Triazolam was redissolved in 360ml of blood to produce a concentration of 5.0 µg/ml, 3.0 µg/ml, 5.0 µg/ml blood and 50 µg/ml of blood. 15ml aliquots were transferred into clean hypovials of 25 ml capacity and sealed with a butyl rubber septa.

3.1.2 TRICYCLIC ANTIDEPRESSANTS

A 20 ml of a stock standard solution of 100 µg/ml of each of Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine in methanol was transferred into a conical flask and evaporated to dryness under a nitrogen stream on a hot plate at 60°C. Each flask containing each of the above drugs was redissolved in 200 ml of blank blood and mixed thoroughly using a magnetic stirrer. The resulting concentration for each of the above drugs, in blood was 10 µg/ml. 5 ml of the 'spiked' blood was transferred into clean 6 ml capacity hypovials which were sealed with a butyl rubber septa.

3.2 DRUGS STORAGE

Two vials for each required measurement of 'spiked' blood for each drug and blank blood were stored at different temperatures and periods of time before re-analysis to measure the effect of the temperature and the time interval on the drug concentration. The interval of time between each analysis was designated as one, two, three and up to twelve months. The drugs were stored at 5°C, 25°C and -20°C. Two samples each of 'spiked' blood and blank blood of each drug were removed from storage, opened and analysed at each designated period of time. These samples were then discarded.

3.3 TEMPERATURE MONITORING OF 'SPIKED' SAMPLES

The storage temperature of the 'spiked' blood (with drug) and blank blood under study were monitored from the day of spiking until the end of the experiment. Storage temperature was monitored over different time intervals (hours, days, months) at the storage temperatures 5°C, 25°C and -20°C.

3.4 DRUGS EXTRACTION

3.4.1 BENZODIAZEPINE

3.4.1.1 Extraction Buffer Preparation

Disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$, m.wt 177.99) buffer was made up by dissolving 1.78 grammes of $Na_2HPO_4.2H_2O$ in deionised water and making up to one litre. The resulting buffer concentration of 0.01M was adjusted with a few drops of orthophosphoric acid (Na_3PO_4) to pH 7.

3.4.1.2 Standard and Internal Standard Solutions:

A stock solution containing the four benzodiazepine drugs (Triazolam, Temazepam, Desmethyldiazepam and Diazepam) was prepared by dissolving 2 mg of each drug in a few drops of methanol and then diluting to 100 ml with deionised water to produce a stock standard solution of 20 µg/ml. Further dilution with deionised water was made to produce a working solution of 9µg/ml.

The internal standard solution (Prazepam) was prepared by dissolving 2 mg in a few drops of HPLC grade methanol and making up to 100 ml with deionised water, the resulting concentration being 20 µg/ml.

The same preparation were used for the method development for all solid phase extraction procedures that follow.

3.4.1.2.1 CN Sorbent/Sample Preparation:

0.9 ml of deionised water was transferred to a 6 ml screw-capped vial.

The following solutions were added:

1. 0.1 ml of the working standard mixture of benzodiazepine drugs (9 µg/ml).
2. 0.1 ml of internal standard (Prazepam 20 µg/ml)
3. 1 ml of 0.01M disodium hydrogen orthophosphate buffer (pH 7)

The vial was mixed well and the samples were extracted as follows:

3.4.1.2.2 **Sample Extraction:**

CN (Cyanopropyl) columns of 3 ml capacity were used. The columns were preconditioned with 3 x 1 ml of methanol, followed by 3 x 1 ml deionised water under a light vacuum (approximately 5mmHg) and followed by 3 x 1 ml (0.01M, pH 7), disodium hydrogen orthophosphate dihydrate buffer. The vacuum was turned off when the phosphate buffer was close to reaching the top of the sorbent bed to prevent the sorbent running dry.

3.4.1.2.3 **Addition of Sample:**

The above mixture (3.4.1.1.1) was transferred to pre-conditioned Bond Elut CN columns and drawn slowly through under vacuum. The vacuum pressure generated by the water pump was kept between 5 - 10 PSI. The cartridge was not allowed to dry out.

The sorbent was washed twice with 1 x 1 ml 0.01M Na_2HPO_4 (pH 7) and dried by passing air for 30 - 40 seconds.

3.4.1.2.4 **Elution of the Sample**

The drugs were eluted from two sorbents using different elution solvents:

1. Acid methanol (25 µl 1% H_3PO_4 in 5 ml methanol).
2. Methanol.

3.4.1.2.5 Analysis of the Sample

The eluent was evaporated to dryness by placing the vial on a hot plate at 60°C under a nitrogen gas stream. The extract was reconstituted in mobile phase (0.01M disodium hydrogen orthophosphate dihydrate) ($Na_2HPO_4.2H_2O$, m.wt 177.99) (pH 8.8) and methanol in the proportions (30 : 70 v/v) was used. 20 µl was injected on to the HPLC system.

3.4.1.3 Bond Elut Certify Sorbent

Another solid phase extraction method was used where Bond Elut Certify columns were used. This column contains a proprietary bonded silica sorbent, which exhibits a unique hydrophobic ion exchange extraction mechanism and will retain benzodiazepine drugs under the proper extraction conditions.

3.4.1.3.1 Sample Preparation

Blood samples (1 ml) were 'spiked' with a working standard mixture of benzodiazepine drugs (9 µg/ml).,was sonicated in a sonic bath for 15 minutes at room temperature. The samples were then diluted with 6ml of phosphate buffer (pH 6.0) and vortex mixed for 30 seconds. The buffered matrix was then

centrifuged at 5000×g for 10 minutes, and then the supernatant was used or further extraction..

3.4.1.3.2 Sample Extraction

Bond Elut Certify Columns of 1 ml capacity were used. They were conditioned with 2 ml of methanol, followed by 2 ml 0.1M phosphate buffer (pH 6.0) under light vacuum, approximately 2 PSI.

3.4.1.3.3 Addition of Sample

The prepared sample (3.4.1.2.2) was applied onto the column and drawn through completely at a flow rate of approximately 1.5 ml/min. The columns were washed with 1 ml deionised water. The columns were acidified by passing through 0.5 ml of 0.01M acetic acid. Then the columns were dried under full vacuum (15 PS.I) for 4 minutes, methanol (50 µl) was added and the columns were dried under full vacuum for 1 minute.

3.4.1.3.4 Elution of Sample

2 ml of 2% ammoniated ethylacetate was added to each column and eluted completely at a flow rate of 0.5 ml/min. The eluates were evaporated to dryness under a nitrogen stream and reconstituted in 100 µl mobile phase.

20 µl was injected into HPLC. Mobile phase is composed of 0.01 M Na_2HPO_4 (methanol 30 : 70 v/v).

3.4.1.4 Cationic Exchange Sorbent (SCX)

The SCX cartridges of 3 ml capacity were conditioned under vacuum with methanol (2 ml), water (1 ml) and 10 mM phosphoric acid (pH 3.4, 0.5 ml). The 'spiked' whole blood samples (9 µg/ml) with the four benzodiazepine drugs (Diazepam, Temazepam, Triazolam and Desmethyldiazepam) and 10 mM phosphoric acid (0.5) were mixed thoroughly and applied to the column. The column was air dried for approximately 30 seconds and then washed with 10 mM phosphoric acid (1 ml), 0.1 M acetic acid (0.5 ml) and methanol (1 ml) .

3.4.1.4.1 Elution of Sample

Ammoniacal methanol (3%) (2ml) was used for elution, it was passed through the column and collected.

The eluate was evaporated to dryness under nitrogen at 45°C and reconstituted in 50 µl of mobile phase, 10 µl of which was analysed by HPLC.

Mobile phase used was Na_2HPO_4 (0.01M) : methanol 30 : 70 v/v.

3.4.1.5 Bond Elut C_8 Sorbent

The method of choice for blood extraction of benzodiazepine drugs is the following. The method was chosen due to its high recovery and its reproducibility. Dry extraction of the blood 'spiked' with benzodiazepine drugs at the different storage conditions (5°C, 25°C and -20°C).

Two vials of the 15 ml aliquots of 'spiked' blood with different drugs and a vial of blank blood is taken out each month from its storage place (5°C, 25°C and -20°C), extracted and analysed for the variation in concentration of drugs at different conditions.

3.4.1.5.1 Extraction Buffer Preparation

The extraction buffer used was a mixture of acetonitrile : methanol : water in the following ratio - acetonitrile : methanol : water (30:35:35 v/v/v).

3.4.1.5.2 Internal Standard Preparation

The internal standard preparation (Prazepam) was prepared by dissolving 2 mg with a few drops of HPLC grade methanol and making up to 100 ml with deionised water, the resultant concentration being 20 µg/ml.

3.4.1.5.3 Sample Preparation

1. 1.0 ml of whole blood samples containing the four drugs (Diazepam, Desmethyldiazepam, Temazepam and Triazolam) at a concentration (9 µg/ml).
2. 0.1 ml of internal standard (Prazepam 20 µg/ml).
3. 1.0 ml of 0.01M disodium hydrogen phosphate (pH 10.4).
4. 1.0 ml of 2% ammonia solution (NH_3 35%).

The tubes were mixed well. Six samples of 'spiked' whole blood were extracted through a Bond Elut C_8 column of 3 ml capacity.

3.4.1.5.4 Extraction Procedure

Bond Elut C_8 cartridges (3 ml capacity) were attached to a Vac-Elut Box. Vacuum was applied using a water pump and the cartridges were conditioned by drawing the following solutions through:

- 3 x 1.0 ml of HPLC grade methanol,
- followed by 3 x 1.0 ml deionised water,
- followed by 3 x 1.0 ml 0.01M disodium dihydrogen orthophosphate pH 10.4]

3.4.1.5.5 Addition of Sample

The prepared sample was transferred to the preconditioned Bond Elut C8 column and was drawn through under vacuum (the vacuum pressure generated by the water pump was kept between 5-10 PSI and the cartridge was not allowed to dry out between applications. Then the column was washed twice with 1 ml of .01M Na_2HPO_4 (pH10.4) followed by 2 ml HPLC grade hexane. The blank sample prepared (3.4.1.4.3) was drawn through under vacuum (pressure 5-10 PSI), then the column was washed as above.

3.4.1.5.6 Elution of Sample

The sorbent was washed twice with 2 ml of .01 M Na_2HPO_4 (pH 10.4) and final elution of the drugs was performed with 1 × 5 ml chloroform.

3.4.1.5.7 Analysis of the sample

The extract was evaporated to dryness by packing the vial on a hot plate 60°C under a stream of nitrogen gas. The residue was redissolved in mobile phase (0.1ml) [3.4.1.1]. 20 µl was injected onto the HPLC.

3.4.1.6 Blood Extraction for Stability Study

Blood extraction of the 'spiked' blood with the four drugs (Diazepam, Desmethyldiazepam, Temazepam and Triazolam) was performed each month

at the three different temperatures (5, 25 and –20°C) by using a C_8 bonded phase column of 3 ml capacity.

Two vials of the 15 ml aliquots of the 'spiked' blood with the different drugs and a vial of blank blood was taken out each month from its storage place (5°C, 25°C and -20°C), extracted and analysed for the variation of drugs at different conditions.

3.4.1.6.1 Sample Preparation

1. 1.0 ml of each 'spiked' blood sample with the four benzodiazepines.
2. 0.1 ml of internal standard (Prazepam µg/ml).
3. 1.0 ml of 0.01M disodium hydrogen phosphate (pH 10.4).
4. 1.0 ml of 2% ammonia solution (NH_3 35%).

Then the tubes were mixed well. Twenty four samples of 'spiked' stored blood at the three different temperatures (5°C, 25°C and -20°C) were extracted through Bond Elut C_8 Columns of 3 ml capacity.

3.4.1.6.2 **Blank Preparation**

1. 1.0 ml of blank blood sample.
2. 1.0 ml of 0.01M disodium hydrogen phosphate (pH 10.4).
3. 1.0 ml of 2% ammonia solution (NH_3 35%).

The tube was mixed well. The three samples taken from the three different storage conditions were extracted through Bond Elut columns of 3 ml capacity.

Extraction, addition, elution and analysis of samples as described in 3.4.1.4.4., 3.4.1.4.5 ,3.4.1.4.6. and 3.4.1.4.7.

3.4.1.7 HPLC System for Benzodiazepine Analysis

The method used for Benzodiazepine drugs (Triazolam, Temazepam, Desmethyldiazepam) analysis was high performance liquid chromatography based on a Pye Unicam pump (Model 4015).This was used to deliver the mobile phase at a flow rate of 1 ml/min. Drugs were detected using a Pye 4025 variable wavelength ultraviolet detector set at 230 nm to monitor the column eluent.

The column used was 25 cm × 4.5 mm internal diameter (I.D) and guard column 5 cm × 4.6 mm (I.D). Both were pre-packed with Hypersil 5 μm

octadecylsilane C18, 20 μl extracts were introduced using a Rheodyne 7125 injection port with a 20 μl loop. The chromatograms were recorded on a BBC Goerz Metrawatt SE120 chart recorder operated at 1 cm/minute and 10mV full scale deflection.

3.4.1.8 Mobile Phase Preparations

Different preparations of mobile phases were tried for the analysis of benzodiazepines on HPLC system:

- 0.1 Na_2HPO_4:methanol (25:75 V/V), as described by reference 172. The pH of the mixture was 8.8.

- 0.1M Phosphate buffer was prepared by dissolving 0.53 grammes of disodium hydrogen phosphate hexahydrate and 8.93 grammes of potassium dihydrogen with a few millilitres of phosphate in water, the pH of the mixture was adjusted with orthophosphoric acid to pH5.4 then made up to 1 litre with deionised water.

The two mixtures tried were:

1. 38 : 62 V/V (Acetonitrile : phosphate buffer) pH .4.
2. 70 : 30 V/V (Acetonitrile : phosphate buffer) pH 5.4.

- 0.1M $Na_2HPO_4.2H_2O$, MW 177.99) Disodium hydrogenorto-phosphate dihydrate; the buffer was made up by dissolving 1.78 grammes of $Na_2HPO_4.2H_2O$ in deionised water. The resulting buffer concentration of 0.01M was adjusted with a few millilitres of 1N sodium hydroxide (NaOH) to pH 10.4, then it was made up to 1 litre with deionised water.

- Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4.2H_2O$, MW 177.99 was made up by dissolving 3.56 grammes of $Na_2HPO_4.2H_2O$ in deionised water, the resulting buffer concentration of 0.2M was adjusted with a few millilitres of orthophosphoric acid to pH 6 and was made up to 1 litre using deionised water, 80 ml of the latter was mixed with 100 ml of methanol and 8 ml of 1-propanol.

- The mobile phase of choice was a mixture of Acetonitrile: Methanol: Water, 30:35:35 (v/v/v).

3.4.1.9 Reproducibility of the HPLC System

A standard solution of 1 µg/ml of each of Diazepam, Desmethyldiazepam, Temazepam and Triazolam was prepared in mobile phase. The reproducibility of the HPLC was determined by injecting six

injections at each of the standards of Diazepam, Desmethyldiazepam, Triazolam and Temazepam (1 µg/ml), each day, over three successive days under the same conditions. Flow rate = 1.0 ml/min, Range (AVFS) = 0.32, Chart Speed 1 cm/min and a wavelength = 230 nm.

3.4.1.10 Reproducibility of the Extraction Method

Thirty samples of blood 'spiked' with the four benzodiazepine drug mixture (Triazolam, Temazepam, Desmethyldiazepam and Diazepam) in a concentration of 0.9 µg/ml of blood were extracted through Bond Elut C_8 columns, 3 ml capacity, and analysed over three successive days. The peak height ratio of drug against the internal standard Prazepam (20 µg/ml) was used to calculate the day-to-day variation of the extraction and analysis method of the four benzodiazepines using HPLC system.

3.4.1.11 Calibration Curves (Linearity)

The peak height ratio of each benzodiazepine drug was plotted against its concentration over a concentration range from low (0.1 µg/gramme), to subtherapeutic (0.2 µg/gramme), and to therapeutic (0.9-2.0 µg/gramme).

Standard mixtures were prepared with concentrations ranging from 0.01 µg to 2 µg/gramme to find the detection limit for each drug. The above

concentration ranges included the subtherapeutic, therapeutic, toxic and fatal levels.

3.4.2 TRICYCLIC ANTIDEPRESSANTS

Introduction

Different solid phase extraction methods have been tried to obtain optimum recoveries of the four drugs of interest (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from whole blood. Some of the methods tried needed a multistep extraction and others had short extraction steps. The best method of extraction that took only a few steps and gave good recoveries was used as the method of extraction for the stability study.

3.4.2.1 Solid Phase Extraction

3.4.2.1.1 C18 Bonded Phase Extraction

3.4.2.1.1.1 Protein Precipitation Methods

Removal of protein by precipitation is an effective method of sample preparation. The advantages of this technique are the speed at which the sample can be prepared and its simplicity. Different sample pre-treatment

procedures were used before extraction of the samples especially when using solid phase extraction methods.[95,98]

3.4.2.1.1.2 Sample preparation

1 ml of whole blood sample 'spiked' with the four tricyclic antidepressants (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) at a concentration of 100 µg/ml for the four drugs was mixed with 3 ml of methanol, vortexed for one minute and then centrifuged for five minutes at 1300 rpm. 1 ml of supernatant was mixed with 200 µl of the internal standard papaverine (0.5 mg/ml) diluted with 1 ml of distilled water and vortex mixed for 20 seconds.

3.4.2.1.1.3 Sample Extraction

The bonded phase columns used were C_{18} extraction columns with a 100 mg capacity and a 40 µm particle size.

The columns were conditioned firstly by washing with 3 ml of 600 µl of diethylamine in 100 ml of HPLC grade methanol under a vacuum of 254 mm Hg. 3 ml of a second conditioning buffer, which was prepared by dissolving 1 g of potassium bicarbonate in 100 ml of 10% acetonitrile in water, was applied to the column and allowed to drain under vacuum. The sample

prepared as in 3.4.2.1.1.2 was applied to the column and allowed to drain under a vacuum of 254 mm Hg. The columns were then washed three times with 1 ml of acetonitrile : water (20 : 80). After the last wash the columns were left under vacuum for two minutes.

3.4.2.1.1.4 Sample Elution

The elution reagent used was prepared by dissolving 600 µl of diethylamine in 100 ml of HPLC grade methanol.

2 x 300 µl portions of the above reagent were used to elute the drugs. The combined eluents were evaporated under a stream of nitrogen. The residue was reconstituted with 50 µl methanol. 1 µl was injected into the gas chromatography unit.

3.4.2.1.2 C_8 Bonded Phase Extraction

3.4.2.1.2.1 Sample pre-treatment

Whole blood samples 'spiked' with the four drugs, Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine, at 100 µg/ml for the four drugs was centrifuged at 3000 rpm for 10 minutes. 1 ml of the plasma obtained was aspirated for further sample preparation procedure.

3.4.2.1.2.2 Sample Preparation

The 1 ml supernatant obtained was prepared by mixing it with a 30 µl aliquot of 500 µg/ml solution of the Papaverine internal standard was added. This mixture was vortexed slightly before proceeding with the extraction.

3.4.2.1.2.3 Extraction Column Preparation

C_8 bonded phase extraction columns were prepared by sequential washing with 1 ml of methanol, 1 ml of distilled water, 1 ml of 0.25% sodium carbonate and, finally, with 1 ml of 0.2 M orthophosphoric acid. The columns were not allowed to dry between preparation and use..

3.4.2.1.2.4 Extraction of Samples

To the prepared plasma samples 50 µl of 3M orthophosphoric acid was added and the solutions were vortexed again briefly. Each sample was pipetted and added into the prepared extraction column. The column was then washed with 1 ml of water and then further washed with 1 ml of 0.25% sodium carbonate.

3.4.2.1.2.5 Elution of Samples

The analytes were eluted with 2 x 150 µl aliquots of methanol. After thoroughly mixing with two aliquots in clean vials. The samples were evaporated to dryness under nitrogen gas. The residue was reconstituted with 50 µl methanol and 1 µl was injected into the gas chromatography unit.

3.4.2.2 Liquid-Liquid Extraction Methods

In order to have a screening procedure by gas chromatography that was simple, quick, comprehensive and required only a small amount of sample, different liquid-liquid extraction procedures were tried before deciding which was the best extraction procedure for the stability study. Some of these procedures needed a multi-step extraction and others needed only a one-step extraction.

3.4.2.2.1 Sample Extraction Using 1-Chlorbutane

A 2 ml sample of blood 'spiked' with each of the four drugs of interest (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) at a concentration of 100 µg/ml was placed in a 10 ml culture tube to which was added 4 ml 1-chlorobutane. Each sample was extracted for five minutes on a rotator. After centrifugation, 4 ml of the upper 1-chlorobutane layer was

transferred to another culture tube to which was added 2 ml (1.0N) HCl. The sample was extracted for five minutes, as before, and, after centrifugation, the upper 1-chlorobutane layer was removed with a Pasteur pipette attached to an aspirator and discarded. To remove any 1-chlorobutane still present the remaining 1.0N HCl was aerated for 1 minute by bubbling air through a Pasteur pipette into the sample. HCl was transferred to a conical centrifuge tube and, after addition of 20 µl concentrated NH_40H, was extracted on a vortex mixer for one minute. After centrifugation for 2 minutes, the aqueous layer was discarded and a 3 µl of the organic layer was injected into the gas chromatography unit.

3.4.2.2.2 One Step Extraction Method Using n-butyl Acetate

Another liquid-liquid extraction method was tried in order to choose the best extraction method with good recoveries. The method tried used 13 x 100 mm screw cap tubes, 1.0 ml of patient whole blood 'spiked' with the four drugs (Amitriptyline, Imipramine, Nortriptyline and Chlorpromazine) at a concentration 100 µg/ml which was combined with 1.0 ml of pH 11.0 bicarbonate buffer and 300µl of n-butyl acetate. The tubes were capped and rotated for ten minutes. Next, the tubes were centrifuged at 3000 rpm for at least five minutes. After centrifugation, 100 µl at the upper organic layer was transferred to 6 x 50 mm tubes. Up to 2.0 µl of the solvent was injected into the gas chromatography system.

3.4.2.2.3 Liquid-Liquid Extraction with Hexane : Butan-1-ol

Blood samples, 'spiked' with the four drugs (Amitriptyline, Imipramine, Nortriptyline and Chlorpromazine) at a concentration of 100 µg/ml, were used. 1 ml of deionised water was added and the tubes were vortex mixed before addition of 1 ml 0.2M Na_2CO_3. Tubes were again vortex mixed and 5 ml of hexane : butan-1-ol (95:5, v/v) were added and the tubes gently agitated for thirty minutes. The samples were then centrifuged at 2500g for five minutes. The organic layer was transferred to a clean set of extraction tubes containing 100µl of 0.2% phosphoric acid. These were gently agitated for thirty minutes and then centrifuged for five minutes. The organic layer was aspirated and the aqueous layer was discarded, 30 µl of the organic layer was injected into the gas chromatographic system.

3.4.2.2.4 Multistep Extraction Using Isoamyl Alcohol in n-Hexane

In separate 16 - x 125 - mm. tubes, 1 ml of blank blood 'spiked' with the four tricyclic drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine at a concentration of 100µg/ml) was combined with 200 µl of 1M Na_2CO_3, 200 µl of the internal standard papaverine (500 µg/ml) and 5 ml of 2% (v/v) isoamyl alcohol in n-hexane. The tubes were vortexed for thirty seconds and centrifuged at 3000 rpm for five minutes. The upper solvent layers were then transferred, using Pasteur pipettes, to clean screw-capped glass vials,

and 0.5 ml of 0.5M HCl was added to each vial. The vials were vortexed for thirty seconds and centrifuged at intermediate speed for five minutes. The upper solvent layers were aspirated to waste and the aqueous layers were alkalinized by adding 0.5 ml of 1M Na_2CO_3 to each vial. The final extraction was accomplished by injecting 50µl of 10% (v/v) isoamyl in chloroform into each vial. The vials were vortexed for five minutes at an intermediate speed.

The aqueous layer was discarded and an aliquot of the organic layer (30 µl) was injected into the gas chromatographic system.

3.4.2.2.5 Single Extraction Procedure With Diethyl Ether

Blood (1.0 ml) 'spiked' with the four drugs (Amitriptyline, Imipramine, Nortriptyline and Chlorpromazine all at a concentration of 100 µg/ml) was mixed with 0.1M sodium hydroxide (0.5 ml) in a glass centrifuge tube. After addition of diethyl ether (3 ml) the tube contents were vortex mixed then centrifuged for five minutes at 3000 rpm. The organic phase was transferred into a second centrifuge tube containing 0.025M hydrochloric acid (0.2 ml). This mixture was vortex mixed then centrifuged. The upper organic layer was aspirated and evaporated to dryness, while the aqueous layer was discarded. The residue was reconstituted with 50 µl methanol containing the internal standard Papverine (500 µg/ml), 1 µl was injected into the gas chromatography unit.

3.4.2.2.6 Extraction Using the Extracting Solvent Hexane:Isoamyl Alcohol

1 ml of blank blood 'spiked' with the four drugs of choice (Amitriptyline, Imipramine, Nortriptyline and Chlorpromazine all at a concentration or 100 µg/ml) was mixed with 0.1M sodium hydroxide Na_4OH (0.2 ml) in a glass centrifuge tube. Two millilitres of hexane : isoamyl alcohol (99 : 1 v/v) was added and the sample was vortexed and centrifuged for 5 minutes at 4000 rpm. After centrifugation, the supernatant was transferred into a 3ml vial and then the solvent was evaporated under a stream of nitrogen at room temperature. The residue was reconstituted with 50 µl methanol containing the internal standard papaverine (500 µg/ml) and 1 µl was injected into the gas chromatography unit.

3.4.2.2.7 Extraction With Ethylacetate

2 ml of 'spiked' blood with each of the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine at a concentration of 100 µg/ml), 2 ml ethylacetate and 50 µl of 10 M potassium hydroxide (KOH) were added to the 'spiked' blood sample. The tube containing the mixture was sealed, mixed on a rock-and-roll machine for ten minutes and centrifuged at 3000 rpm. The supernatant (1 ml of the top acetate layer) was transferred to a clean 3 ml vial, and was evaporated to dryness under nitrogen. The residue

was reconstituted with methanol containing the internal standard Papaverine at a concentration of 0.5 mg/ml. 1 µl was injected into the GC system.

3.4.2.2.8 Reproducibility of the GC System:

A standard solution of 3 µg/ml of each of Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine was prepared in methanol. The reproducibility of the GC system was determined by injecting five injections of each of the standards each day, over three successive days, under the same conditions, to determine the day-to-day variation in retention times of the four drugs. The initial temperature was 120°C. The final temperature was 290°C. The initial time was two minutes. The final time was twelve minutes. He oven rise was 8°C/minute. The injector temperature was 290°C. The detector temperature was 290°C.

3.4.2.2.9 Minimum Detectability:

Standard solutions of 1 µg/ml of the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) was made up in methanol. A serial dilution of the four standard drug solutions in methanol was used to determine the minimum detectability of the four drugs on the GC system.

3.4.2.2.10 Reproducibility of the Extraction Method:

Thirty samples of human post-mortem blood 'spiked' with the four tricyclic antidepressant drugs mixture (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine in a concentration of 2 µg/ml) were liquid-liquid extracted and analysed over three successive days. The peak height ratio of each drug against the internal standard (papaverine 500 µg/ml) was used to calculate the day-to-day variation of the extraction method of the four tricyclic antidepressants using the GC system.

3.4.2.2.11 Calibration Curve

The calibration curves for the four tricyclic drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) were constructed over the concentration range 0.5-10 µ/ml prepared in blank human post-mortem blood. Six samples at each concentration for each drug was liquid-liquid extracted as the method described earlier in 3.4.2.3.7. The samples were reconstituted with 50 µl methanol containing the internal standard papaverine (50 µg/ml). 1 µl was injected into the GC system. The peak height ratios of drug over internal standard were plotted against concentration to construct a calibration curve for each tricyclic antidepressant drug.

3.4.2.3 Method of Extraction used in Stability Study

3.4.2.3.1 Introduction

The method of extraction that was chosen after doing the studies to find out which method of extraction with good recoveries for the four drugs of choice for the stability study was the best. The best method was the liquid-liquid extraction method using ethyl acetate and KOH, as explained previously.

3.4.2.3.2 Materials

3.4.2.3.2.1 Chemical and Reagents

The chemicals and reagents were HPLC grade methanol. Ethyl acetate was used in extraction and 10 M KOH which was prepared by dissolving 56g of KOH in 100 ml distilled water.

3.4.2.3.2.2 Internal Standard Solution Preparation

The internal standard solution (papaverine) was prepared by dissolving it in a few drops of HPLC grade methanol until the drug is dissolved and making up to 100 ml with HPLC grade methanol, the resulting concentration being 500 µg/ml.

3.4.2.3.3 Drugs Extraction

Two vials of each of the blood spiked samples (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) and blank was removed from its storage place, at each storage temperature (5, 25, –20° C), this was carried out each month between each analysis. The extraction was done by adding the samples each month at the three storage temperature, and extracted as in section 3.4.2.2.7.

3.4.2.3.4 Drugs Analysis

1 µl of reconstituted sample was injected into the gas chromatography unit. The GC conditions and the size of column used is described below.

3.4.2.3.5 Equipment

The separation of derivatized Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine for all the analyses performed was achieved using Chrompack/Packard model 438A GC fitted with a capillary column (Chrompack CP-sil 5, 25m - x - 0.32 mm internal diameter with 0.4 um film thickness). The injector temperature was 290°C. The initial oven temperature was 120°C. This was maintained for 2 minutes and then increased at

8°C/minute to 290°C. The final temperature was maintained for 12 minutes. The detectors were FID/NPD detectors.

Gas Flow Rate on the GC

Helium was used as a carrier gas. The linear velocity was approximately 60 cm/sec through the column, achieved by maintaining a column head pressure at 5psi.

CHAPTER 4 - RESULTS AND DISCUSSION

4.1 'SPIKED' SAMPLES, STORAGE, EXTRACTION AND ANALYSIS PROCEDURES

The 'spiked' blood samples with the drugs under study was prepared at a concentration level consistent with toxicity in patients since most of the forensic toxicological cases involve measuring the drugs in samples following overdose. Therefore, when investigating the stability of the drugs of forensic interest in post-mortem blood,[33,37-40] it is of importance to the toxicologist interpreting results that the study should be in accordance with the actual case.

The storage temperatures were chosen to reflect laboratory practice. The 5°C refrigeration represent the first storage temperature for samples received from the mortuary until a full drug screening is completed, usually days. Sometimes, as a result of further enquiries, weeks may pass between acquisition and drug quantitation. Then the samples must be kept at -20°C prior to analysis. The 25°C represents the realistic temperature to which the corpse may have been exposed for a few days after death and as an upper limit for room temperature exposure. Therefore, it is important and necessary at the same time to ensure that the drugs are sufficiently stable at different storage temperatures and time intervals. The blood solutions of drugs were kept under the same storage conditions and then analysed at the

designated time. The concentrations of each drug in blood solution was analysed at the designated time. Freshly prepared drug standard samples were used to calculate the concentration of the drug in 'spiked' samples. In calculating the recovered drug the initial quantitation (day zero) was assigned a value of 100%. Successive quantitations were made and '% of original present' was calculated. The decrease in drugs concentration with time was obtained from the linear regression curve constructed between concentrations (x) and the storage times (y). The linear regression was established for all drugs under study and at the three storage temperatures for blood samples. Figure 14 shows the procedure of storage, extraction and analysis of the specimen for drug stability.

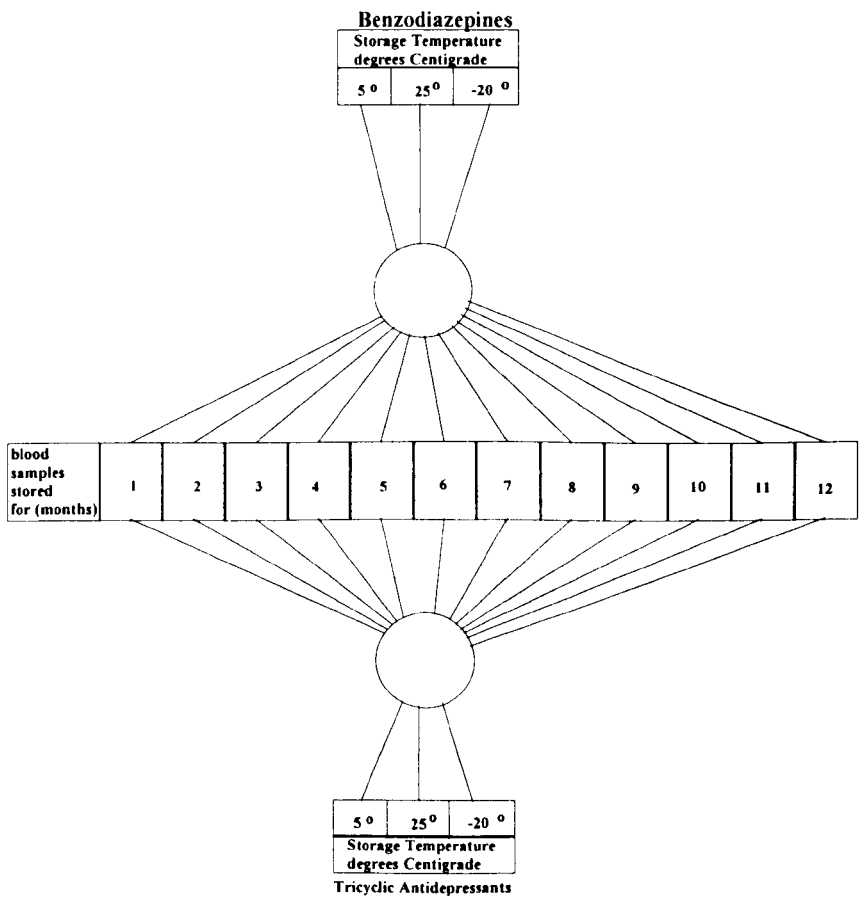


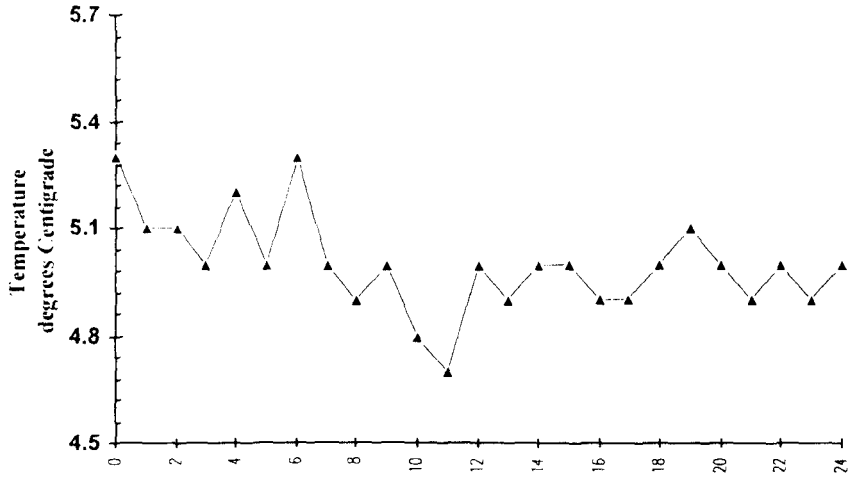
Figure 14: **The 'spiked' samples, storage, extraction and analysis procedure.**

4.1.1 TEMPERATURE MONITORING OF 'SPIKED' SAMPLES

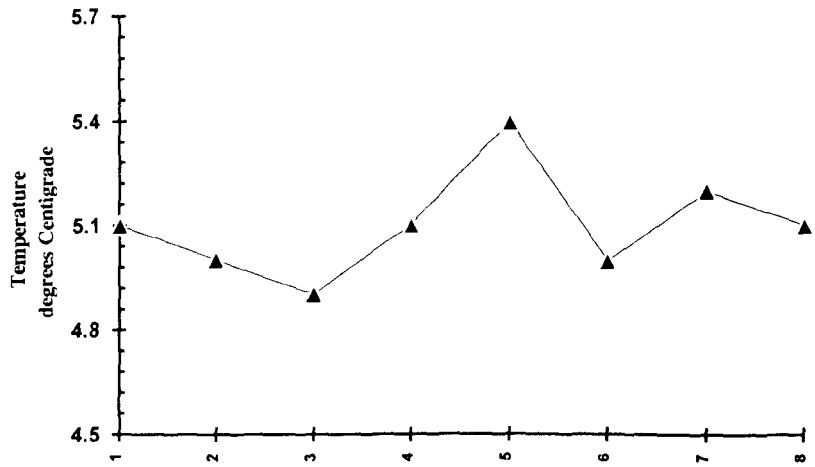
Sample of whole blood were 'spiked' with benzodiazepines and tricyclic antidepressants and stored in a refrigerator (5°C), and incubator (25°C) and a deep freeze (-20°C). Storage temperatures were monitored regularly over 24 hours throughout the course of the experiment which started after one month from spiking date (day zero) and ended at twelve months for both benzodiazepines and tricyclic antidepressants.

Results and Discussion

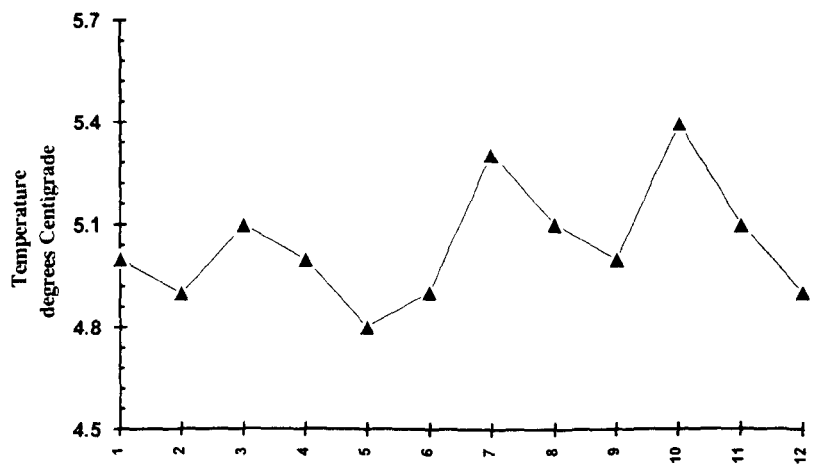
Figures 15, 16 and 17 demonstrate the fluctuation of the set storage temperatures 5, 25, -20°C monitored over different time intervals (hours, days, months). The average of each storage temperature over the different time intervals and the variation in temperatures over the different time intervals and the variation in temperature are summarised in Table 1. It shows there was a ± 2.6% variation at 5°C, a ± 1.1% variation at 25°C and a ± 7% variation at -20°C over a 24 hours period. Over 7 days the fluctuation was a ± 2.8% at 5°C, a ± 1.4% variation at 25°C and a ± 2.5% variation at -20°C. Over the 12 months there was a ± 3.4% variation at 5°C, a ± 1.1% variation at 25°C and a ± 5.5% variation at -20°C. The measured temperatures are not significantly different from the set temperature at any given time throughout the course of the experiment.



(a) Time (hours)

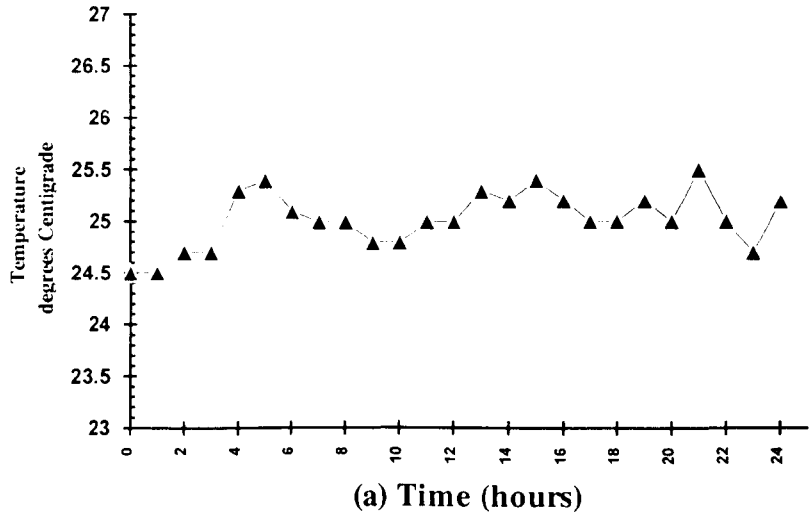


(b) Time (days)

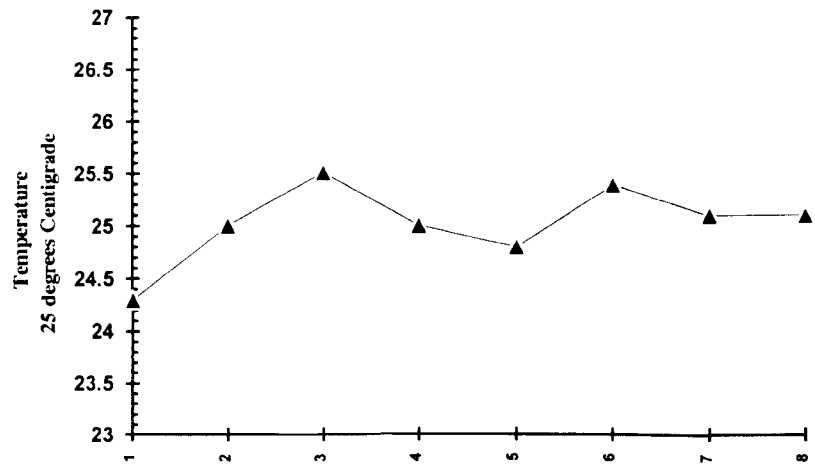


(c) Time (months)

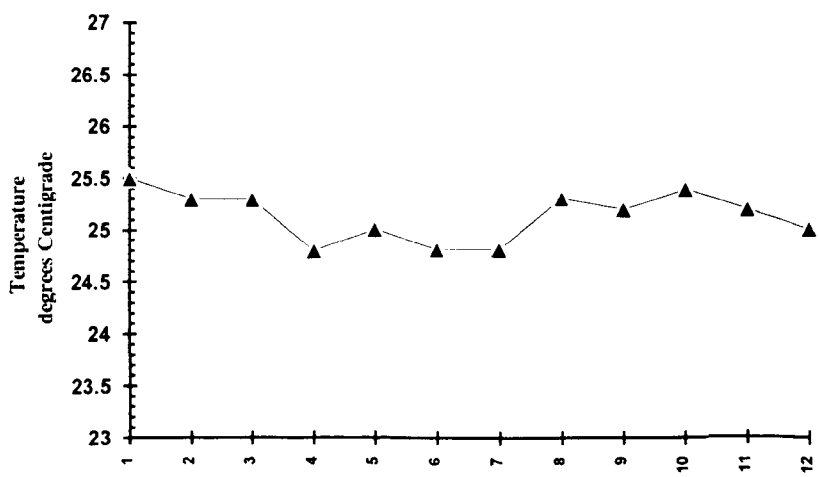
Figure 15: Fluctuation of Storage Temperature 5°C Monitored Over: (a) Twenty-Four hours; (b) Seven days; (c) Twelve months.



(a) Time (hours)



(b) Time (days)



(c) Time (months)

Figure 16:Fluctuation of Storage Temperature 25°C Monitored Over:
(a) Twenty-Four hours; (b) Seven days; (c) Twelve months.

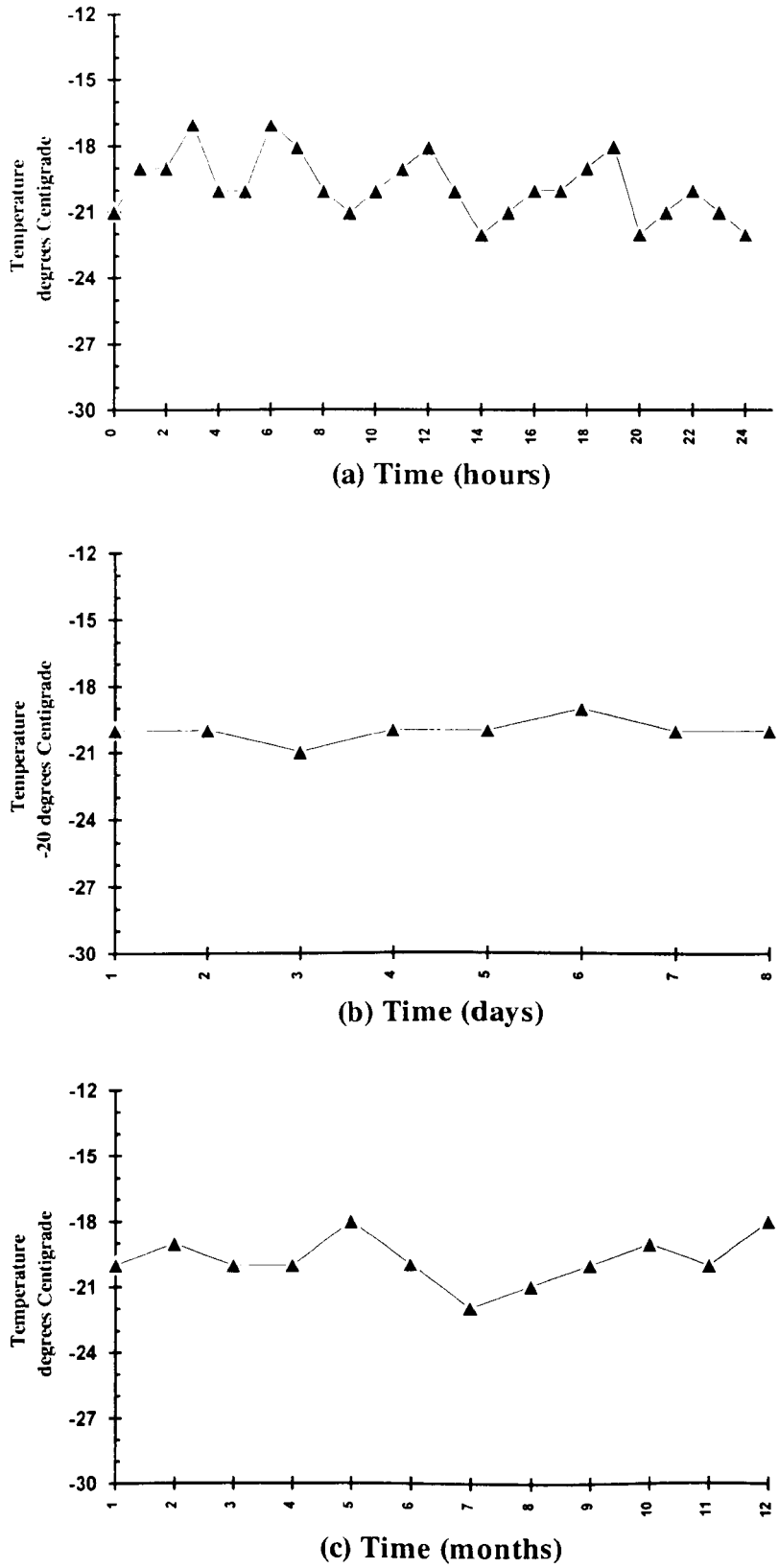


Figure 17: Fluctuation of Storage Temperature -20°C Monitored Over: (a) Twenty-Four hours; (b) Seven days; (c) Twelve months.

Table 1: The Average Measured Temperature and Standard Deviation of the Three Storage Conditions Monitored Over Different Time Intervals (24 hours, 7 days, 12 months).

Monitoring Intervals	Refrigeration Temperature set at 5°C	Incubator Temperature set at 25°C	Deep Freeze Temperature set at -20°C
24 hours	5.2 ± 0.13	26.1 ± 0.27	-20 ± 1.41
7 days	5.1 ± 0.14	25.0 ± 0.35	-20 ± 0.5
12 months	5.0 ± 0.17	25.1 ± 0.28	-19.8 ± 1.1

Conclusions

The temperature fluctuation of the three storage conditions are shown to be minimal and not significant, and within the normal temperature fluctuation range of refrigerators, incubators and freezers, with not more than 0.5°C differences between set temperatures and actual temperatures of the three storage media.

4.2 RESULTS AND DISCUSSION OF SORBENT EXTRACTION

4.2.1 CYANOPROPYL (CN) SORBENTS

The absolute recoveries of the four benzodiazepine drugs (Triazolam, Temazepam, Desmethyldiazepam and Diazepam) from 'spiked' deionised water using the sorbent cyanopropyl (CN) were calculated by comparing the peak height ratio of the extracts from deionised water with unextracted standard solutions prepared in mobile phase at the same concentration and under identical conditions.

Since cyanopropyl sorbent is a derivatised short propyl hydrocarbon chain placed near the surface of the silica, it will give the opportunity for the analyte to interact with the underlying silica through hydrogen bonding. In the case of benzodiazepines (weak bases) they will interact with the silica. Hydrogen bonding was the greatest in Temazepam and the lowest in Desmethyldiazepam. Elution of benzodiazepines from the cyanopropyl sorbent was facilitated with polar solvent that can disrupt the hydrogen bonding between functional groups on the analyte and the sorbent surface. Acidic methanol was more polar than 100% methanol which eluted the drug better.

Table 2 lists a recovery of above 88% for all benzodiazepine drugs using the acidic methanol eluent and low recoveries using the 100% methanol (less than 74%).

Table 2: **Percentage Recoveries of Benzodiazepine Drugs (0.9 µg/ml) from 'spiked' Water using Cyanopropyl Sorbents (n = 4).**

	Diazepam	Temazepam	Triazolam	Desmethyl-Diazepam
Acidic Eluent	91 ± 5.2%	99 ± 2.2%	95 ± 3.1%	90 ± 3.8%
Basic Eluent	69 ± 2%	71 ± 2.9%	51 ± 2.8%	74 ± 4.%

Column: Hypersil 5 µm C_{18} Cartridge (25 cm x 4.6 mm i.d.)
Mobile Phase: 0.01M disodium hydrogen orthophosphate (pH 8.8):Methanol(30:70)
Detection Wavelength: 230nm Flow Rate: 1 ml/minute

Extracting whole post-mortem blood samples with benzodiazepine drugs using Bond Elut CN columns of 6 ml capacity using the same eluting solvent as for 'spiked' deionised water samples, gave low recoveries and sample to sample variation.

4.2.2 BOND ELUT CERTIFY SORBENT

The absolute recoveries of the four drugs (Diazepam, Temazepam, Triazolam and Desmethyldiazepam) from 'spiked' whole blood samples were calculated by comparing the peak height ratio of the extracts from whole blood with unextracted standard solutions prepared in mobile phase at the same concentration and under identical conditions.

The results of this study indicate that the developed procedure is an effective extraction method but it depends on the physical properties of the drugs and the eluting solvent used. Those drugs that have pKa close to the pH of the eluting system will give good recoveries. Clean extracts from whole blood were obtained.

Table 3 shows the results of the four benzodiazepines obtained from 'spiked' whole blood samples.

Table 3: **Recoveries of the Four Benzodiazepine Drugs Extracted from 'spiked' Whole Blood at a Concentration of 9 µg/ml using Bond Elut Certify Sorbent (n = 5).**

Drug	Recovery (%)
Diazepam	30.2%
Desmethyldiazepam	39.6%
Temazepam	74.2%
Triazolam	78.3%

Column: Hypersil 5 µm C_{18} Cartridge (25 cm x 4.6 mm i.d.)
Mobile Phase: 0.01M disodium hydrogen orthophosphate (pH 8.8):Methanol(30:70)
Detection Wavelength: 230nm Flow Rate: 1 ml/minute

4.2.3 STRONG CATION EXCHANGE (SCX) SORBENT

Recoveries of the four benzodiazepine drugs (Diazepam, Desmethyldiazepam, Temazepam and Triazolam) were determined in 'spiked' whole blood samples by comparison of peak heights with unextracted standards and were determined over a concentration of 9 µg/ml.

The method of sorption is a mixed mode sorption isolation of benzodiazepines using a combination of reversed-phase C_8 and cation exchange using a benzesulfonic acid cation exchange resin in hydrogen form. Applying the sample mixed with sodium phosphate buffer at pH 8.8, converts the resin into sodium form, and the pH 8.8 ionises the carboxyl group of benzodiazepines, thus, the drugs are sorbed in their ionic form by reversed-phase, with sodium ion acting as a counter ion. Applying acetic acid displaces the cations present on cation exchange resin and converts the carboxyl anion to carboxylic acid, eluting the drugs with 2% ammoniated ethylacetate. 2% ammonium removes any protonated group and unlocks the ion exchange step of sorption and the ethylacetate elutes the drugs.

The study was made of the absolute drug recoveries obtained by analysis after a concentration (evaporation and reconstitution) step. The recoveries for the four drugs were relatively good.

The technique has advantages over liquid phase ion paired extraction in that it allows the removal of extraneous sample material by washing the support with an ionic solvent (phosphoric acid, acetic acid) to remove ionic material while the drugs are retained by nonpolar interaction with the benzene - solufonyl-proply moiety. The final eluent (3% ammoniacal methanol) disrupts both ion unpaired and non-polar interactions and allows elution of all the drugs

in a relatively small volume. This washing results in the production of a cleaner extract.

Table 4 shows the results of the extraction of four benzodiazepine drugs from 'spiked' blood sample using SCX columns.

Table 4: **Absolute Recoveries (n = 4) of the Four Drugs Extracted from Whole Blood at a Concentration of 9 µg/ml.**

Drug	Absolute Recovery (%)
Diazepam	85.8%
Desmethyldiazepam	88.6%
Temazepam	86.9%
Triazolam	84.1%

Column: Hypersil 5 µm C_{18} Cartridge (25 cm x 4.6 mm i.d.)
Mobile Phase: 0.01M disodium hydrogen orthophosphate (pH 8.8):Methanol(30:70)
Detection Wavelength: 230nm Flow Rate: 1 ml/minute

Mobile Phase for HPLC Use for Stability Study

In reversed phase chromatography the mobile phases used are based on water mixed with different proportions of organic solvents, different proportions of mobile phases were tried for the analysis of benzodiazepines on HPLC system. The results are shown in Table 5.

Table 5: Comparison of the Results of Different Mobile Phase Preparations

Drugs	K′ (Capacity by Factor)	Mobile Phase	Solvent Ratios
1 2 3 4	5.43 4.93 3.92 2.42	0.1M Na_2HPO_4: Methanol pH 8.8	**25:75 (v/v)**
1 2 3 4	5.63 4.75 4.21 2.60	0.1M mixture of 2 phosphate buffer pH5.4 (disodium hydrogen phosphate hexahydrate and potassium dihydrogenphosphate)	**(0.53g+8.939) in 1 litre water**
	(a) (b)	Acetonitrile:phosphate buffer pH5.4	**a. 38:62 (v/v)** **b. 70:30 (v/v)**
1 2 3 4	6.32 5.43 3.42 4.92 3.75 3.62 2.34 2.28		
1 2 3 4	8.42 7.63 4.9 3.4	0.1M $Na_2HPO_4.2H_2O$ pH10.4	
1 2 3 4	3.29 2.75 1.83 0.73	$Na_2HPO_4.2H_2O$: Methanol:1-propanol	**80:100:8(v/v/v)**
1 2 3 4	9.47 8.62 6.75 5.38	Acetonitrile:Methanol: water	**30:35:35(v/v/v)**

1 = Diazepam: 2 = Desmethyldiazepam: 3 = Temazepam: 4 = Triazolam

4.2.4 BOND ELUT C_8 SORBENT

4.2.4.1 Recoveries

The absolute recoveries of the four benzodiazepine drugs (Diazepam, Temazepam, Desmethyldiazepam and Triazolam) using Bond Elut C_8 columns

of 3 ml capacity, containing C_8 packing material were calculated by comparing peak height ratios of the extracted samples with unextracted standards prepared in mobile phase at the same concentration.

The absolute recoveries of the four benzodiazepine drugs are shown in Tables 6, 7, 8 and 9.

The recoveries for the four benzodiazepine drugs were considerably higher since benzodiazepine drugs are weakly basic compounds and they have the affinity to interact hydrophobically to the long C_8 chain. The interaction was disrupted using chloroform as the eluting solvent.

Table 6: **The Recovery of Triazolam from 'spiked' Blood Extracted Through a C_8 Column and analysed by HPLC**

Concentration (µg/ml)	Recovery (%)	Number of Samples
0.05	98	6
0.2	96	6
0.9	84	6
2.0	92	6

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

Table 7: **The Recovery of Temazepam from 'spiked' Blood Extracted Through a C_8 Column and Analysed by HPLC**

Concentration (µg/ml)	Recovery (%)	Number of Samples
0.05	84	6
0.2	96	6
0.9	86	6
2.0	90	6

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

Table 8: **The Recovery of Desmethyldiazepam from 'spiked' Blood Extracted Through a C_8 Column and Analysed by HPLC**

Concentration (µg/ml)	Recovery (%)	Number of Samples
0.05	84	6
0.2	98	6
0.9	93	6
2.0	94	6

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

Table 9: **The Recovery of Diazepam from 'spiked' Blood Extracted through a C8 Column and analysed by HPLC**

Concentration (µg/ml)	Recovery (%)	Number of Samples
0.05	94	6
0.2	97	6
0.9	94	6
2.0	92	6

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

4.2.5 CALIBRATION CURVES

The calibration curves for the four benzodiazepine drugs (Triazolam, Temazepam, Desmethyldiazepam and Diazepam) were drawn over the concentration range 0.05 – 2 μg/ml prepared in blank blood. Six samples of each concentration for each drug were extracted through Bond Elut C_8 columns. The internal standard (Prazepam) was added to all samples. The peak height ratios of drug over internal standard at each concentration was plotted to construct a calibration curve for each benzodiazepine drug. A standard calibration curve was constructed using the peak height ratios (std/I.std) and the benzodiazepine drug concentrations. The relation between benzodiazepine drugs (Diazepam, Desmethyldiazepam, Temazepam, Triazolam) concentration and average peak height ratio (std I.std) of samples analysed by HPLC are highlighted in Table 10.

Table 10 Statistical Data for the Four Calibration Curves of Benzodiazepines

Drug	Concentration (µg/ml)	Average Peak Height Ratio n = 6
Diazepam	0.05	0.02
	0.2	0.25
	0.9	1.3
	2.0	2.4
Desmethyldiazepam	0.05	0.028
	0.2	0.29
	0.9	1.2
	2.0	2.4
Temazepam	0.05	0.048
	0.2	0.24
	0.9	1.2
	2.0	2.1
Triazolam	0.05	0.04
	0.2	0.23
	0.9	1.33
	2.0	2.8

Column: Hypersil 5 µm C_{18} Cartridge (25 cm x 4.6 mm i.d.)
Mobile Phase: 0.01M disodium hydrogen orthophosphate (pH 8.8):Methanol(30:70)
Detection Wavelength: 230nm Flow Rate: 1 ml/minute

The equations of the lines are:

$y = 1.19 x + 0.04$, $y = 1.17 x + 0.04$, $y = 1.04 x + 0.07$, $y = 1.40 x + 0.02$

for the Diazepam, Desmethyldiazepam, Temazepam and Triazolam, respectively, and the regressions are:

0.9966, 0.9977, 0.9988 and 0.9999 for Diazepam, Desmethyldiazepam, Temazepam and Triazolam, respectively.

A linear relation was obtained for each drug as shown in Figures 18, 19, 20 and 21. A 50 µg/ml of benzodiazepine drug extracted solution chromatogram at range 0.02 is shown in Figure 22.

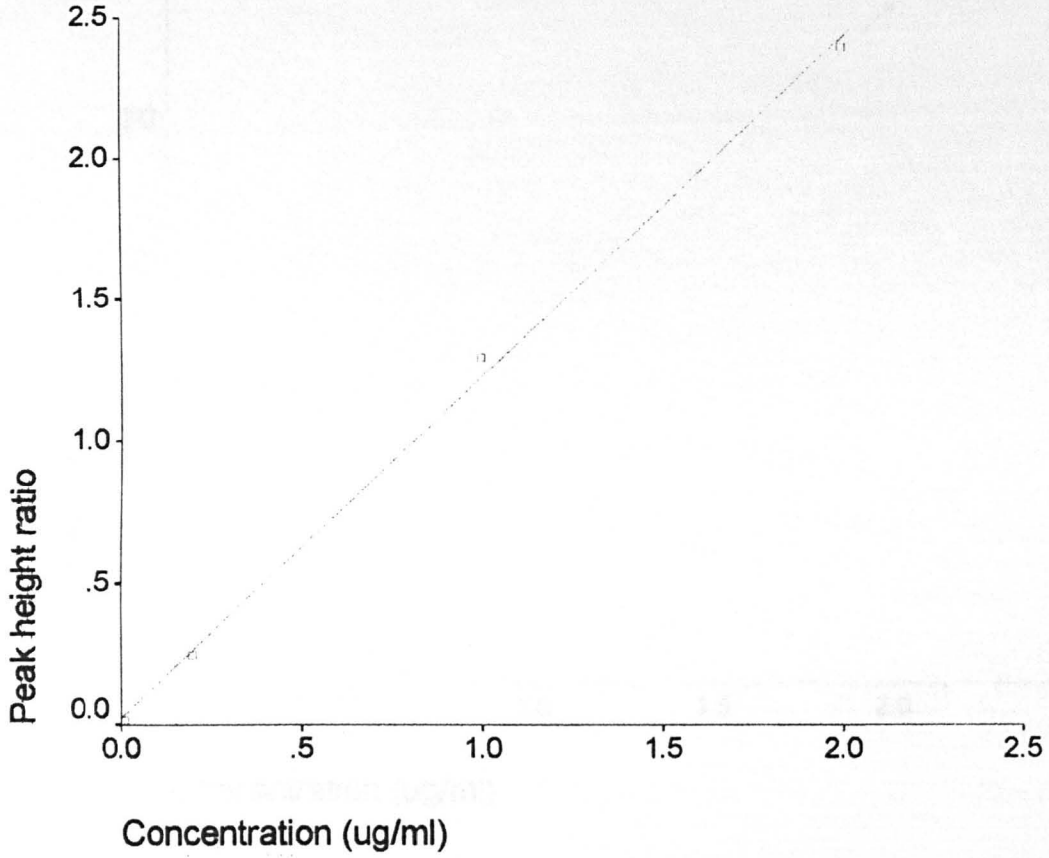


Figure 18: Calibration Curve of Diazepam

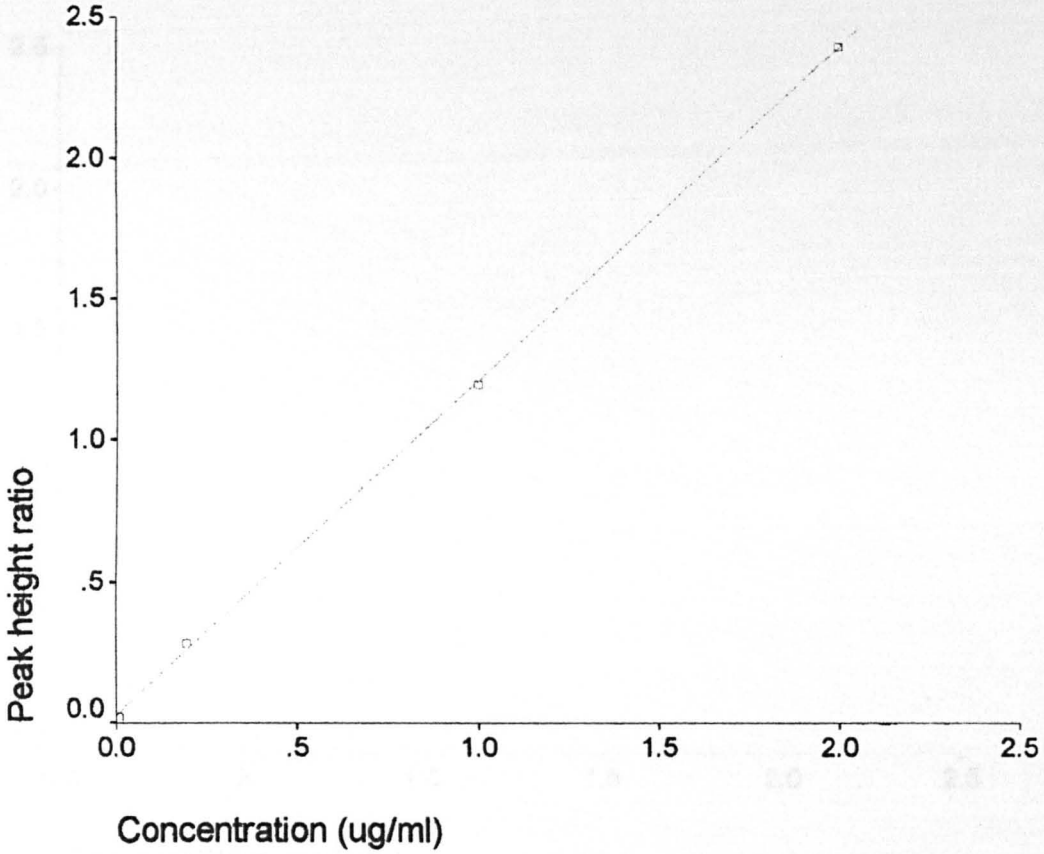


Figure 19: **Calibration Curve of Deamethyldiazepam**

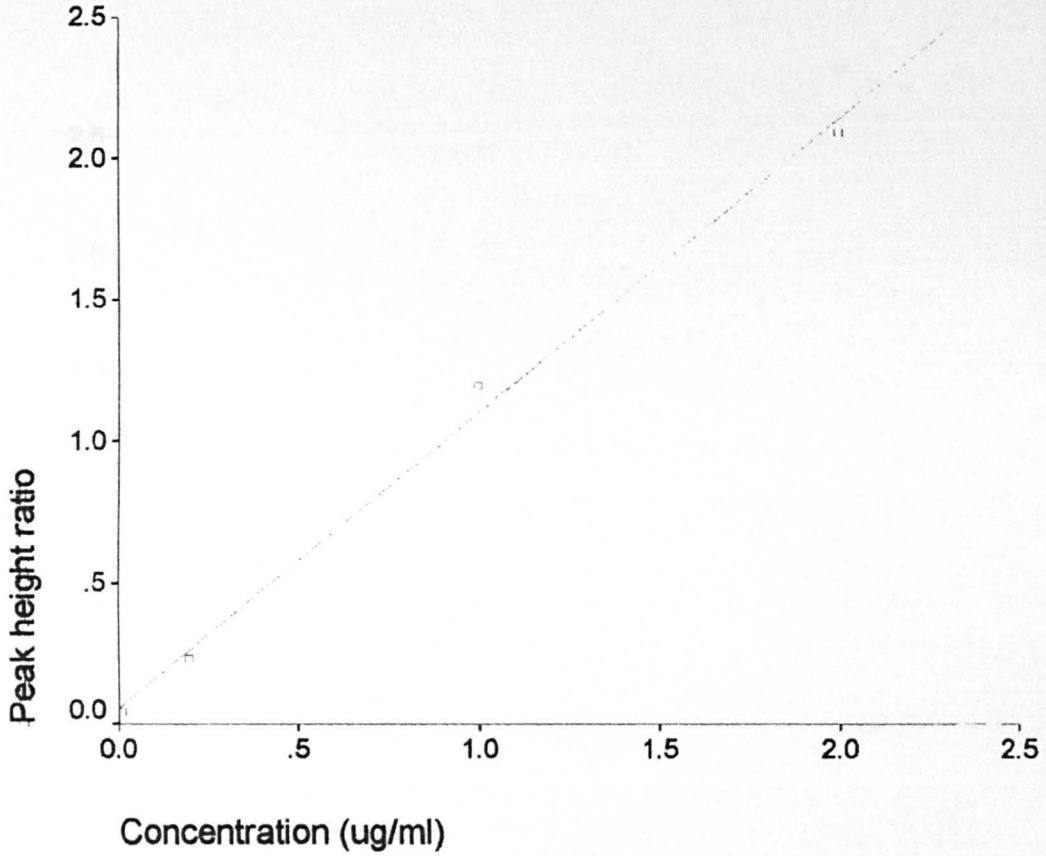


Figure 20: **Calibration Curve of Temazepam**

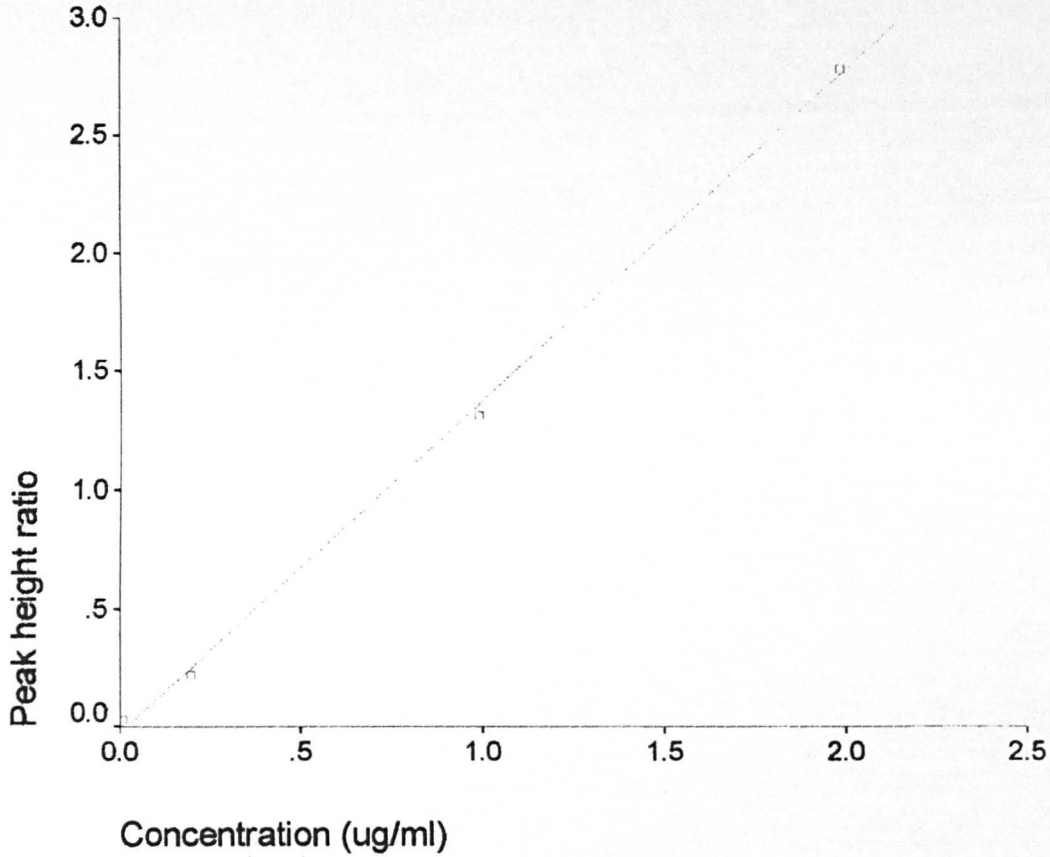


Figure 21 Calibration Curve of Triazolam

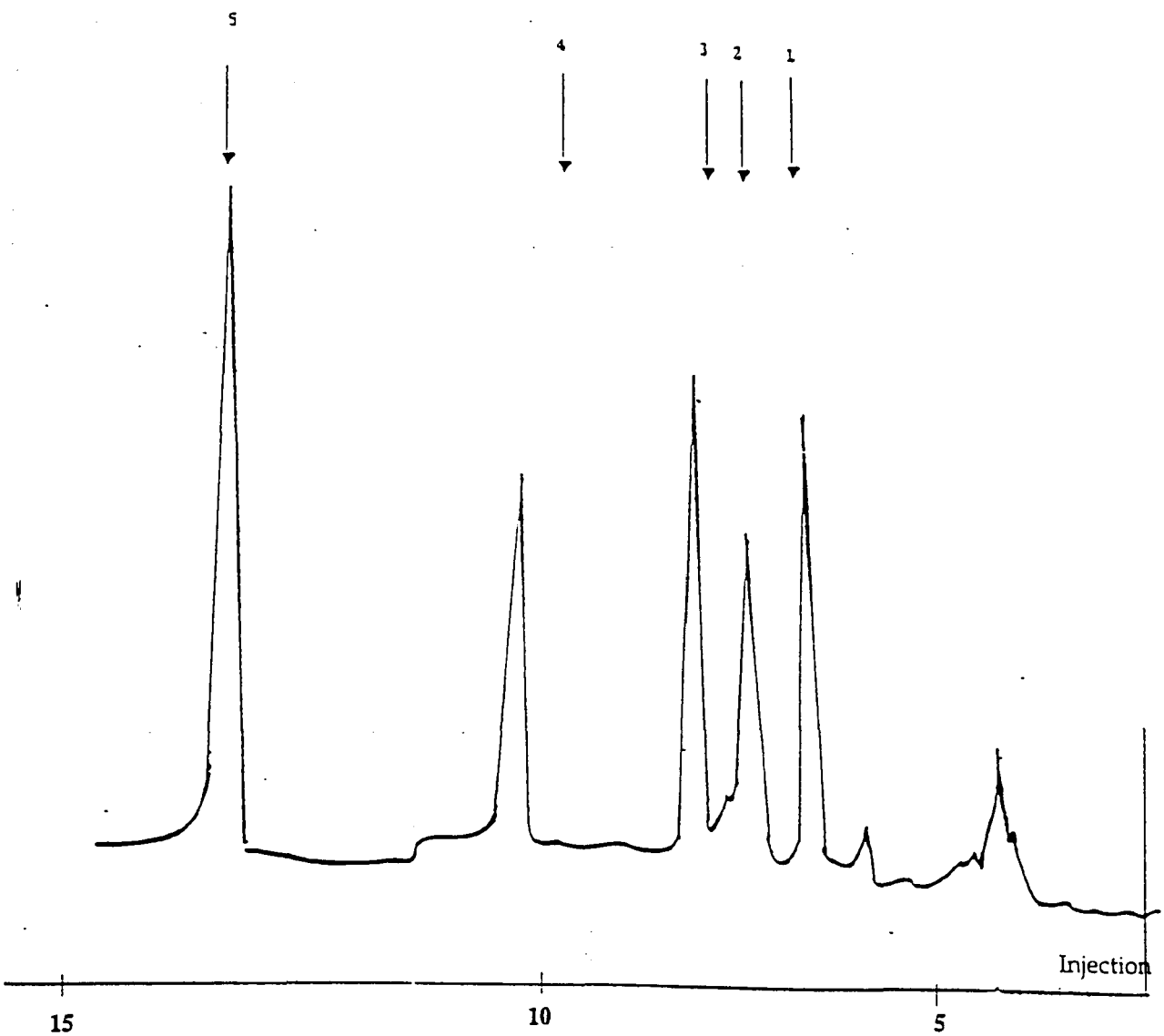


Figure 22: Separation of Benzodiazepine Drugs from Spiked Whole Blood Sample (50 μg) by HPLC System

1. Triazolam
2. Temazepam
3. Desmethyldiazepam
4. Diazepam
5. Prazepam

4.2.6 THE REPRODUCIBILITY OF THE EXTRACTION METHOD

Thirty samples of human blood 'spiked' with the four benzodiazepine drugs mixture (Triazolam, Temazepam, Desmethyldiazepam and Diazepam) in a concentration of 0.9 µg/ml of blood were extracted through Bond Elut C_8 column of 3 ml capacity and analysed over three successive days. The peak height of each drug was used to calculate the day to day variation of the extraction and analysis method of the four benzodiazepines using HPLC system.

Table 11 shows the average recoveries, co-efficient of variation and number of samples used each time. Excellent recoveries were obtained for the four benzodiazepine drugs. The recovery was calculated by comparing the peak height of unextracted standard benzodiazepine drugs with extracted samples.

4.2.7 REPRODUCIBILITY OF THE HPLC SYSTEM

The reproducibility of the HPLC system for the four benzodiazepine drugs (Diazepam, Temazepam, Desmethyldiazepam, Triazolam) was tested over three days. A summary of the reproducibility of the four benzodiazepine drugs is summarised in Table 12.

Table 11: Reproducibility of Recovery of Benzodiazepine Drug over a 3 day period using C_8 columns for extraction.

Drug	Days	Average Recoveries	Coefficient Variation	No of Samples Extracted
Diazepam	1	93	1.10%	10
	2	93	0.98%	10
	3	93	1.30%	10
Desmethyl-Diazepam	1	92	1.02%	10
	2	92	0.98%	10
	3	91	0.89%	10
Temazepam	1	88	0.88%	10
	2	88	0.88%	10
	3	87	1.20%	10
Triazolam	1	87	0.89%	10
	2	87	0.89%	10
	3	87	0.88%	10

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

Table 12: The Reproducibility of the Four Benzodiazepine Drugs by HPLC

	Within Day Variation COV (6 samples analysed)	Day-to-Day Variation COV over three days (24 samples analysed)
Diazepam	2.2%	**2.9%**
Desmethyldiazepam	1.4%	**2.3%**
Temazepam	3.3%	**3.9%**
Triazolam	1.6%	**2.1%**

Column: Hypersil 5 µm C_{18} Cartridge (25cm x 4.6 mm i.d.)
Mobile Phase: acetonitrile:methanol:water (30:35:35 v/v/v)
Detection Wavelength: 230nm Flow Rate: 1ml/minute

4.2.8 DRUG EXTRACTION 'SPIKED' SAMPLES

Samples of whole blood 'spiked' with drugs were extracted and analysed at designated time. In each analysis a standard and blank were prepared to

calculate the concentration of drugs under study. Data of each study were accumulated and tabulated at the end of the experiment.

The decline in drug concentrations were represented here by *xy* curve where *x* represents the drug concentration and *y* represents the storage time. From the curves presented it will be noticed that the drug concentration decreases at six months then increases the following month. This could be explained by the drug being bound with blood protein and therefore a decrease in drug concentration would be expected. When the drugs were released from the protein a higher concentration would be expected.

The standard for significant breakdown of drug to have occurred was a level measured which ranged between 0.45 - 5.5 SD from day zero concentration.

4.2.8.1 Benzodiazepines

4.2.8.1.1 Diazepam Stability

Diazepam concentration in blood solution samples at the three different storage temperatures and time periods are summarised in Table 13. The percentage standard deviation for blood Diazepam for the entire method of extraction and analysis was 6.7%

Table 13: The Concentration of Diazepam (μg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at -20°C
Zero Day	5.0	5.0	5.0
1 month	4.8	4.7	5.0
2 months	4.6	4.8	4.8
3 months	4.9	5.9	4.8
4 months	4.6	4.8	4.7
5 months	4.7	4.6	4.7
6 months	4.7	4.8	4.8
7 months	4.9	3.9	4.6
8 months	3.8	4.2	4.6
9 months	3.4	4.9	4.8
10 months	3.0	3.5	3.9
11 months	2.8	3.2	3.8
12 months	2.7	2.9	3.4

The decline in blood Diazepam concentration at 25°C was low until the seventh month of storage. At the eighth month the decrease in blood Diazepam concentration in samples stored at 24°C was significant, with a recovery of 76%. After twelve months of storage, the recovery was 54% at the storage temperature 25°C.

The blood Diazepam concentration was relatively stable up to nine months of storage at the storage temperature 5°C and -20°C, the recoveries of drug being 80% and 96%, respectively. After twelve months of storage, more than 50% of the drug could be recovered.

An average decrease in concentration of 200 ng/ml was observed at up to six months of storage regardless of storage temperature. An average decrease in concentration of 400 ng/ml was observed from the seventh up to twelve months at the storage temperature 25°C. A 900 ng/ml decrease in concentration was observed for drug Diazepam samples stored at 5°C after seven months of storage. At eight months the concentration rises to 500 ng/ml from previous months and at nine months rises to 700 ng/ml from the eighth month. Eventually it drops again to reach 2100 ng/ml of the original concentration. At -20°C after six months of storage the decrease was uniform. 200 ng/ml at up to eight months then drops to reach 1600 ng/ml of the original concentration.

From the above findings, Diazepam was shown to follow the same pattern of decrease in concentration regardless of the storage temperature or the storage media and a reasonable amount of Diazepam would be recovered even after twelve months of storage.

A comparison between blood Diazepam samples stored at the three storage temperatures is shown in Figure 23.

Figure 24 shows the chromatogram of Diazepam after six months storage at 25°C.

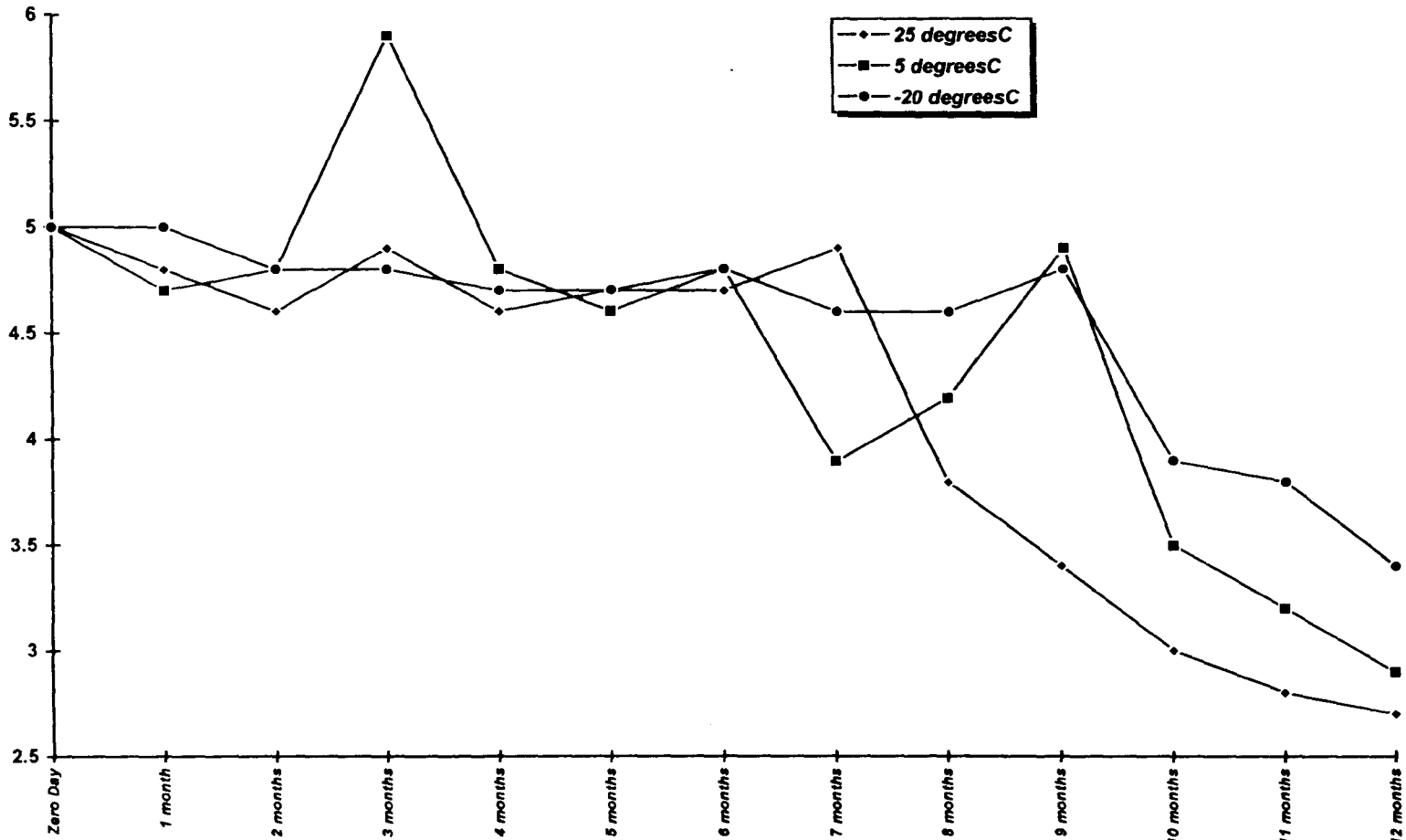


Figure 23: Changes in Diazepam Concentration with Time in Blood Samples Stored at 25, 5 and -20 Degrees Centigrade from Day Zero up to 12 months.

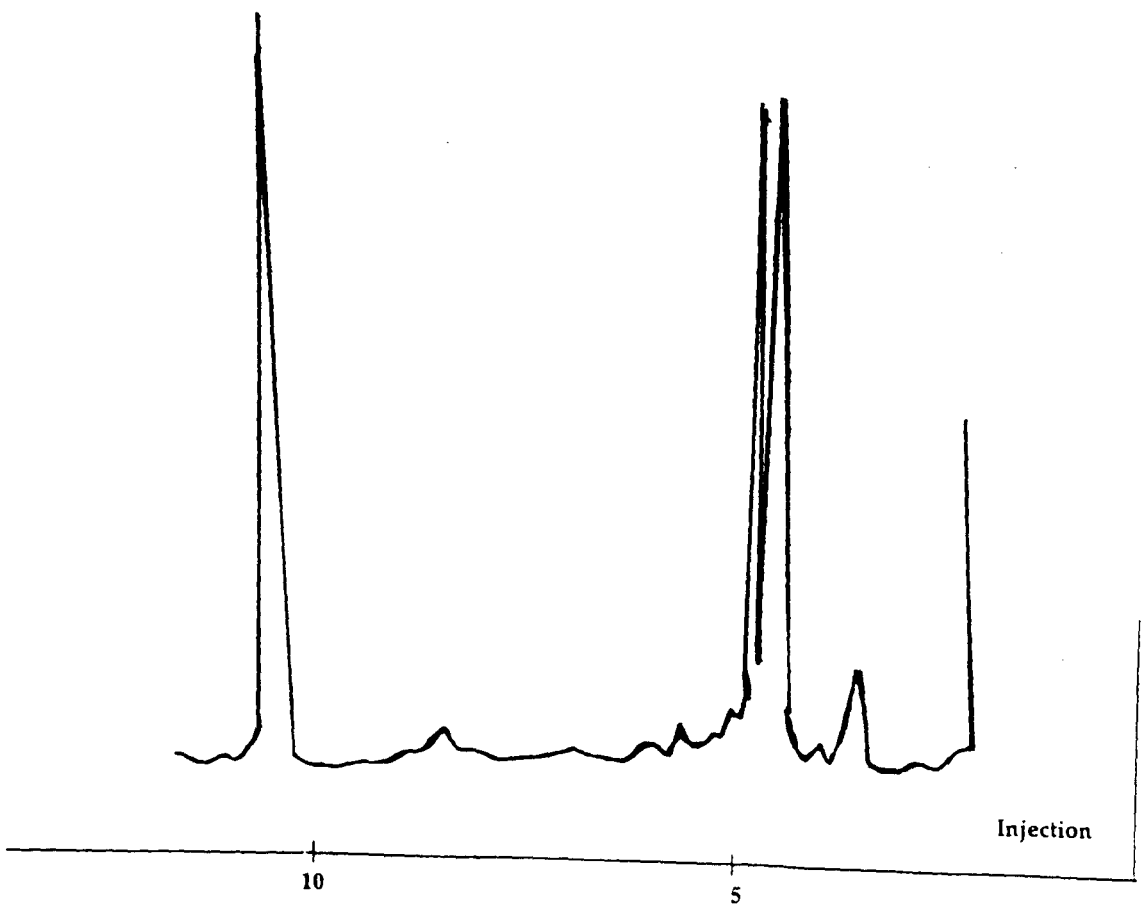


Figure 24: Chromatogram of Diazepam at 25°C after 6 months of Storage
By HPLC system
Range: 0.32
RT of Diazepam: 10.6

4.2.8.1.2 Desmethyldiazepam Stability

The results of blood solution 'spiked' with Desmethyldiazepam stored at the three different storage temperatures and analysed at the designated times are summarised in Table 14. The percentage standard deviation of the Desmethyldiazepam was 5.3% of blood Desmethyldiazepam for the entire experiment.

Table 14: **The Concentration of Desmethyldiazepam (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.**

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at -20°C
Zero Day	3.0	3.0	3.0
1 month	2.6	2.7	2.8
2 months	2.1	2.4	2.9
3 months	2.1	2.5	2.7
4 months	1.9	2.1	2.6
5 months	1.7	1.9	2.7
6 months	1.9	1.9	2.5
7 months	1.5	2.0	2.2
8 months	1.1	1.7	2.4
9 months	1.3	1.8	2.1
10 months	0.9	1.6	1.8
11 months	0.6	1.3	1.6
12 months	0.9	1.4	1.7

Figure 25 shows the decline in concentration for blood samples stored at 5°C, 25°C and -20°C for Desmethyldiazepam. A significant decline in blood concentration was observed for those samples stored at 25°C. After six months

of storage 63% of the drug could be recovered from the samples stored at 25°C. After twelve months of storage 30% of the drug could be recovered. Only 46% of the Desmethyldiazepam was recovered after twelve months of storage, while for those samples stored at -20°C, up to 83% of the drug could be recovered after six months of storage, and after twelve months of storage 57% of the Desmethyldiazepam could be recovered.

At 25°C, after two months of storage, a decrease of 500 ng/ml was observed on the concentration of Desmethyldiazepam. At up to six months of storage the decrease was steady at 200 ng/ml in concentration, and after six months the decrease was also steady but higher, an average of 400 ng/ml in concentration. At 5°C an average decrease of 300 ng/ml was observed up to six months. The decrease was 600 ng/ml between the seventh and the twelfth months. A decrease of 500 ng/ml was observed between day zero and the sixth month at -20°C. The decrease was noticed to be steady between the sixth and the twelfth months, the average decrease being 300 ng/ml.

Figure 26 shows the decrease in blood Desmethyldiazepam concentration at 5°C after twelve months of storage.

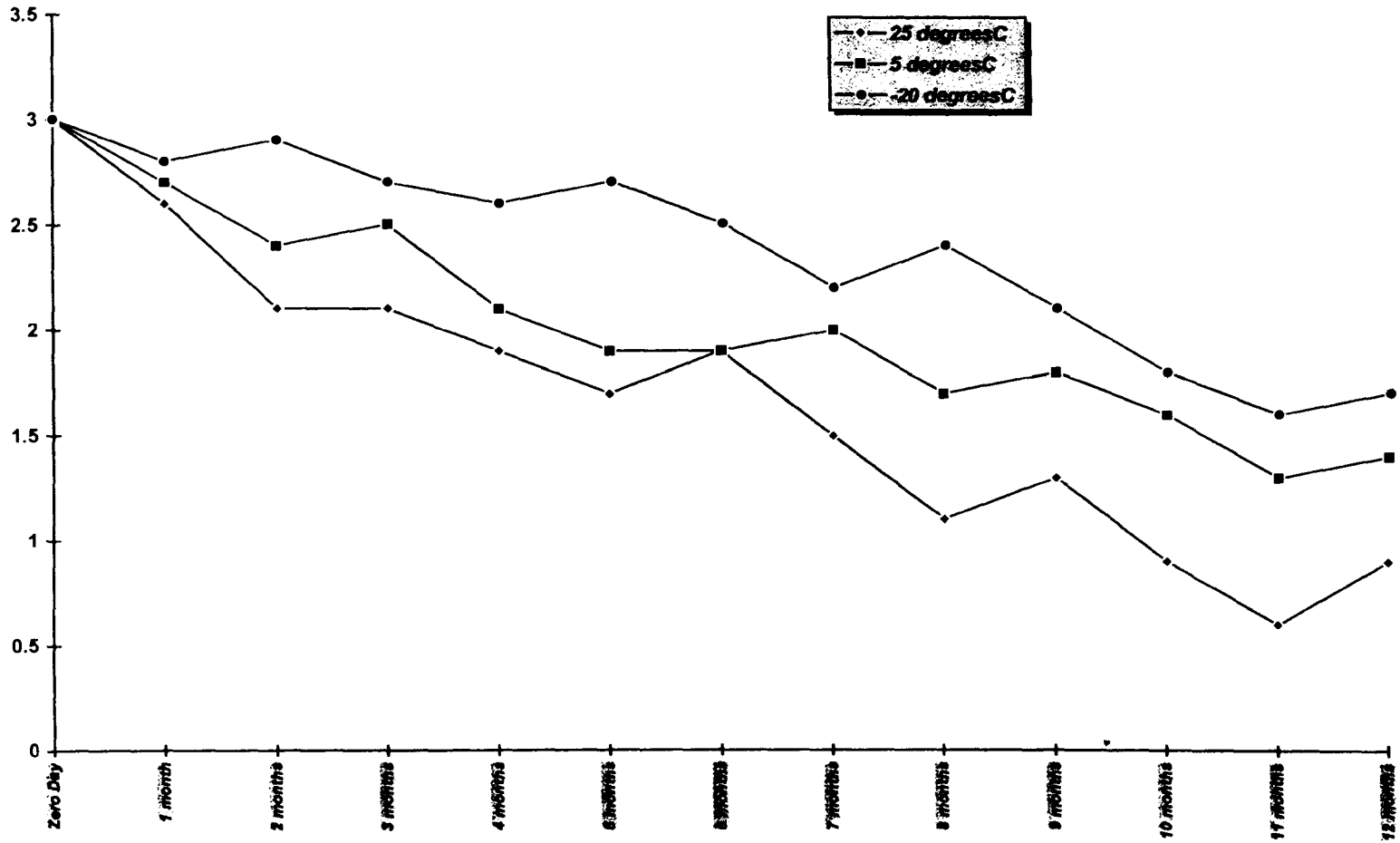


Figure 25: Changes in Desmethyldiazepam Concentration with Time in Blood Samples Stored at 25, 5 and -20 Degrees Centigrade from Day Zero up to 12 months.

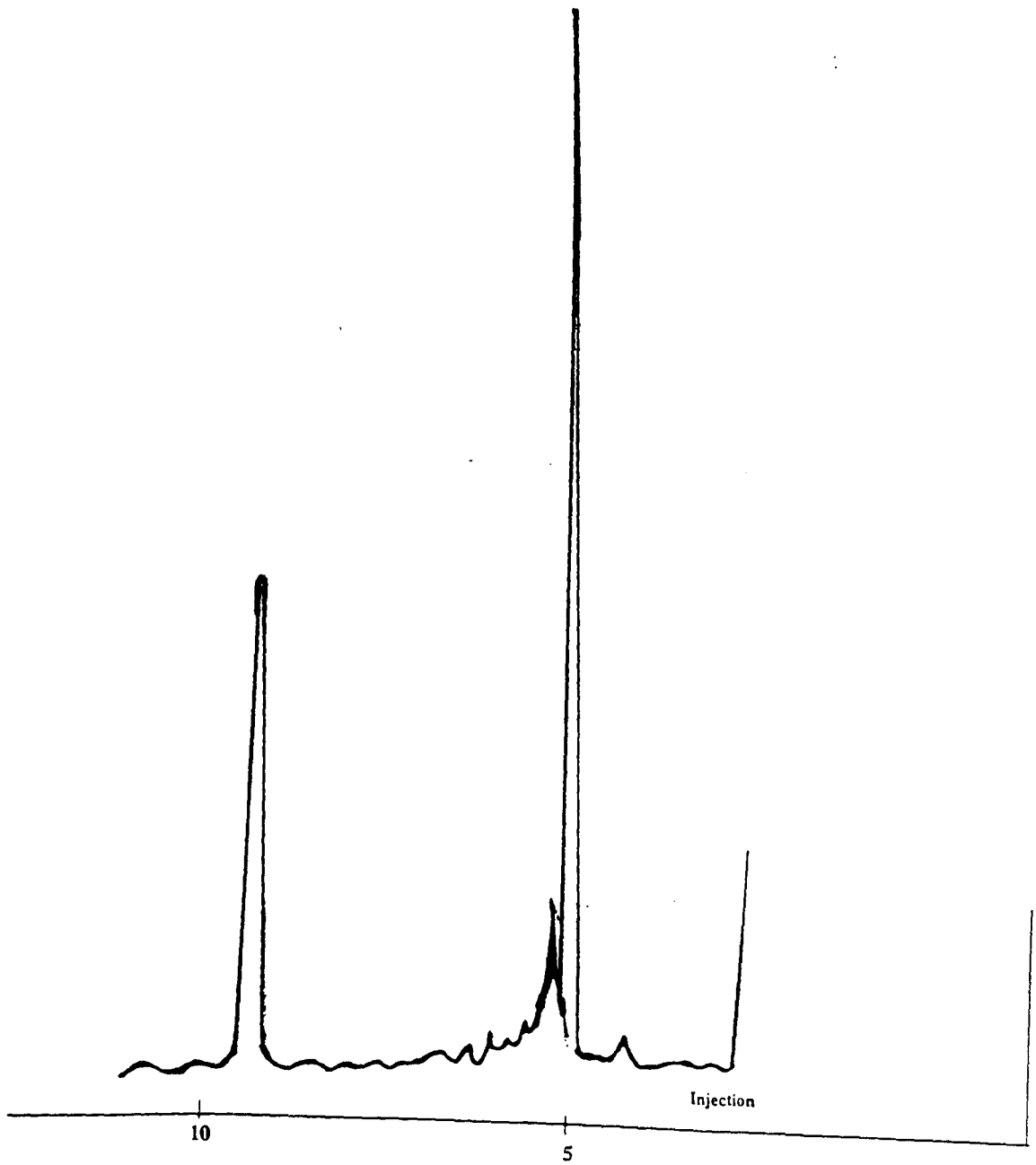


Figure 26: Chromatogram of Desmethyldiazepam at 5°C after 12 months Of Storage by HPLC System
Range: 0.04
RT of Desmethyldiazepam: 7.8

Conclusion

Desmethyldiazepam showed the best stability for those samples at -20°C. After one year of storage the drugs can still be detected at a storage temperature of -20°C. A significant decrease in Desmethyldiazepam concentration was observed for samples stored at both 25°C and 5°C temperatures, with the greater and more rapid decrease occurring in the blood stored at room temperature.

4.2.8.1.3 Temazepam Stability

The stability of Temazepam in blood was investigated. 'Spiked' samples of blood with Temazepam, at a concentration of 5.0 μg/ml from day zero until twelve months, was studied.

The results of the blood solution 'spiked' with Temazepam stored at the three storage temperatures were analysed at the designated times and are presented in Table 15.

Table 15: The Concentration of Temazepam (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at -20°C
Zero Day	5.0	5.0	5.0
1 month	4.9	4.8	5.0
2 months	4.6	4.5	4.8
3 months	4.4	4.4	4.8
4 months	4.2	4.5	4.8
5 months	4.2	4.6	4.7
6 months	4.4	4.5	4.4
7 months	4.1	4.4	4.6
8 months	4.2	3.9	4.2
9 months	3.7	3.8	4.2
10 months	3.4	3.6	3.8
11 months	3.1	3.4	3.5
12 months	2.8	3.1	3.5

The percentage standard deviation for the entire Temazepam analysis at the three temperatures was 5.7%. The standard for significant breakdown of Temazepam to have occurred was a level measured which had decreased by ±0.57 from day zero concentration.

Figure 27 shows the decline in Temazepam concentration from day zero up to twelve months when stored at the three different temperatures (5°C, 25°C and -20°C). Samples of blood stored at 25°C declined steadily. At twelve months 56% of the drug could still be detected. For the samples that were refrigerated at 5°C, at six months 90% ±2 of the drug could be detected, the same as for those samples stored at -20°C for the same time. For those samples stored up to twelve months at 5°C, 25°C and -20°C, the recovery was 62%±8.

An average decrease in concentration at 200 ng/ml was observed at up to six month of storage regardless of the storage temperature. A decrease of 500 ng/ml was observed between the eighth and the ninth months of storage. Then a steady decrease of an average of 300 ng/ml was observed at up to a year for the samples stored at 25°C. At 5°C the decrease was high between the seventh and eighth month, 500 ng/ml in concentration. Afterwards the decrease was steady, an average of 200 ng/ml in concentration at up to twelve months. At -20°C the decrease was steady after six months of storage, an average of 400 ng/ml in concentration.

A chromatogram showing the decrease in blood Temazepam concentration after 8 months at -20°C is shown in Figure 28.

An average decrease in concentration of 2000 ng/ml was observed for the samples stored at 25°C and -20°C, while at 5°C the average decrease in concentration was less at 1000 ng/ml. After the sixth month the decrease was steady. The decrease in concentration was an average of 2000 ng/ml at up to the twelfth month for the samples stored at 25°C and -20°C and 3000 ng/ml in concentration of the samples stored at 5°C.

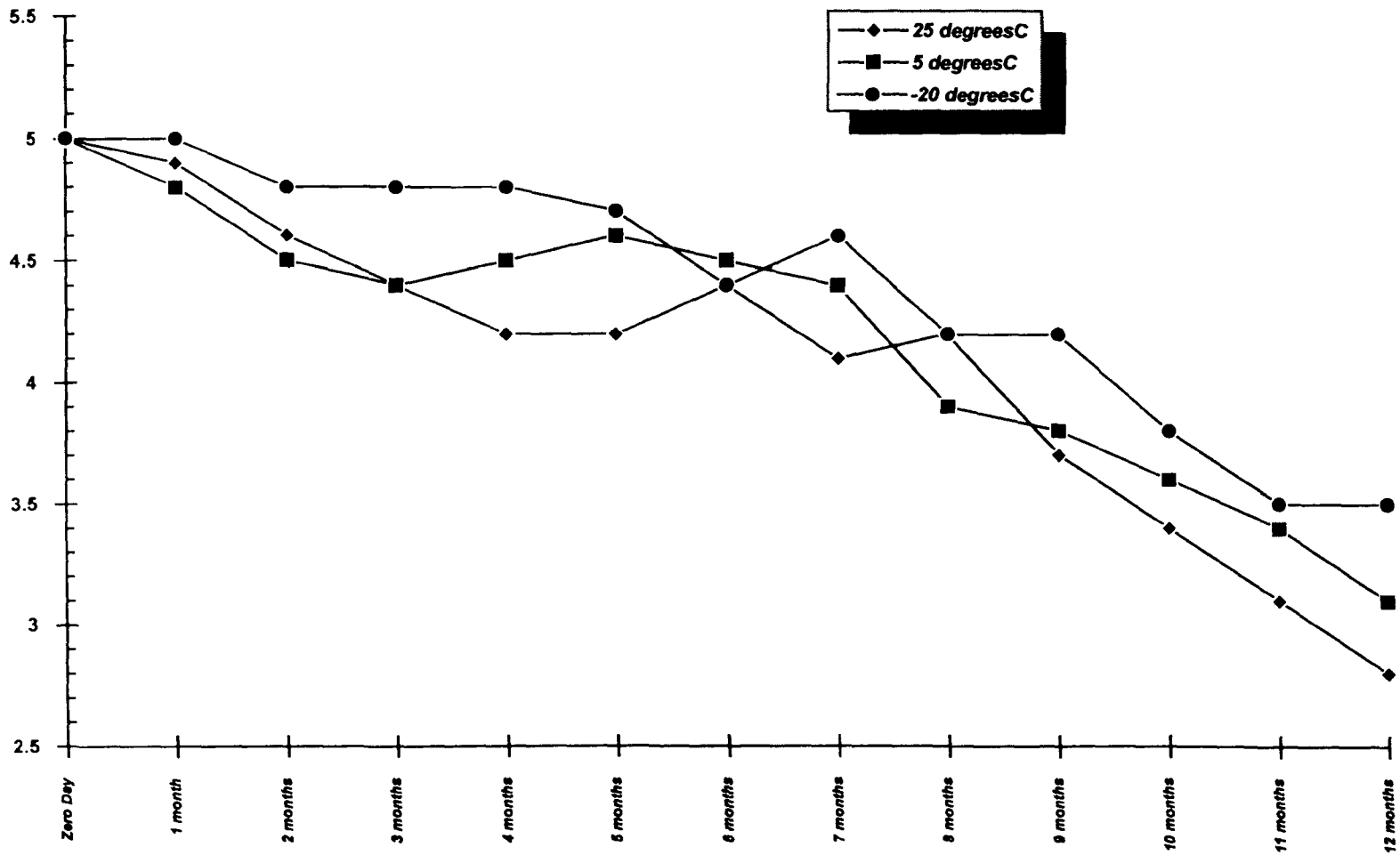


Figure 27: Changes in Temazepam Concentration with Time in Blood Samples Stored at 25, 5 and -20 Degrees Centigrade from Day Zero up to 12 months.

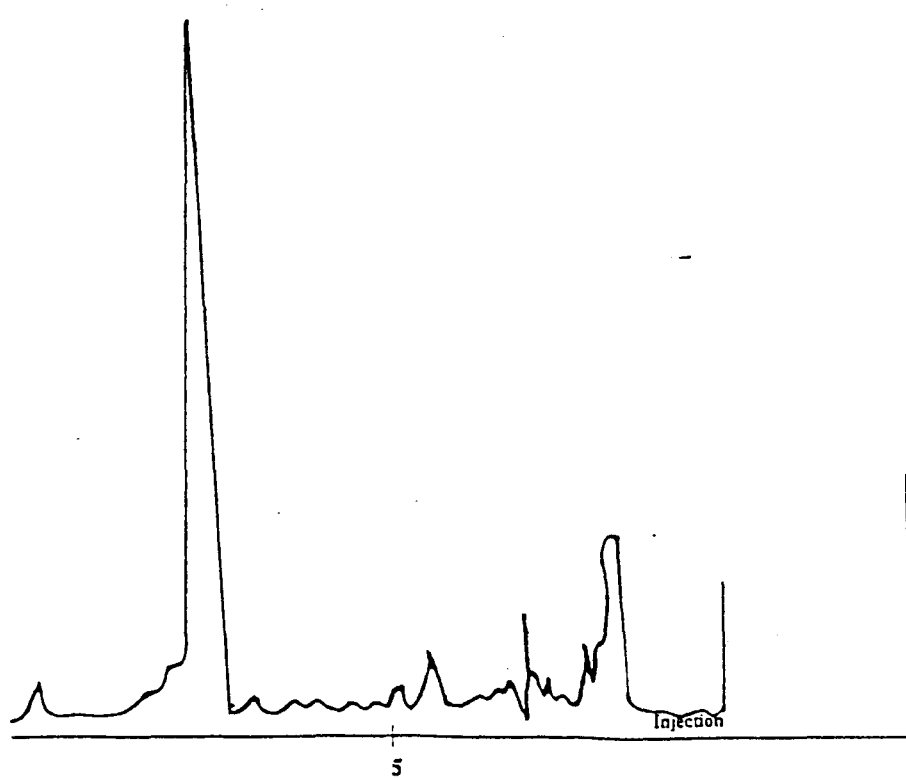


Figure 28: Chromatogram of Temazepam at -20°C after 8 months of Storage by HPLC System.
Range: 0.32
RT of Temazepam: 7.4

Conclusion

Blood Temazepam is stable when stored at 5°C and -20°C. It could be detected up to a year when stored at any temperature, being able to have 56% recovery when stored at room temperature.

4.2.8.1.4 Triazolam Stability:

The results of the blood solution 'spiked' with Triazolam stored at three storage temperatures and analysed at designated times are summarised in Table 16. The percent standard deviation of the Triazolam was 5.1% for blood Triazolam for the entire experiment.

Table 16: **The Concentration of Triazolam (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.**

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at -20°C
Zero Day	50	50	50
1 month	47	48	48
2 months	46	47	49
3 months	47	47	48
4 months	45	45	48
5 months	47	46	49
6 months	45	45	47
7 months	44	46	48
8 months	40	44	46
9 months	38	38	46
10 months	36	36	44
11 months	34	34	44
12 months	32	31	42

Figure 29 shows the decline in Triazolam concentration in blood samples stored at 5°C, 25°C and -20°C.

In concentration an average decrease of 2000 ng/ml was observed for the samples stored at 25°C and -20°C, while at 5°C the average decrease in concentration was less (1000 ng/ml). After the sixth month the decrease was steady, the decrease in concentration was an average of 2000 ng/ml up to the twelfth month for the samples stored at 25°C and -20°C and 3000 ng/ml in concentration for the samples stored at 5°C.

A steady decrease in Triazolam concentration is shown at the three different storage temperature. Up to six months the percentage of recovery of Triazolam was 94% for drugs stored at -20°C and 90% for those stored at 5 and 25°C. After one year 65% was recovered of Triazolam blood at 25°C, 74% for the blood sample stored at 5°C and 84% of the blood samples stored at -20°C. A chromatogram showing the decrease in Triazolam concentration after 4 months of storage at 25°C is shown in Figure 30.

Conclusion

Triazolam showed the best stability over the other benzodiazepines studied since blood solution at the three storage temperatures showed average recoveries 91% ±3 for blood after six months of recovery. After one year of

storage about 74% ±10 of Triazolam was recovered regardless of the storage temperature in blood.

Figure 30: shows the change in concentration of Triazolam drug after four months of storage.

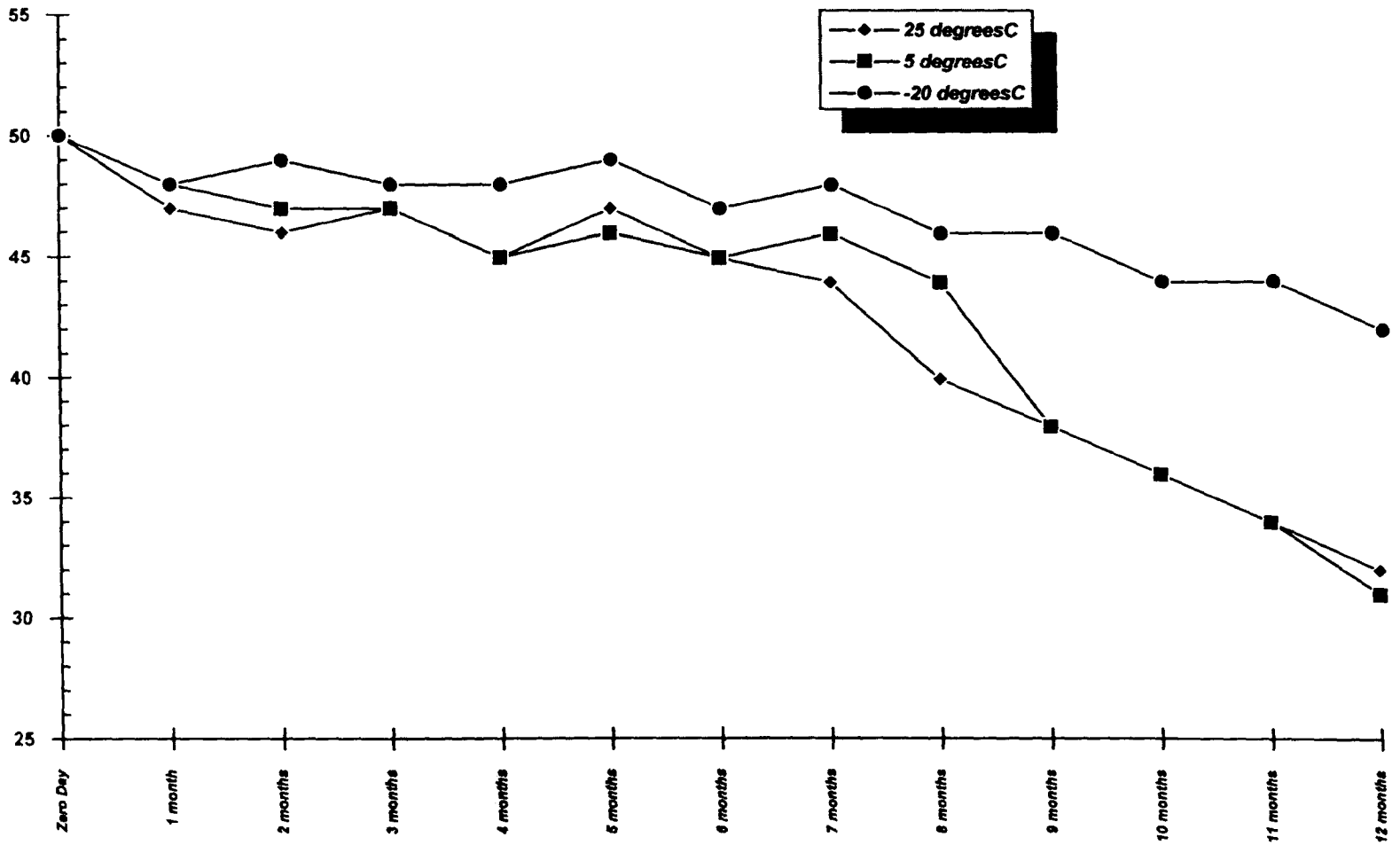


Figure 29: Changes in Triazolam Concentration with Time in Blood Samples Stored at 25, 5 and -20 Degrees Centigrade from Day Zero up to 12 months.

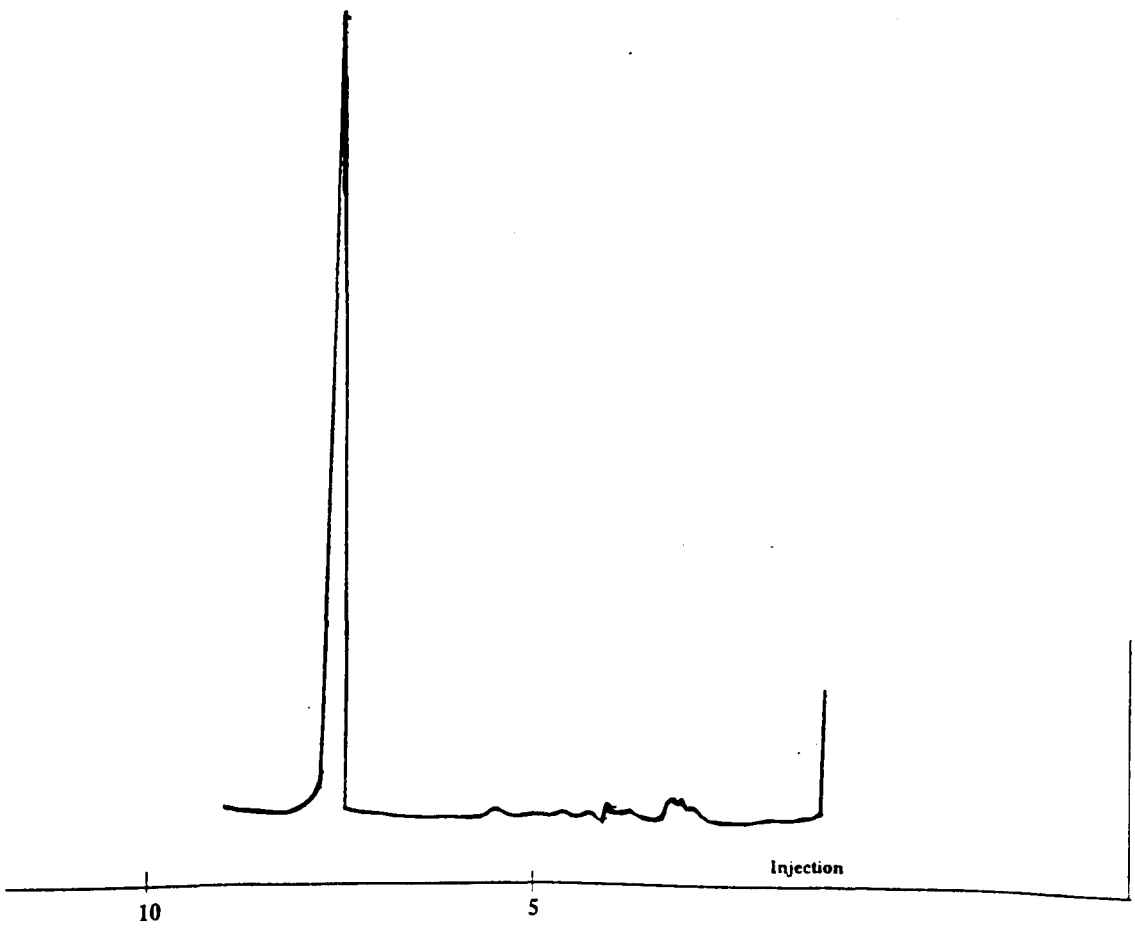


Figure 30: Chromatogram of Triazolam at 25°C after 4 months of Storage
Analysed by HPLC System
Range: 0.32
RT of Triazolam 7.0

4.4 RESULTS AND DISCUSSION OF LIQUID-LIQUID EXTRACTION

4.4.1 SAMPLE EXTRACTION USING 1-CHLOROBUTANE

This method has an advantage over other methods in that with the extraction step using 1-chlorobutane as the extracting solvent with gas chromatographic analysis, the complete analysis can be accomplished in one to one-half hours.

Most solvents in use for drug extraction also extract interfering substances, making phase separation and evaporation difficult by this procedure by using 1-chlorobutane as an extracting solvent eliminated the time-consuming steps of evaporation.

Recoveries of the four tricyclic antidepressants (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) were determined in 'spiked' whole blood samples by comparison of peak height ratio of the extracts with unextracted standards and were determined over a concentration of 100 µg/ml, shown in Table 17.

Table 17: Retention Times and Recoveries of Tricyclic Drugs (100 µg/ml) for 'spiked' Blood.

Drug	Retention Time (minutes)	% Recovery
Amitriptyline	14.7	69.2
Nortriptyline	13.2	59.8
Imipramine	14.9	79.8
Chlorpromazine	18.1	56.9

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

4.4.2 ONE STEP EXTRACTION METHOD USING N-BUTYLACETATE

The absolute recoveries of the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from 'spiked' whole blood samples were calculated by comparing the peak height ratio of the extracts from whole blood with unextracted standard solutions at the same concentration and under identical conditions. The percentage recovery for each drug and its retention time is shown in Table 18.

Table 18: The Absolute Retention Time and Drug Recoveries of the Four Extracted Drugs at a Concentration of 100 µg/ml.

Drug	Absolute Retention Time (RT) min.	% Recovery
Amitriptyline	15.16	73
Nortriptyline	15.83	75
Imipramine	15.62	77
Chlorpromazine	18.83	65

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

This procedure has been highly successful with whole blood because it exhibits less emulsification and relatively good recoveries for the four drugs.

The success of the described extraction is that it meets the requirement of today's toxicology laboratory, that is, small sample volume, short analysis time, ease of sample preparation and sensitive detection. This procedure has replaced a more time consuming multi-step extraction procedure that required evaporation and reconstitution. Butylacetate was chosen as the solvent in this procedure for its lack of volatility and its polarity, which is essential in a single-step extraction without evaporation.

4.4.3 LIQUID-LIQUID EXTRACTION WITH HEXANE : BUTAN-1-OL

Extraction recoveries of the four tricyclic antidepressant drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) was determined by comparing the peak heights of extracted blood with peak heights of the standard papaverine prepared in methanol at the same concentration and under identical conditions.

The method provides an advantage over previously reported methods by allowing quantitation of antidepressants in a single extraction.

Clean extracts were obtained from extracting the blood samples but could not guarantee the absence of interferences.

The absolute recoveries of the four drugs are shown in Table 19.

Table 19: **Percentage Average Recoveries (n = 4) of Tricyclic Drugs (100 µg/ml) from Blood.**

Drugs	% Average Recoveries
Amitriptyline	85.5
Nortriptyline	74.3
Imipramine	84.0
Chlorpromazine	70.0

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

The highest recovery was for the drug Amitriptyline and the lowest was for Chlorpromazine.

4.4.4 MULTI-STEP EXTRACTION USING ISOAMYL ALCOHOL IN N-HEXANE

The major advantage of this method for tricyclic antidepressant extraction from blood is the low volume of sample used. The described extraction procedure requires only 1 ml of blood. The procedure is clean, sensitive, reliable, rapid and inexpensive.

The resulting extracts, when analysed using capillary columns with nitrogen-selective, yielded valuable qualitative data.

The absolute recoveries of the four tricyclic drug (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from 'spiked' blood were calculated by comparing the peak height ratio of the extract with unextracted standard solution under identical conditions and are shown in Table 20.

Table 20: **Percentage Average Recoveries (n = 4) for the Four Tricyclic Antidepressants (100 µg/ml)**

Drug	% Average Recoveries
Amitriptyline	60.8
Nortriptyline	64.3
Imipramine	60.3
Chlorpromazine	58

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

The recoveries of the four drugs ranged from a high of 64.3% for Nortriptyline to a low of 58% for Chlorpromazine.

4.4.5 SINGLE EXTRACTION PROCEDURE WITH DIETHYLETHER

This procedure is a successful method of extraction due to the small initial volume of blood taken (1 ml). It is a convenient, simple and relatively rapid method of extraction.

Extraction with diethylether gave a cleaner extract than did other solvents. The direct injection of an organic aliquot from such an extraction gave good peak shapes of the compounds when analysed.

The absolute recoveries of the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from 'spiked' blood were calculated by comparing the peak height ratio of the extract with the unextracted standard solution under identical conditions. The results are shown in Table 21.

Table 21: **Percentage Average Recoveries (n = 4) for the Four Tricyclic Antidepressant Drugs (100 µg/ml)**

Drug	% Average Recoveries
Amitriptyline	56.5
Nortriptyline	61.8
Imipramine	54.3
Chlorpromazine	53.5

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

The average recoveries of the drugs ranged between 53.5 - 61.8% which is considered to be low.

4.4.6 LIQUID-LIQUID EXTRACTION USING THE EXTRACTING SOLVENT HEXANE : ISOAMYL ALCOHOL

This procedure was an efficient one in terms of the low number of procedural stages in achieving the extraction.

Samples high in lipid often emulsify during liquid-liquid extraction. This is especially true with a solvent such as hexane. The addition of isoamyl alcohol increases the polarity of hexane which inhibits emulsion formation and minimises adsorption of tricyclic antidepressants onto the surface of the glassware.

With all the advantages that this procedure has, still the percentage recoveries were not high.

The absolute recoveries of the four tricyclic antidepressant drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from 'spiked' blood were calculated by comparing the peak height ratio of the extracts from blood with the unextracted standard solution under identical conditions. These are shown in Table 22.

Table 22: **Percentage Average Recoveries of Tricyclic Antidepressant Drugs (100 µg/ml) from 'spiked' Blood**

Drug	% Average Recoveries
Amitriptyline	59.5
Nortriptyline	61
Imipramine	55.3
Chlorpromazine	50.5

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

4.4.7 LIQUID-LIQUID EXTRACTION WITH ETHYLACETATE

This method was chosen for its good recoveries for the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) from 'spiked' blood samples over a concentration range of 2.5 - 50µg/ml.

The absolute recoveries of the four TCA drugs were calculated by comparing peak height ratios of the extracted samples with the unextracted standards under identical condition.

The recovery of Amitriptyline was from 'spiked' blood using liquid-liquid extraction and analysed by GC (Table 23).

Table 23: Recovery of Amitriptyline from 'spiked' Blood by Liquid-Liquid Extraction and Analysed by GC

Concentration (µg/ml)	Recovery (%)	Number of Samples
2.5	78.4 ± 4.0%	4
5.0	86 ± 2.5%	4
10	95 ± 3.0%	4
50	106 ± 6.0%	4

The recovery of Nortriptyline was from 'spiked' blood using liquid-liquid extraction and analysed by GC (Table 24).

Table 24: Recovery of Nortriptyline from 'spiked' Blood by Liquid-Liquid Extraction and Analysed by GC

Concentration (µg/ml)	Recovery (%)	Number of Samples
2.5	72.6 ± 3.0%	4
5.0	84 ± 3.5%	4
10	92 ± 4.0%	4
50	108 ± 4.5%	4

The recovery of Imipramine was from 'spiked' blood using liquid-liquid extraction and analysed by GC (Table 25).

Table 25: Recovery of Imipramine from 'spiked' Blood by Liquid-Liquid Extraction and Analysed by GC

Concentration (µg/ml)	Recovery (%)	Number of Samples
2.5	73 ± 2.0%	4
5.0	84 ± 3.0%	4
10	93.5 ± 3.0%	4
50	102 ± 5.0%	4

The recovery of Chlorpromazine from 'spiked' blood using liquid-liquid extraction and analysed by GC (Table 26).

Table 26 **Recovery of Chlorpromazine from 'spiked' Blood by Liquid-Liquid Extraction and Analysed by GC**

Concentration (µg/ml)	Recovery (%)	Number of Samples
2.5	71.2 ± 3.0%	4
5.0	80.7 ± 2.5%	4
10	90.5 ± 3.0%	4
50	104 ± 4.5%	4

4.4.8 THE REPRODUCIBILITY OF THE EXTRACTION METHODS

Thirty samples of human post-mortem blood 'spiked' with the four tricyclic antidepressant drugs mixture (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) in a concentration of 2 µg/ml were liquid-liquid extracted and analysed over three successive days. The peak height of each drug was used to calculate the day to day variation of the extraction and analysis method of the four tricyclic antidepressants using GC systems.

Table 27 shows the average recoveries, co-efficient of variation and number of samples used each time. Good recoveries were obtained but were not very high at a concentration of 2 µg/ml but is shows that the method was reproducible. The recovery was calculated by comparing the peak height ratio of the unextracted standard tricyclic antidepressants with extracted samples.

Table 27: Reproducibility of Recovery of Tricyclic Antidepressant Drugs over a 3 Day Period using Liquid-Liquid Extraction.

Drug	Days	Average Recoveries	Coefficient Variation %	No of Samples Extracted
Amitriptyline	1	81.3	1.2	10
	2	81.7	1.51	10
	3	81.5	1.51	10
Nortriptyline	1	75.6	1.2	10
	2	75.7	1.1	10
	3	75.5	0.660	10
Imipramine	1	75.9	1.4	10
	2	75.4	0.74	10
	3	75.5	0.97	10
Chlorpromazine	1	72.2	1.4	10
	2	72.9	1.6	10
	3	73	1.2	10

4.4.9 REPRODUCIBILITY OF THE GC SYSTEM

The reproducibility of the GC system for the four tricyclic antidepressant drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) was tested over three days. A summary of the reproducibility for the four TCA drugs is shown in Table 28.

Table 28: **Reproducibility of the GC System for the Analysis of the Four Tricyclic Antidepressant Drugs**

	Within Day Variation COV (6 samples analysed)	Day-to-Day Variation COV over three days (24 samples analysed)
Amitriptyline	4.2%	4.9%
Nortriptyline	3.9%	3.5%
Imipramine	2.3%	3.4%
Chlorpromazine	2.5%	3.2%

Column: Chrompack CP-sil5, 25 m x 0.32 mm i.d.0.4 µm film thickness
Oven Temperature: Initial temp. 120°C Final temp. 290°C FID/NPD detector

4.4.10 CALIBRATION CURVES

The calibration curves for the four tricyclic antidepressant drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) were constructed over the concentration range 0.5 - 10 µg/ml prepared in blank blood. Six samples at each concentration were liquid-liquid extracted. The internal standard papaverine was added to all samples. The peak height ratios of drugs over the internal standard at each concentration were plotted to construct a calibration curve for each tricyclic antidepressant drug. The relationship between the tricyclic antidepressant drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine), concentration and average peak height ratio (std/I.std) of samples analysed by GC are highlighted in Table 29.

Table 29: Statistical Data for the Four Calibration Curves of Tricyclic Antidepressants

Drug	Concentration (µg/ml)	Average Peak Height Ratio On = 6
Amitriptyline	0.5	1.0
	2.0	3.7
	4.9	6.1
	8.0	14.1
	10.0	17.1
Nortriptyline	0.5	1.1
	2.0	4.0
	4.0	7.1
	8.0	13.8
	10.0	18.0
Imipramine	0.5	1.2
	2.0	3.8
	4.0	6.6
	8.0	13.8
	10.0	18.1
Chlorpromazine	0.5	1.4
	2.0	4.2
	4.0	6.8
	8.0	14.4
	10.0	17.2

A linear relation was obtained for each drug as shown in Figures 31, 32, 33 and 34.

The equations of the lines were:

$y = 1.72 x + 0.03$, $y = 1.74 x + 0.27$, $y = 1.76 x + 0.07$, $y = 1.68 x + 0.57$

for Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine, respectively, and the regressions were:

0.9991, 0.9970, 0.9982 and 0.9981 for Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine, respectively.

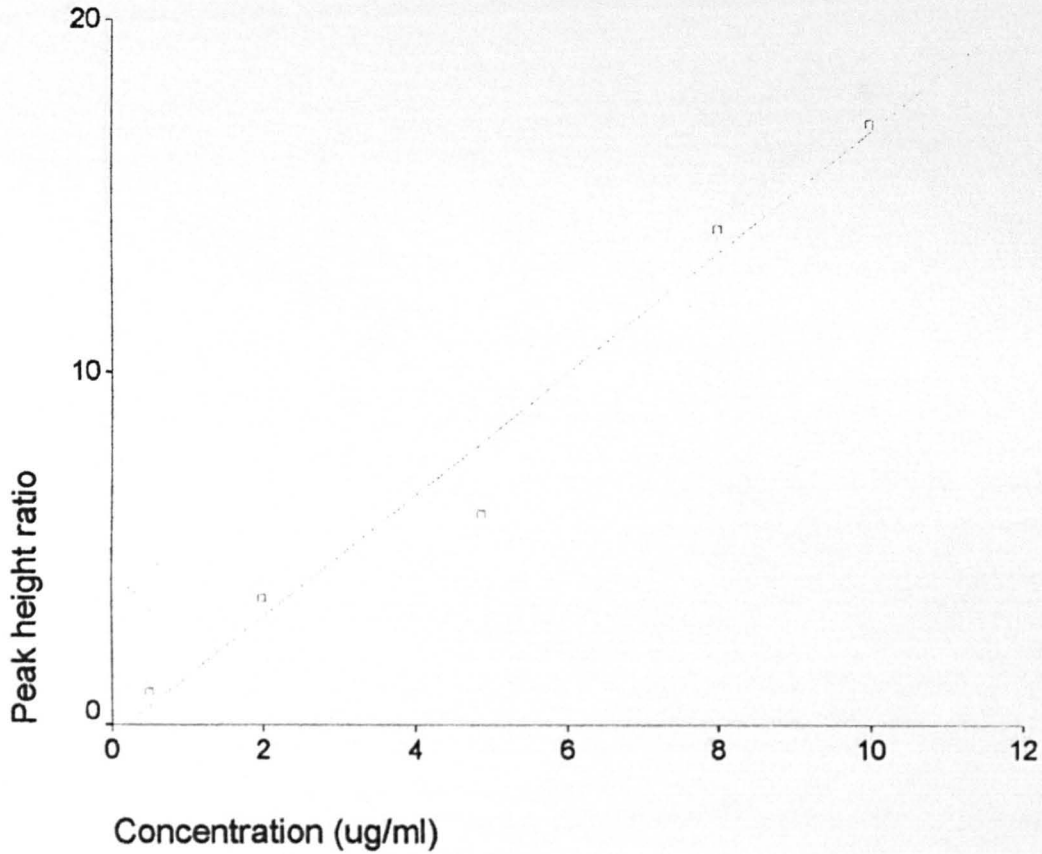


Figure 31: **Calibration Curve of Amitriptyline**

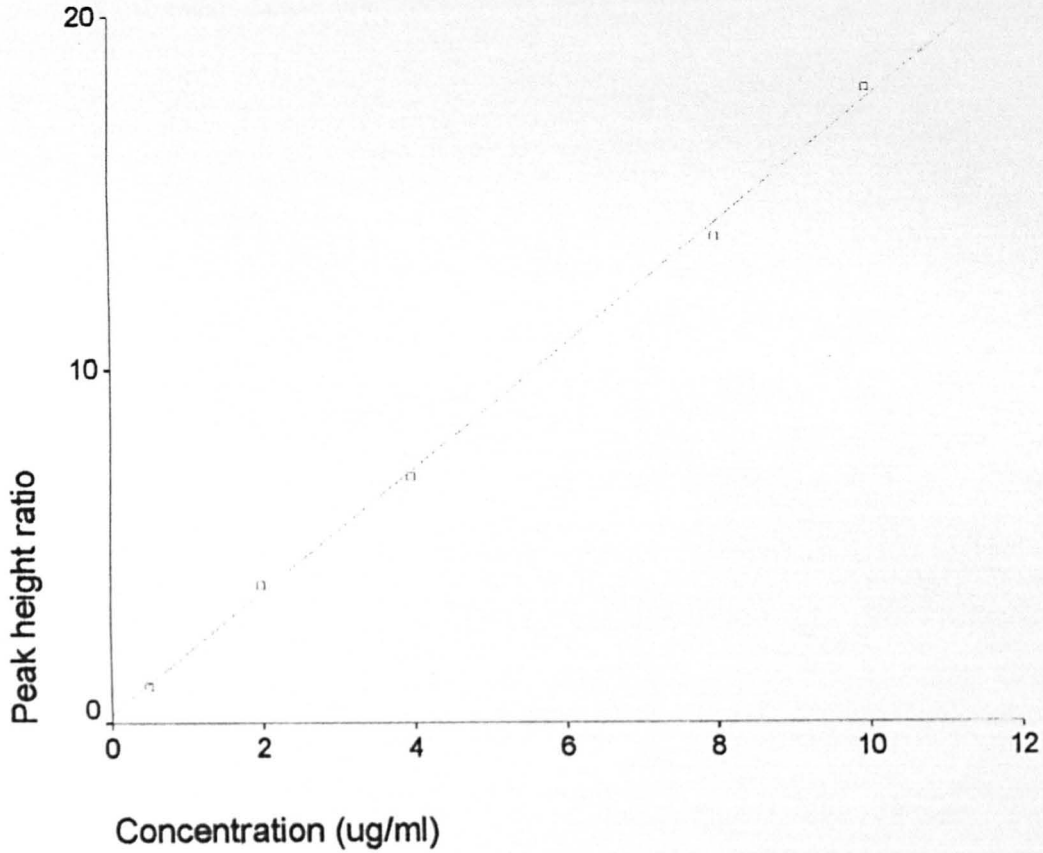


Figure 32: **Calibration Curve of Nortriptyline**

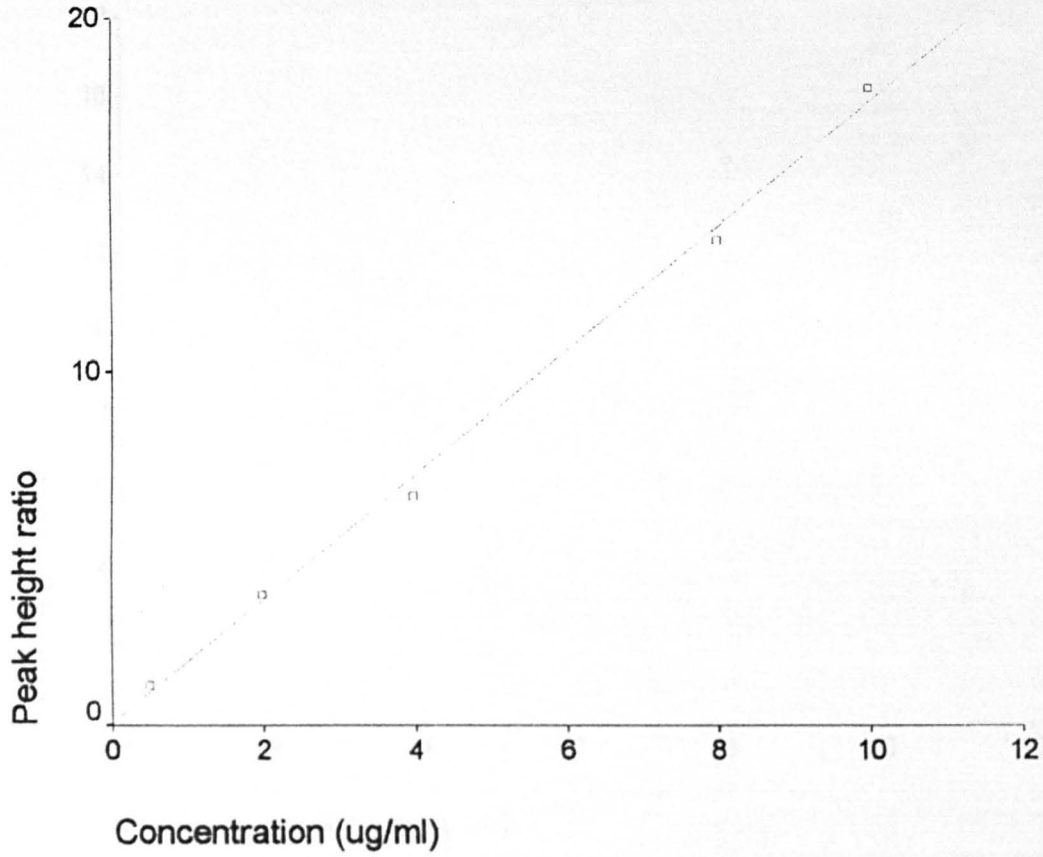


Figure 33: **Calibration Curve of Imipramine**

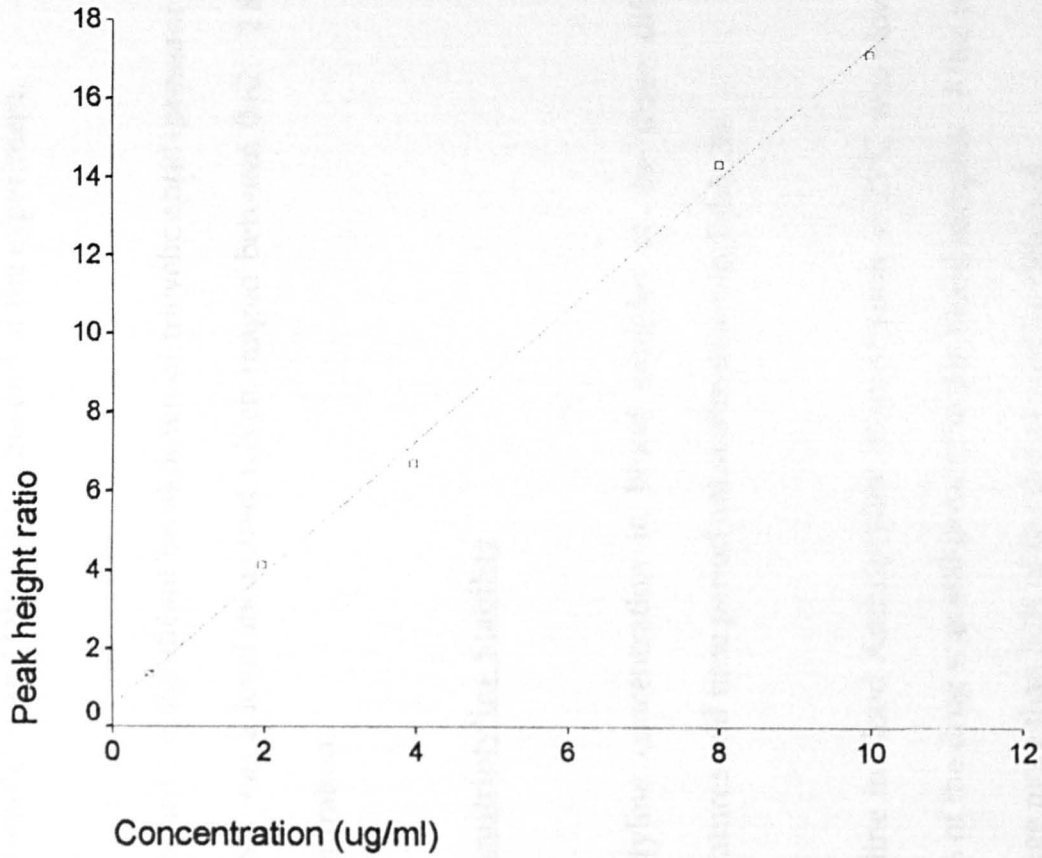


Figure 34: **Calibration Curve of Chlorpromazine**

4.4.11 DRUG EXTRACTION OF 'SPIKED' SAMPLES WITH TRICYCLIC ANTIDEPRESSANTS

Samples of whole blood 'spiked' with the antidepressant drugs were extracted and analysed at the designated times. In each analysis a standard and blank were prepared to calculate the concentration of drugs. The data of each study were accumulated and tabulated at the end of the experiment.

The standard for significant breakdown of tricyclic antidepressant drugs to have occurred was a level measured which ranged between 0.62 - 2.8 from day zero concentration.

4.4.11.1 Amitriptyline Stability

Amitriptyline concentration in blood samples at the three different storage temperatures and time period are summarised in Table 30.

The decline in blood Amitriptyline concentration at 25°C was slow. At six months 68% of the drug was still present in the blood samples. After twelve months of storage more than 50% of the drug could be detected.

At 5°C blood Amitriptyline was stable even after twelve months of storage, 76% of the drug can be detected.

Table 30: The Concentration of Amitriptyline (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at –20°C
Zero Day	9.5	9.8	10
1 month	9.7	10	10
2 months	10	10	10
3 months	9.2	9.6	8.7
4 months	10	7.6	8.6
5 months	7.6	10	10
6 months	6.8	7.2	8,7
7 months	5.6	8.0	8.5
8 months	5.9	8.2	9.2
9 months	6.0	8.6	9.0
10 months	6.2	8.3	8.9
11 months	6.5	7.9	8.5
12 months	5.5	7.6	8.4

At 5°C and -20°C up to six months the loss in drug concentration was 3900 ng/ml and 2900 ng/ml, respectively. The loss in concentration reached up to 5400 ng/ml and 4600 ng/ml from the original concentration at 5°C and –20°C, respectively after one year of storage.

At -20°C the recoveries of blood Amitriptyline at six months (being half the period of storage) was that 87% of the drug could be detected and at twelve months 84% of the drug could be detected.

From day zero up to the fourth month at the storage temperature 25°C Amitriptyline there was almost 100% recovery and the fluctuation in the drug concentration showed that in the first month 97% of the drug could be detected

while in the second month 100% of the drug could be detected. This could be due to the drug being bound to blood proteins and released the second month. A drop in concentration was noticed after the fourth month. A drop of 2400 ng/ml in concentration between the fourth and fifth month. The drop in concentration reached an average of 2100 ng/ml in concentration at up to the twelfth month. At 5°C a fluctuation in the drop of concentration was noticed at up to the fifth month. At some months a drop was noticed but the following month it levelled off to the original concentration. The loss in concentration reached 2700 ng/ml at up to one year of storage but drug was more stable than at 25°C. At -20°C the drug was very stable; the loss in drug concentration was 1600 ng/ml of the original concentration after one year of storage.

From the above findings blood Amitriptyline is relatively stable at the three different temperatures (5°C, 25°C and -20°C). Even after twelve months of storage, the drug can still be detected.

Amitriptyline concentration after four months of storage at 25°C is shown in Figure 35.

A comparison between blood Amitriptyline samples stored at the three different temperatures is shown in Figure 36.

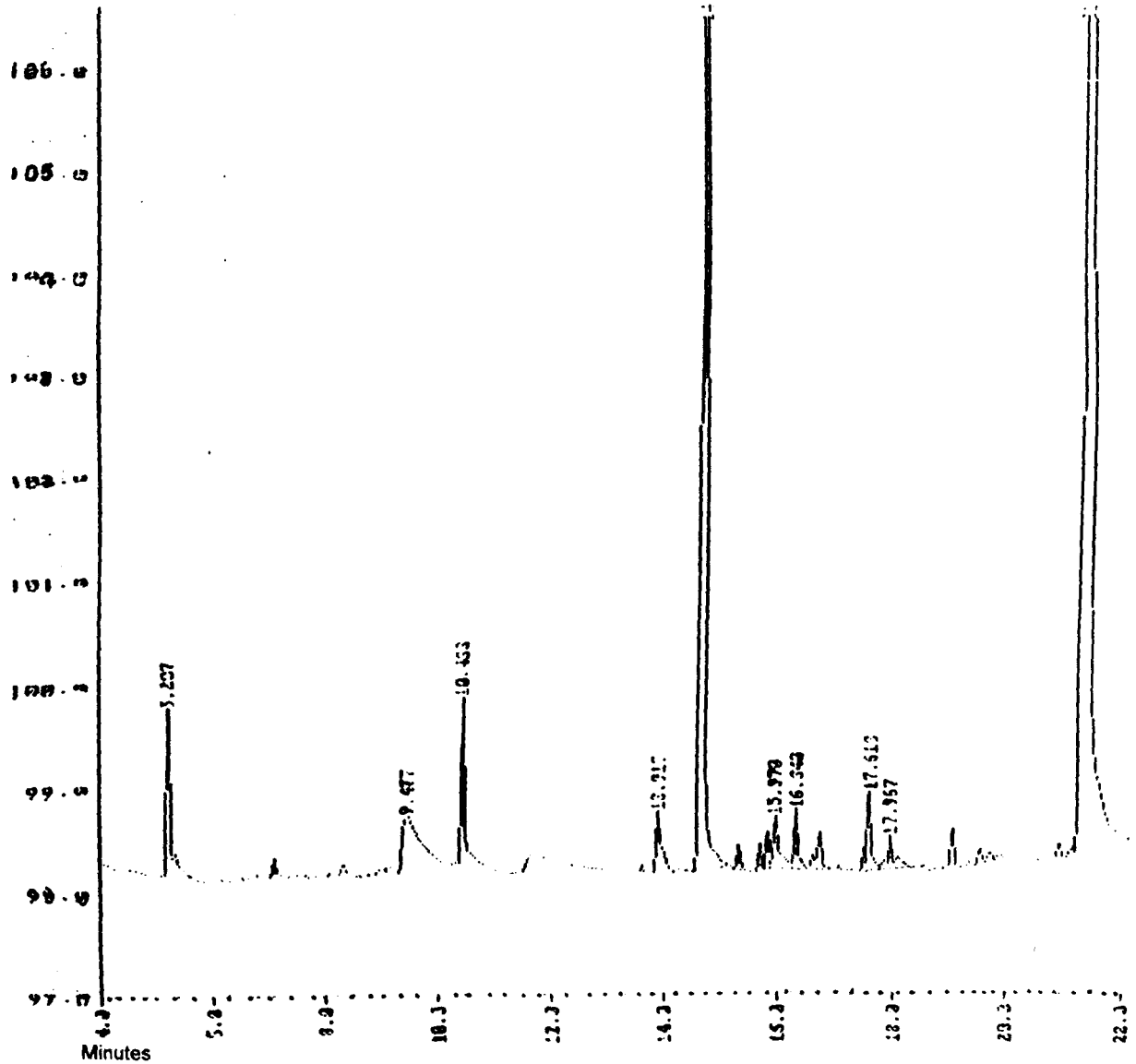


Figure 35 Chromatogram of Amitriptyline after four months storage at 25°C by GC.

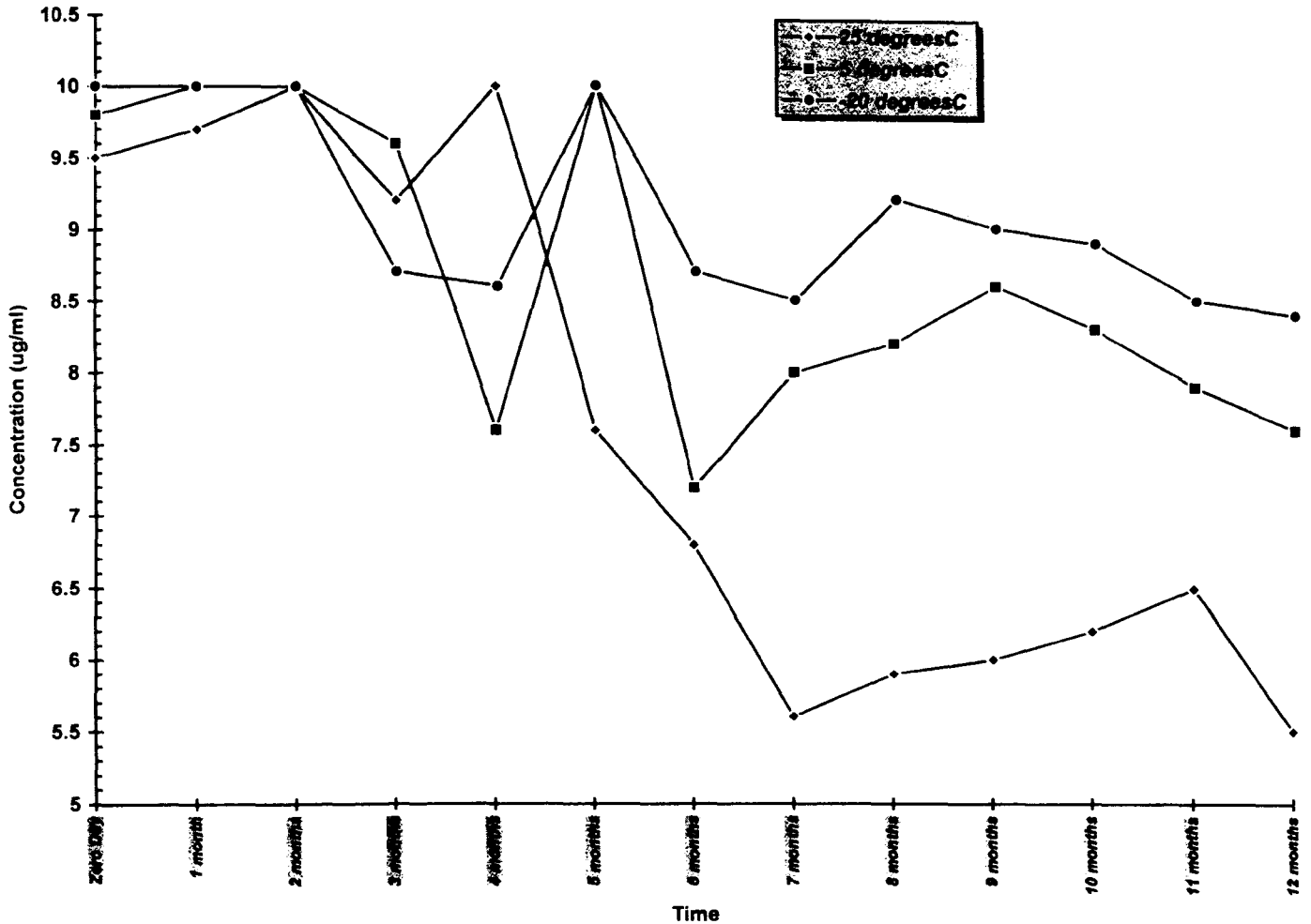


Figure 36: Changes in Amitriptyline Concentration with Time in Blood Samples Stored at 25, 5 and -20°C

4.4.11.2 Nortriptyline Stability

The result of blood solution supplied with Nortriptyline stored at the three different storage temperatures and analysed in the designated time are summarised in Table 31.

Table 31: **The Concentration of Nortriptyline (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.**

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at –20°C
Zero Day	9.4	10	9.6
1 month	6.3	8.7	10
2 months	5	10	10
3 months	5.0	8.4	8.7
4 months	4.7	6.9	7.9
5 months	4.3	6.5	6.9
6 months	4.3	6.1	7.1
7 months	4.0	5.3	6.5
8 months	3.9	5.3	6.5
9 months	4.1	5.1	6.1
10 months	3.5	4.9	5.5
11 months	3.1	4.9	5.7
12 months	2.5	4.6	5.4

At 25°C the blood Nortriptyline showed no stability. After six months only 43% of the drug could be recovered and after one year only 25% could be detected.

From the first month, the drop in concentration was noticed. 3100 ng/ml of the drug was lost. Up to the eighth month the loss in concentration reached 5500 ng/ml and it continued up to a year to reach 6900 ng/ml from the original concentration.

With 5°C and -20°C being more stable, more than 62% of the drug could be recovered after six months and after one year 46% and 54% were recovered at 5°C and -20°C, respectively.

Up to six months at 5°C and -20°C, the loss in drug concentration was 3900 ng/ml and 2900 ng/ml, respectively. The loss in concentration reached up to 5400 ng/ml and 4600 ng/ml from the original concentration at 5°C and -20°C, respectively, after one year of storage.

As a result of this study Nortriptyline was shown to be unstable, being most stable for those samples stored at -20°C. A significant decrease in Nortriptyline concentration was observed for samples stored at both 25°C and 5°C temperatures, with the greater and more rapid decrease occurring in the blood samples stored at room temperature.

Figure 37 shows the decline in Nortriptyline concentration for blood samples at 5°C, 25°C and -20°C.

Figure 38 shows the chromatogram of Nortriptyline after six months of storage at 5°C

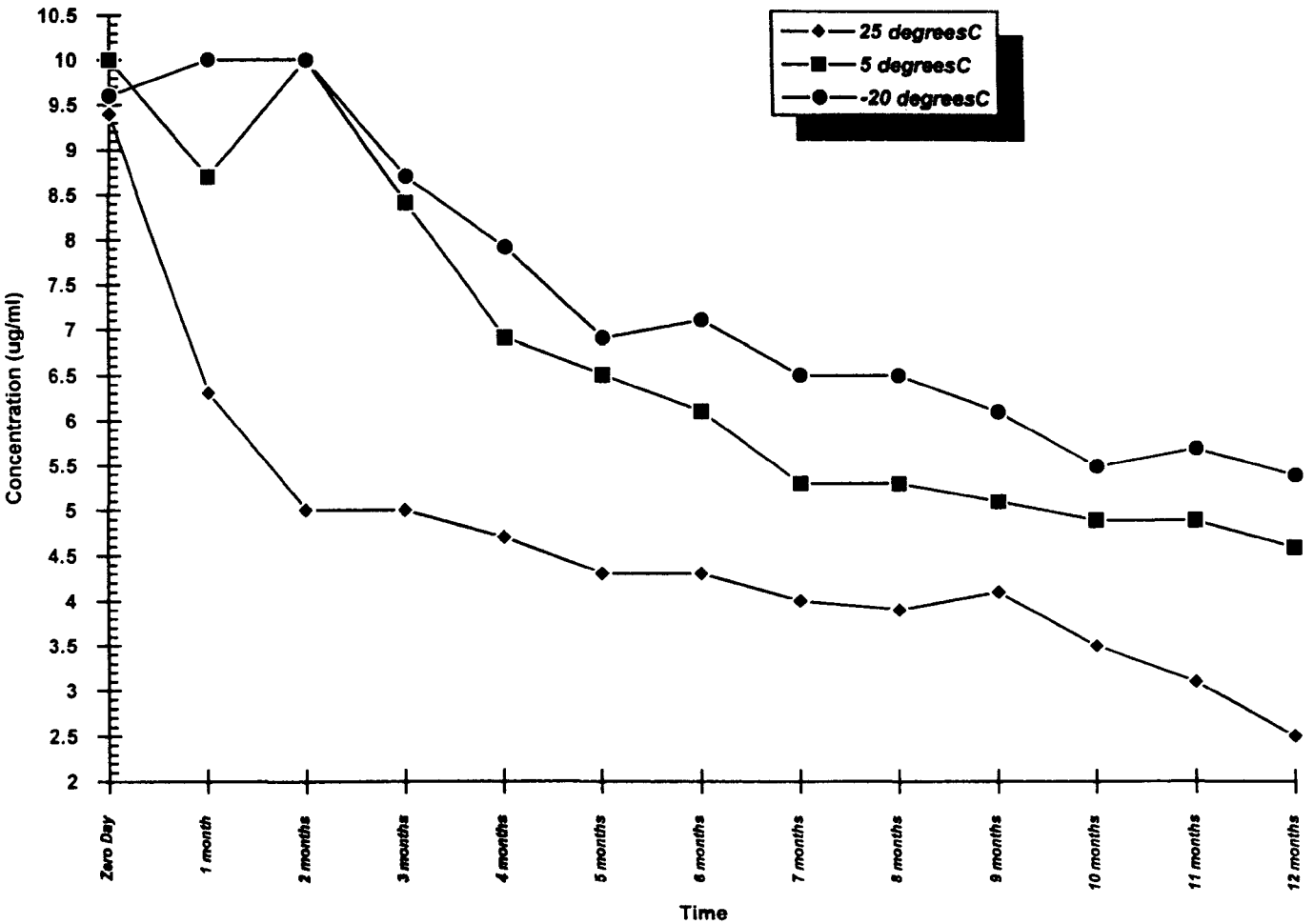


Figure 37: Changes in Nortriptyline Concentration with Time in Blood Samples Stored at 25, 5 and -20°C

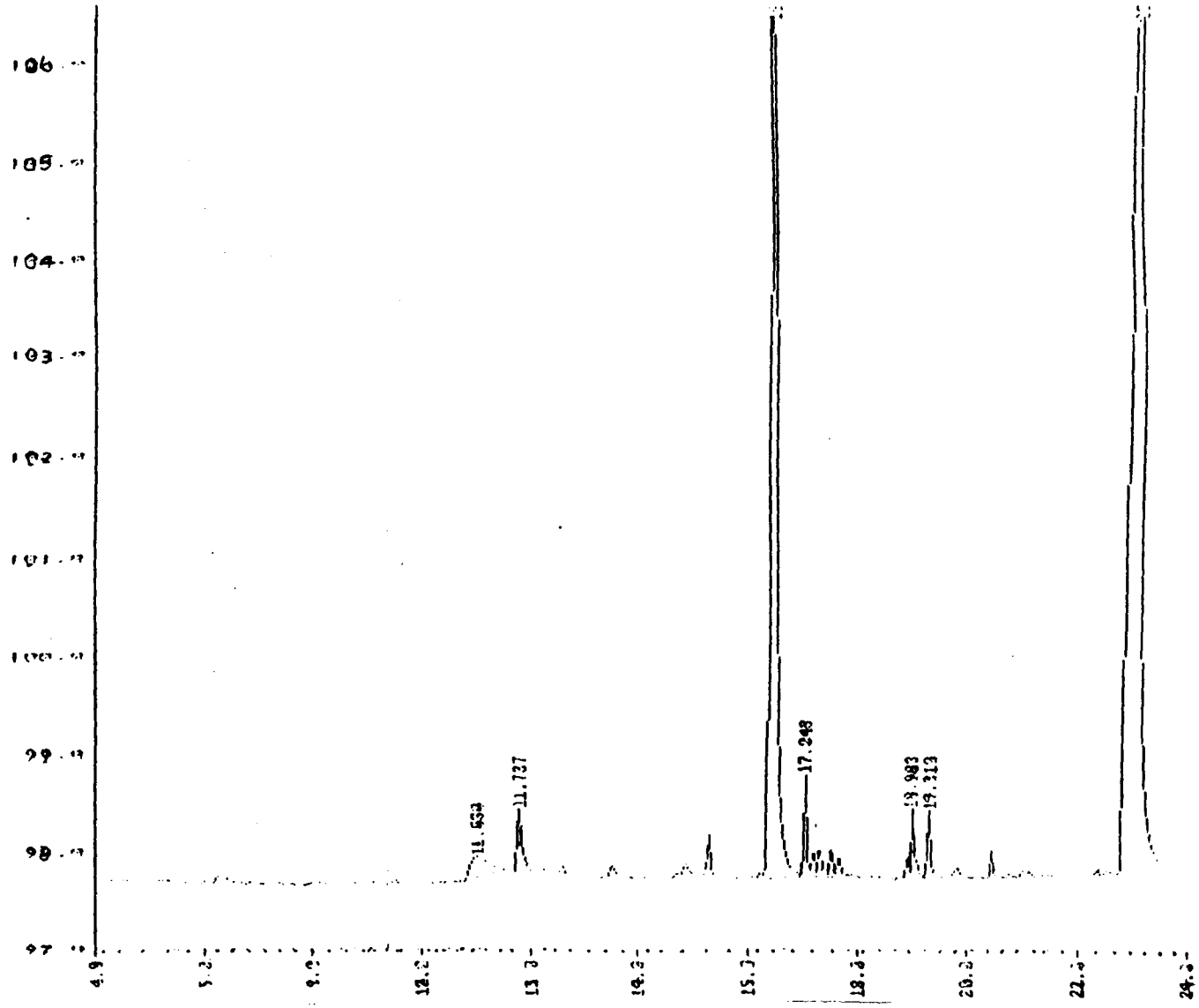


Figure 38 Chromatogram of Nortriptyline after six months of storage at 5°C by GC.

4.4.11.3 Imipramine Stability

Imipramine stability in blood was investigated where 'spiked' samples of blood with Imipramine at a concentration of 10 µg/ml from day zero until twelve months.

The results of the blood solution 'spiked' with Imipramine stored at the three storage temperatures was analysed at the designated time and are presented in Table 32.

Table 32: **The Concentration of Imipramine (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.**

Storage	Concentration When Stored at 25°C	Concentration When Stored at 5°C	Concentration When Stored at –20°C
Zero Day	9.5	9.7	10
1 month	6.6	10	10
2 months	4.8	6.7	10
3 months	3.3	10	8.4
4 months	9.1	6.6	7.8
5 months	6.9	7.3	8.4
6 months	6.6	6.4	7.7
7 months	5.3	7	8
8 months	5.1	6.7	7.8
9 months	4.9	6.4	7.5
10 months	4.3	6.1	6.9
11 months	4.6	5.5	7.1
12 months.4	4.4	5.4	6.9

Figure 39 shows Imipramine concentration after nine months of storage at -20°C.

Figure 40 shows the decline in Imipramine concentration from day zero up to twelve months when stored at the three different temperatures (5°C, 25°C and -20°C). Samples stored at 25°C declined unsteadily. At twelve months only 44% of the drug could be detected. The samples that were refrigerated showed stability up to the seventh month where 70% of the drug could still be recovered and even after one year of storage more than 50% of the drug could still be recovered. The same is the case for the samples stored at -20°C where 69% of the drug could be recovered after one year of storage.

At 25°C a very fast drop in concentration was noticed for the first three months to reach 6200 ng/ml in concentration. A sudden rise in concentration was noticed at the fourth month, then the concentration dropped again to reach an amount of 5100 ng/ml of the original concentration. The high drop in concentration at the start might be due to a high binding of the drug to the blood proteins especially at the second and third months. At 5°C and -20°C the drop in concentration was not as high as the sample stored at 25°C. At 5°C the loss in concentration reached 4600 ng/ml of the original concentration when stored for up to a year while at -20°C the loss in concentration was less; it reached 3100 ng/ml after twelve months of storage.

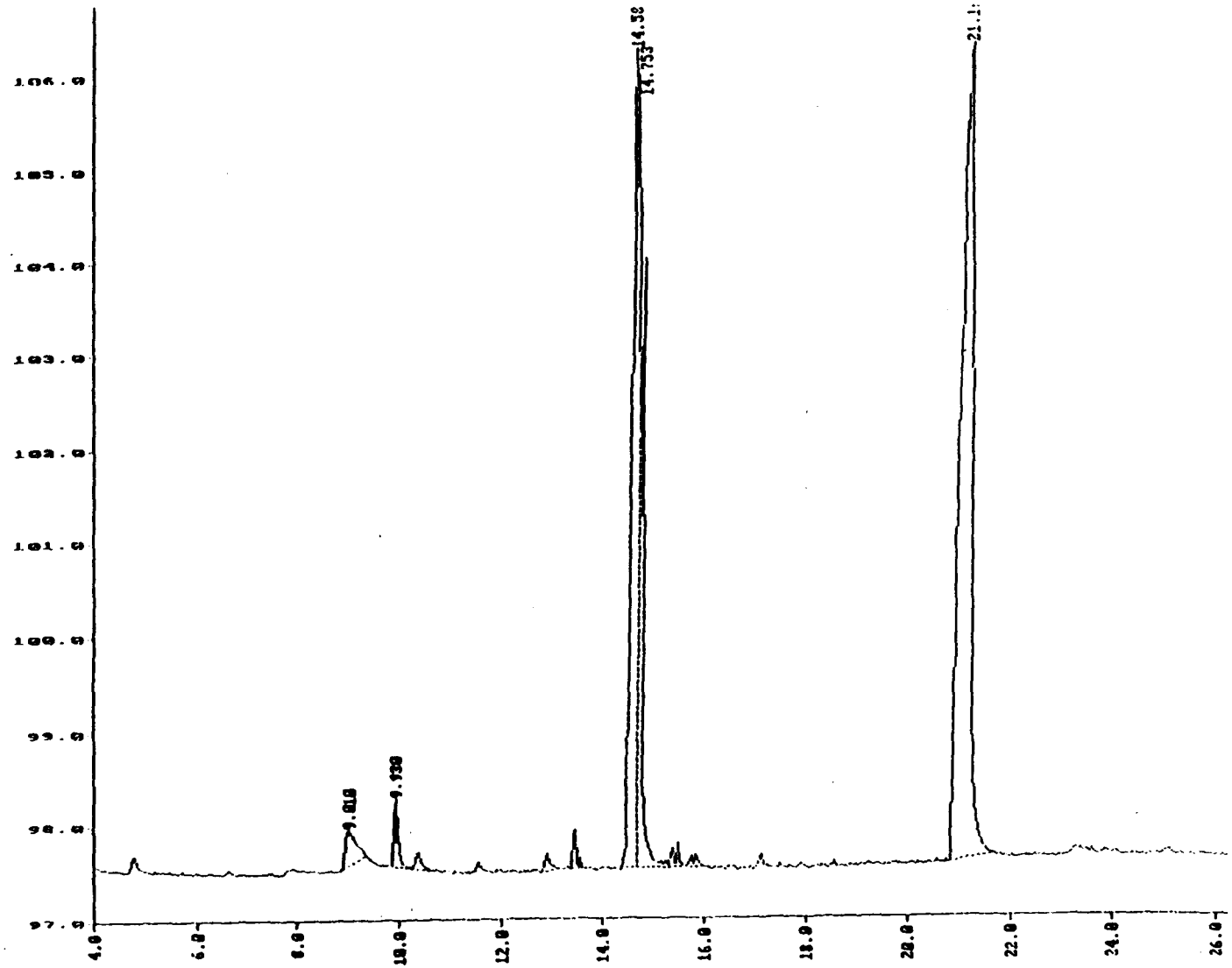


Figure 39 **Chromatogram of Imipramine after nine months of storage at -20°C by GC.**

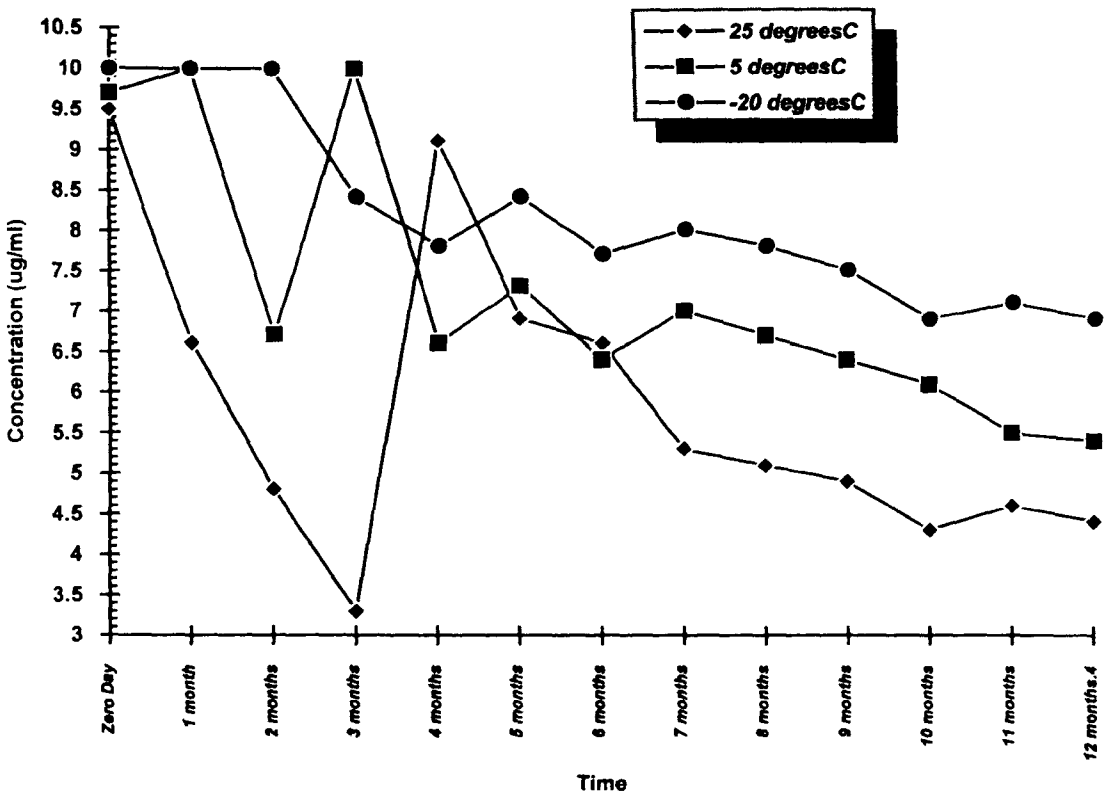


Figure 40: Changes in Imipramine Concentration with Time in Blood Samples Stored at 25, 5 and -20°C

It is concluded from this study on Imipramine that this drug is relatively stable when stored at 5°C and -20°C. It could be detected for up to one year at any stored temperature.

4.4.11.4 Chlorpromazine Stability

Blood samples 'spiked' with Chlorpromazine at a concentration 10 µg/ml stored at the three storage temperatures were analysed at designated times. The results are tabulated in Table 33.

Table 33: **The Concentration of Chlorpromazine (µg/ml) in Blood Solution Stored at 5, 25 and -20 Degrees Centigrade from Day Zero up to 12 months.**

Storage	Concentration When Stored at 25°C	Concentration When Stored At 5°C	Concentration When Stored at –20°C
Zero Day	9.1	9.3	9.1
1 month	10	10	10
2 months	10	10	10
3 months	5.5	8.5	10
4 months	7.6	8.8	9.1
5 months	4.5	7.9	10
6 months	4.7	7.5	8.8
7 months	3.7	7.3	10
8 months	3.6	6.9	8.7
9 months	3.4	6.4	8.5
10 months	2.9	6.7	7.1
11 months	2.5	5.3	6.9
12 months	2.5	4.9	6.4

A steady decrease was noticed for blood Chlorpromazine at the three storage conditions.

Figure 41 shows Chlorpromazine concentration after twelve months of storage at 5°C. After six months of storage 47% of the drug could be recovered and after one year only 25% could be detected. At 5°C and 20°C the drug was relatively stable being able to detect 75% and 88% at 5°C and -20°C, respectively, after six months of storage and still after one year 64% could be detected for the samples stored at -20°C.

At 25°C the loss in drug concentration was 2400 ng/ml from day zero up to the fourth month. After the fourth month the drop in drug concentration was very fast and reached a level of loss of 7500 ng/ml of the original concentration after one year of storage. The loss in concentration was lower for the samples stored at 5°C and -20°C. The loss after ten months was 3300 ng/ml for the samples stored at 5°C and 2900 ng/ml for the samples stored at -20°C.

Figure 42 shows the decline in Chlorpromazine concentration in blood samples stored at 5°C, 25°C and -20°C.

The conclusion from the Chlorpromazine study is that the best blood stability is for the samples stored at -20°C, being followed by those stored at 50°C and the least stable being the samples stored at room temperature.

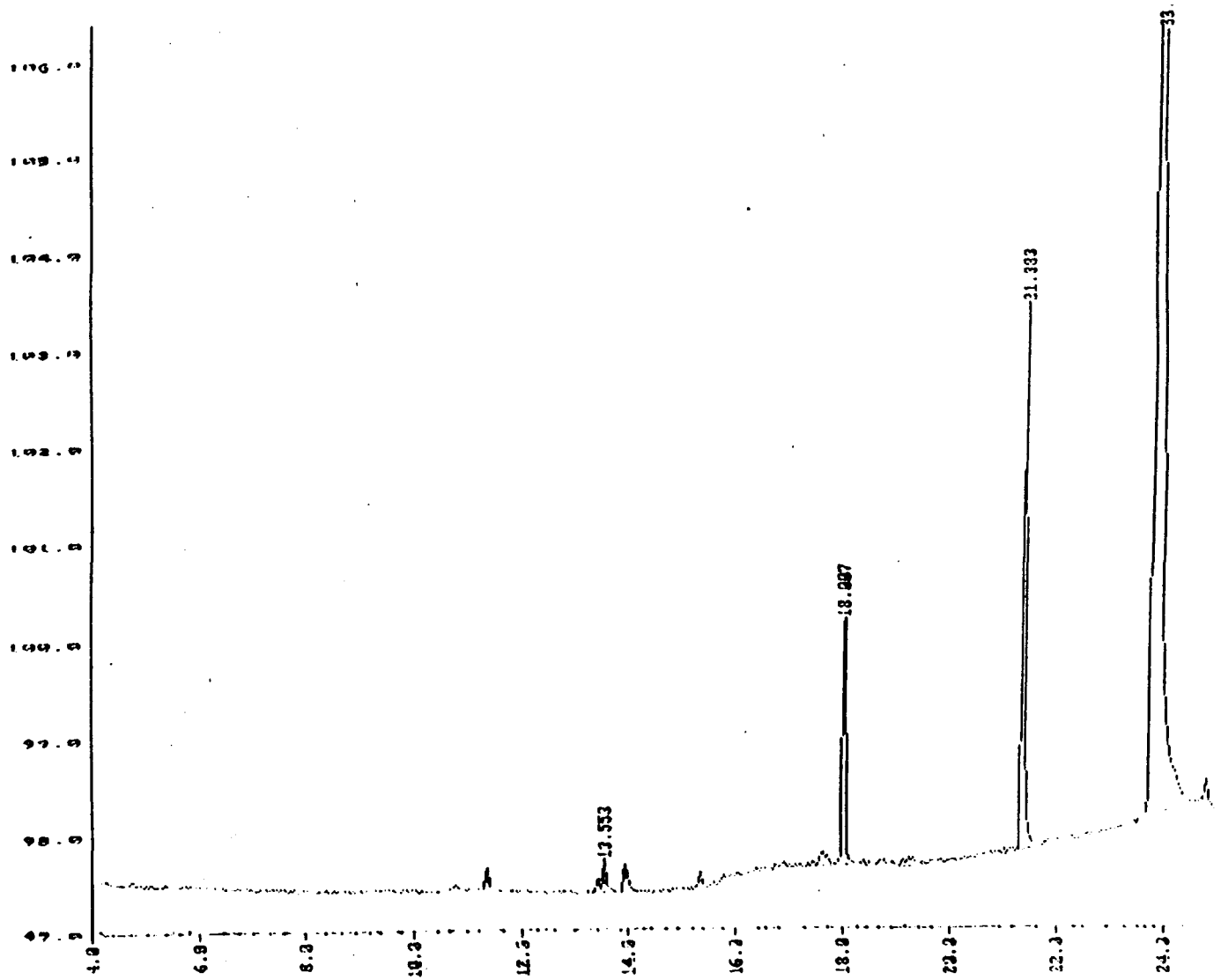


Figure 41 **Chromatogram of Chlorpromazine after twelve months of storage at 5°C by GC.**

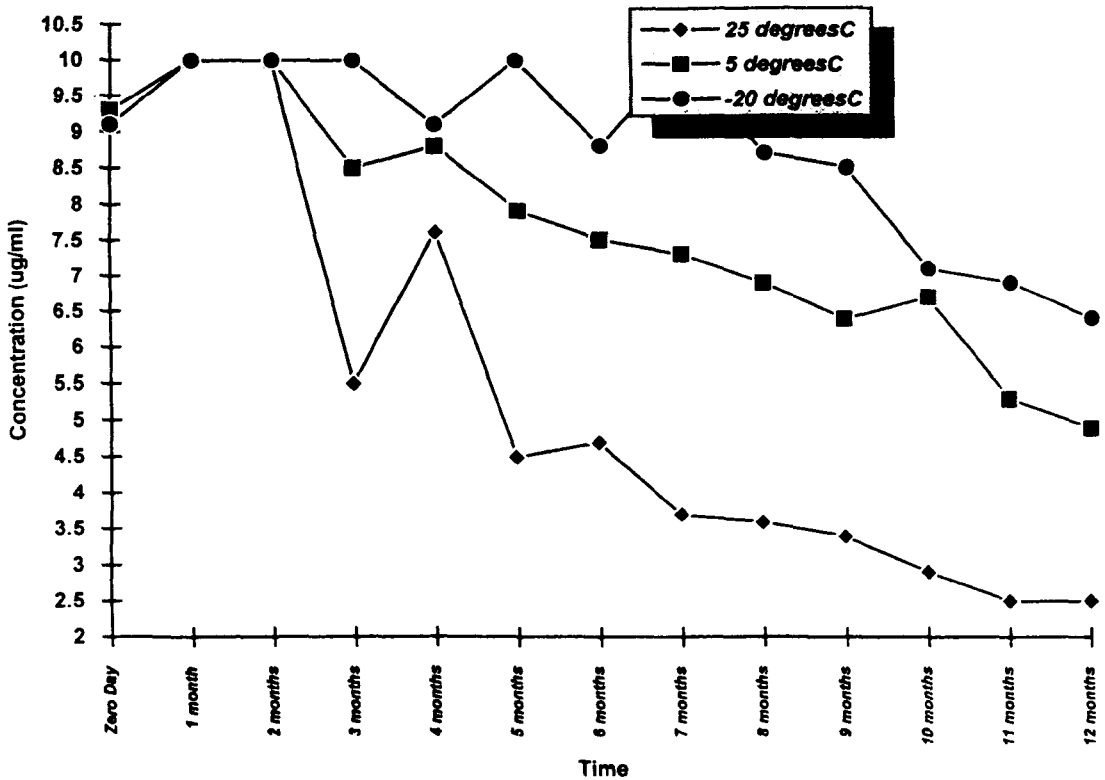


Figure 42: **Changes in Chlorpromazine Concentration with time in 1 blood samples stored at 25, 5 and -20°C**

4.4.12 TESTING FOR BREAKDOWN PRODUCT OF TCA THROUGH GC-MS SYSTEM

Samples taken at different times and temperatures for the four drugs (Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine) were tested after they had been tested for its change in concentration and tested for any breakdown products of its drugs. Figure 43 shows the mass spectrum of Amitriptyline at 25°C after seven months of storage. The peaks obtained are the principal peaks at m/z:58, 59, 202, 42. No unknown peaks were obtained.

Figure 44 shows the mass spectrum of Imipramine at -20°C after nine months of storage. The peaks obtained again are the principal peaks at m/z:58, 235, 85, 234, 236, 195, 193. No unknown peaks were obtained.

This shows that none of the samples had any breakdown products when analysed through GC-MS.

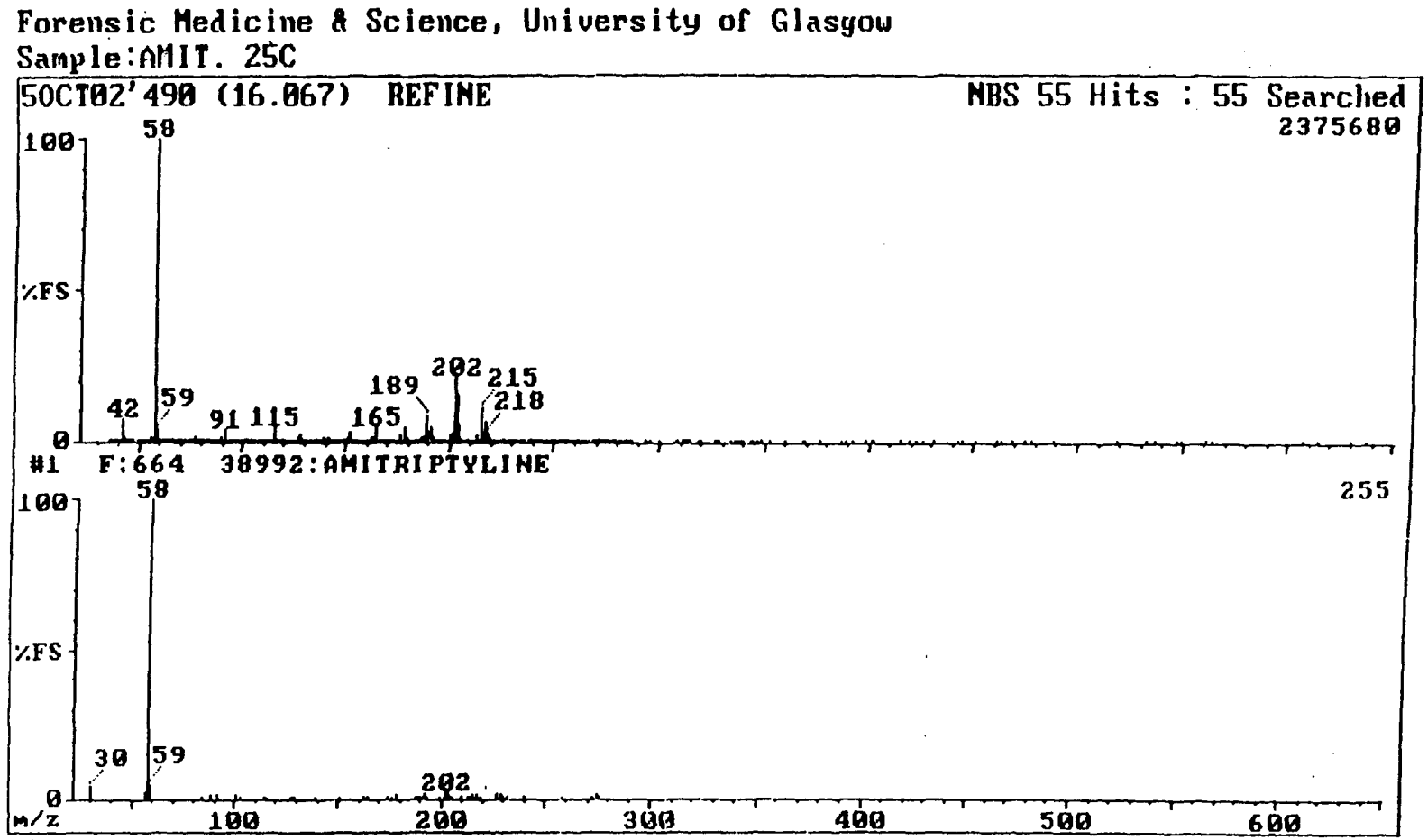


Figure 43 Mass Chromatogram of Amitriptyline at 25°C after seven months of storage.

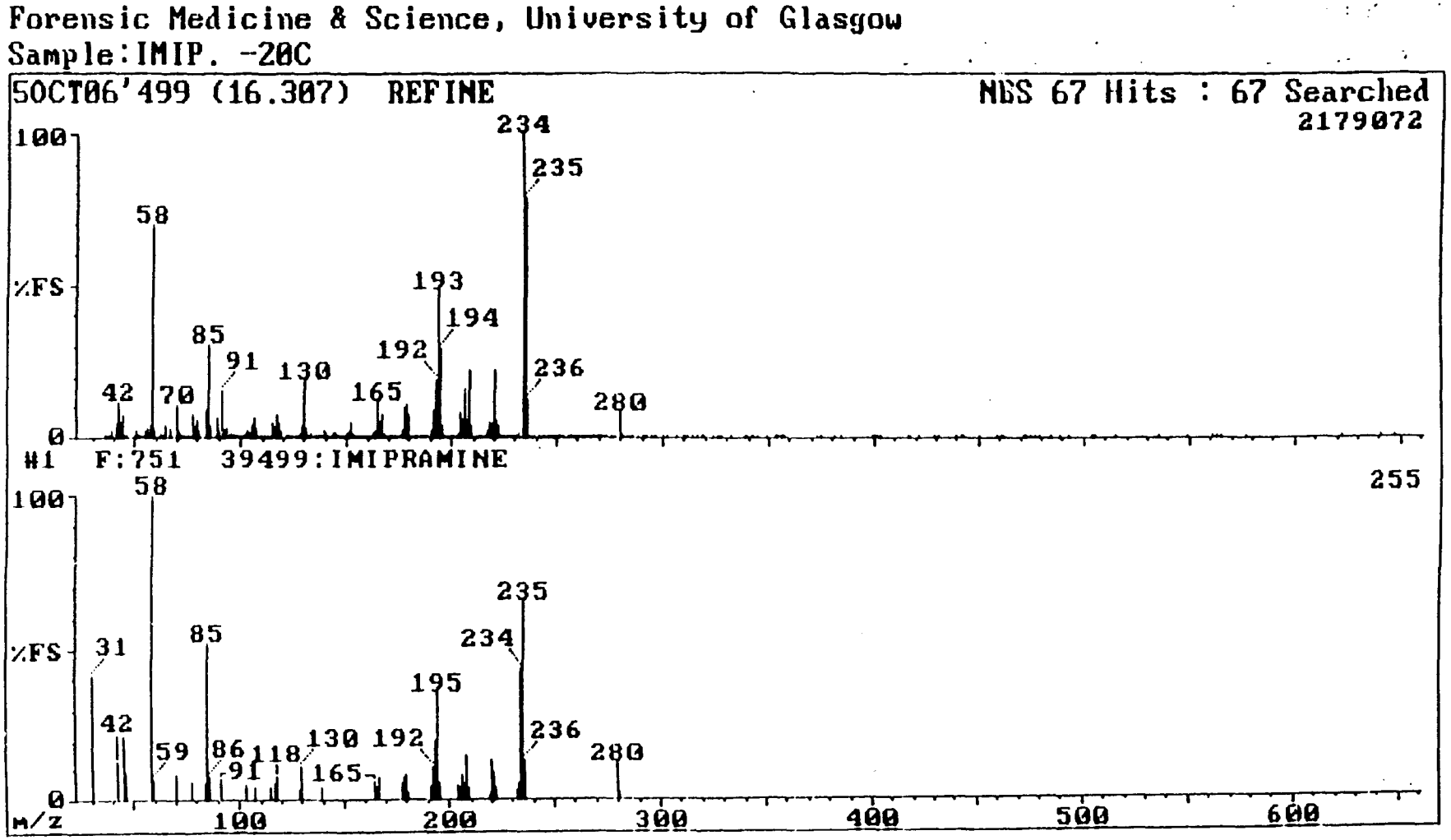


Figure 44 Mass Chromatogram of Imipramine at –20°C after nine months of storage

CHAPTER 5

CONCLUSION

This study was primarily concerned with the stability of drugs in post-mortem blood and was constructed for different periods of time under three different storage conditions. Differences in stability was noticed for different drugs due to the difference in chemical structures. The main aim of this study was to estimate the time of persistence of the drugs in post-mortem blood before they ceased to be recognised by initial screening and assay procedures. In this work the stability of some commonly encountered benzodiazepines and tricyclic antidepressants was investigated for one year of storage at three different temperatures (5°C, 25°C and -20°C). Diazepam was found to be relatively stable over a period of one year with more than 50% recovered regardless of the storage temperature. Desmethyldiazepam, Temazepam and Triazolam were stable being able to be detected at up to a year when stored at any temperature.

Amitriptyline, Nortriptyline, Imipramine and Chlorpromazine were also found to be stable under the storage conditions studied, with a steady decline in their concentration with time at up to a year. 50% of the drugs Amitriptyline and Imipramine were recovered regardless of the storage temperature.

Nortriptyline and Chlorpromazine could be detected up to a year when stored at any temperature.

The study showed that at therapeutic and toxic concentrations of the studied drugs, a reasonable amount of the drugs were still detectable after one year of storage, regardless of the temperature. This finding provides significant information for the forensic toxicologist to assist with the interpretation of results even in one year old samples.

To undertake the study, published methods for the analysis of the drugs in biological matrices were investigated. An optimised procedure for the determination of benzodiazepine drugs by reversed-phase high pressure liquid chromatography was developed. It required an initial extraction of the drugs using a solid phase extraction based on a C_8 bonded phase sorbent. Tricyclic antidepressants were determined by gas chromatography with FID/NPD detectors following an extraction method based on liquid-liquid extraction. This combination was demonstrated to be the most effective procedure to analyse for these drugs and Chlorpromazine in blood samples.

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