A TOXICOLOGICAL STUDY OF THE EFFECT OF PUTREFACTION ON THE ANALYSIS OF DRUGS OF FORENSIC INTEREST IN BIOLOGICAL MATERIAL

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by

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DEDICATION

For my father (R.I.P)

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LIST OF ABBREVIATIONS

ACD	Anticonvulsant Drugs
AC	Acid Drugs
NP	Normal-Phase
RP	Reverse-Phase
CI	Chemical ionisation
DETMDS	Diethyltetramethyldisilazine
EI	Electron impact
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectometry
HPLC	High performance liquid chromatography
MI	Molecular ion
M.Wt	Molecular weight
M/Z	Mass to charge ratio
MeoH	Methanol
SIR	Selective ion recording
ng	Nanogram
ug	Microgram
mg	Milligram
w/v	Weight/volume
ul	Micro-litre
ml	Millilitre
SPE	Solid phase extraction
DE	Diatomaceous earth
I.S	Internal standard
DEE	Diethylether

IPA	Isopropyl Alcohol
WCOT	Wall Coated Open Tubular
SCOT	Support Coated Open Tubular
PLOT	Porous-Layer Open Tubular
PEA	2-Phenethylamine
TRY	Tryptamine
TYR	Tyramine

SUMMARY

The effect of putrefaction on drug and poison determinations is a well known problem stemming from the beginning of modern forensic toxicology more than 150 years ago. The problem arises from two main sources, the occurrence of endogenously produced interfering substances and the potential destruction by the putrefactive processes of a drug or poison present in the tissue. Most of the data available was obtained using older methods for drug and poison determinations like colour tests or thin layer chromatography. Also, putrefaction was studied separately from the stability of drugs. Studies involved the accumulation of data on the occurrence of putrefactive products in different types of tissues at 5°C, 25°C or else they involved the study of the stability of drugs in putrefied tissues without regard to the concentration of the interfering substances in the specimens under investigation.

More modern studies on the effect of putrefaction on drug determination and stability have been conducted for a short time. Periods varied from a few days to a maximum of 90 days. Again, the measurement of the amounts of putrefactive products formed in the samples when they were analysed for drug stability was not done. None of the published work in this field studied the effect of putrefaction on drug determination and stability at the three different temperatures 5°C, 25°C, -20°C, where samples could be kept for a long time, ie refrigerated, room temperature or deep frozen. In this thesis, the effect of putrefaction on drug determination in whole blood spiked with the acidic drugs, phenobarbitone, phenytoin and carbamazepine and the non-acid drugs, temazepam, morphine and buprenorphine over a period of one year at three

different temperatures is studied. The occurrence of the four main interfering substances, indole, 2-phenethylamine, tyramine and tryptamine was investigated. The drug stability without putrefaction was also studied at identical storage conditions for identical periods for reference purposes.

The interference of the four putrefactive amines with drug extraction, separation and detection using HPLC with ultraviolet detection for phenobarbitone, phenytoin, carbamazepine, temazepam and was investigated.

The liquid-liquid extraction methods used for the determination of putrefactive amines, Indole, 2-phenethylamine, tyramine and tryptamine is known to be tedious and time-consuming. Methods based on solid phase extraction was developed to extract the four amines. The extraction methods showed a good reproducibility and high efficiency. A method was developed to separate these putrefactive amines by HPLC using a normal-phase column for non-acid interferences. A reversed phase system was required to achieve Indole separation and determination of acid interferences.

The formation of the four purefactive amines was monitored in blood samples spiked with drug and found to be reduced dramatically regardless of the storage temperature, when blood samples were stored in sealed vials.

At a toxic level of each drug under study a reasonable amount of the drug was found to be detectable after one year of storage regardless of the storage temperature or media. The decrease rate of each drug concentration with time at the three storage tempereatures (5, 25, -20^{o}C) was established and the feasibility of using this rate to predict the concentration after storage was investigated using authentic post-mortem samples. The possible interference from the four

putrefactive amines with the analytical methods was investigated and showed a possible interference from Indole with amylobarbitone and carbamazepine determination by HPLC system.

CHAPTER ONE

PUTREFACTION

1.1 - INTRODUCTION

Putrefaction is the result of bacterial and enzyme activity. The process begins immediately after death in the cellular structure but visible evidence is delayed for several hours under normal conditions. It becomes apparent after 48 to 72 hours. It may be hastened or delayed by one or more factors, such as atmospheric temperature and humidity [1].

The initial spread of putrefaction is largely influenced by two factors:

(a) The cause of death.

Where a person dies from acute intestinal obstruction, there is usually a frank bacteraemia before death, so that organisms from the bowel have already spread through the body.

(b) Residual Body Temperature.

The period of time that the internal temperature of the body remains above $70^{o}F$ is of paramount importance.

Both the cited factors would hasten the onset of putrefaction. The first sign of putrefaction in the cadaver is the appearance of greenish discolouration of the skin (lower right anterior abdominal wall), followed by the appearance of blue or purplish/red lines over the trunk, the root of the neck and upper arms and thighs. These lines are due to the decomposition and haemolysis of blood in the veins. As putrefaction continues, gases of putrefaction are produced in the internal organs. The composition of the gases varies according to the post-mortem interval and environment of the body [2].

Bacteria produces a large variety of enzymes and these break down the various tissues of the body, different enzymes acting on carbohydrate, fats and protein.

1.2 - POST-MORTEM CHEMISTRY OF BLOOD

With the development of automation in the hospital laboratory, ease of performance and low cost make routine post-mortem examinations of blood fluids both practical and desirable. A sufficient body of published knowledge has now been accumulated to enable an investigator to interpret post-mortem values for a wide variety of substances [3].

The glucose and total reducing substances in post-mortem blood samples collected from five sites showed no significant difference in the values between the specimens grouped according to the time interval between death and collection (12-48 hours). The values for blood from the liver were highest and those for the peripheral blood the lowest [4].

Post-mortem urea nitrogen from individuals who died suddenly were all within the normal range. The stability of urea nitrogen was demonstrated by a variation of less than 3 mg per 100 ml in over 90% of the cases [5].

A sharp post-mortem increase of the free amino acid concentration in the blood was caused by enzymatic breakdown of proteins [3]. Values of less than 14 mg per 100 ml were usually found when less than 10 hours had elapsed. Post-mortem concentration continued to increase until enzyme exhaustion with values frequently over 30 mg per 100 ml by 48 hours after death [6]. Ammonia showed a sharp rise in concentration after eight hours.

Other organic compounds

Serum fatty acid, total lipoproteins, and beta lipoproteins were all markedly stable post-mortem, showing little reduction due to autolysis, 0.5% per hour [7, 8].

In a study of 94 individuals with normal antemortem bilirubins, it was found that there was a small but definite increase in average bilirubin concentration with increasing post-mortem time (0.2 mg per 100 ml in 2 hours, 0.7 mg per 100 ml in 20

hours). The post-mortem levels of both total protein and albumin closely approximated known antemortem values [5].

Enzymes

Acid phosphatase, alkaline phosphatase, glutamic oxalic transaminase and lactic dehydrogenase showed a marked post-mortem elevation (of up to 20 times in the case of acid phosphatase) by 48 hours after death [9]. The values of alkaline phosphatase was tripled in 20 hours post-mortem [5]. Amylase glutamic oxaloacetic transaminase and lactic dehydrogenase showed post-mortem increases in their concentration with time [9] while blood cholinesterase remained stable [10]

Hormones:

Cortisol

Finlayson showed that post-mortem concentrations were the same as those during life and remained stable for at least 18 hours after death [11].

17-hydroxycorticosteroids

A study from 64 individuals on blood samples obtained between 5 to 20 minutes after death showed an elevated level of 17-hydroxycorticosteroids [12].

Epinephrine and norepinephrine

The post-mortem catecholamines in the blood of normal individuals was found to be in higher concentration than those in the blood of living patients with pheochromocytomas and as high as those in blood from cases of accidental fatal adrenalin poisoning [13].

Thyroxine and thyroid stimulating hormone

The values of thyroxine tend to fall. In contrast, thyroid stimulating hormone (TSH) remained in the normal range for one to two days after death [6].

Insulin

It has been shown that post-mortem serum insulin values are higher than those of healthy individuals and that in the same body there can be great variation in concentration depending on the source of the serum [14].

Chorionic gonadotropin

In cases of choriocarcinomas or related tumours, the post-mortem level of the chorionic gonadotropin concentration corresponded well with antemortem values [15].

Electrolytes

Chloride showed an average rate of fall (0.97 meq/litre per hour) and the same time sodium declined immediately after death with average rate of 0.9 meq/litre per hour. In both cases there was a great deal of individual variation [5]. Within an hour after death, a marked increase in potassium values was noted followed by further gradual increase in the levels due to release of potassium from the cells [16].

Calcium Phosphate

Calcium remains constant in the early post-mortem period whereas the organic phosphates and inorganic phosphates show an increase in their concentrations [17].

Sulphur Magnesium

A study of the post-mortem stability using serial sampling revealed that the inorganic sulphur in the serum remained unchanged for the first 24 hours, and then decreased by 20% in the next two days [18]. Plasma magnesium concentration values showed a mild increase, but when haemolysis occurred, the plasma magnesium increased rapidly [17].

Hydrogen ion concentration [pH]

The acidity of the blood shows an increased after death. pH values averaged 6.73 for the first 12 hours post-mortem and 6.43 for the next 12 hour period [17, 19].

CHAPTER TWO

LITERATURE REVIEW

2.1 PRODUCTION OF INTERFERING SUBSTANCES

2.1.1 - Introduction

As the autolysis of the human body progresses releasing different putrefactive products, the presence of these materials in post-mortem specimens to be analysed for the presence of drugs and poisons has been a challenge for the forensic toxicologist. Some of these substances were encountered in the spectrometric analysis of alkaloids in autopsy materials.[20, 21].

The main interfering substances have been listed by Kaempe. He described some important characteristics of these compounds like infrared and ultraviolet spectra [20].

The interfering substances are classified by Williams as either acidic or non-acidic [22]. Acidic interfering substances are those that extract or theoretically should extract from aqueous acid solution into organic solvents.

They are substances that are co-extractable with acidic drugs. Non-acidic interfering substances (non-acids) are those extracted from ammoniacal or alkaline solutions by organic solvents. Some substances like Indole are extractable at any pH and are included in both the acid data accumulation and the non-acid data accumulation.

The effective quantitative and qualitative analysis of a drug in biological material can be hindered by the occurrence of interfering substances. These interfering substances can be divided into three major groups:

1. **Naturally occurring**

These are present in the biological material as normal components. This category includes vitamins, hormones, products of diet and the end of products of various biochemical cycles.

2. **Products of putrefaction**

Putrefaction implies an intensive rotting of tissue or blood. This decay may be connected with autolysis, bacterial growth, photochemical effects or fungal growth.

3. **Artefacts**

An artefact is anything made by human workmanship. This broad definition of an artefact can describe substances produced on extraction or substances originating from a sample container.

2.1.2 Acidic Interferences

Table 1 lists the main interfering acids compounds with their predominant source.

TABLE 1.

List of acid interfering substances.

COMMON NAME	PREDOMINANT SOURCE
Butyric acids	Product of putrefaction
Cholesterol	Naturally occurring
4,4́-Dihydroxychalcone	Naturally occurring
p-Hydroxybenzaldehyde	Artefact, Product of putrefaction
p-Hydroxybenzoic acid	Product of putrefaction
5-Hydroxymethylfurfural	Artefact
p-Hydroxyphenylacetic acid	Product of putrefaction
p-Hydroxyphenylethanol	Product of putrefaction
p-Hydroxyphenylpropionic acid	Product of putrefaction
Phenylacetic acid	Product of putrefaction
Stearic acid	Naturally occurring
Succinic acid	Naturally occurring
Indole	Product of putrefaction

Phenolic acid has been noted to interfere with the determination of barbiturates [23]. Phenylacetic acid imparts a mousy odour to human urine if present. Butyric acid is found in blood when a bacterial production of alcohol has occurred [24].

Indole

Indole is the most commonly used name for the benzopyrrole in which the benzene ring is fused at the 2- and 3- positions of the pyrrole ring. The NH group of Indoles is relatively acid [25] and forms the anion in the presence of strong bases. The low melting point and moderate polarity of Indole afford good solubility in a wide range of solvents including petroleum ether, benzene chloroform and alcohol and hot water.

Indole itself has been obtained from many naturally occurring materials by methods which suggest that the Indole is in many cases the product of decomposition of its derivatives. Indole is found in human and animal faeces.

Indole is a tertiary amine and is a putrefactive base produced by the decay of protein [26]. Putrefactive bases with tertiary amine structure are important interfering substances, since they may be related to a whole series of drugs such as ergotamine and ergometrine which are derivatives of Indole. Its formation in the putrefaction of proteins is presumed to be the result of the decomposition of Tryptophan by an enzyme called 'tryptophanase' which catalyzes the direct removal of the side chain to form Indole [27], Figure 1. The first work mentioned Indole and measured its concentration in putrefied post-mortem material published in 1977. The study also showed that Indole production can be retarded by refrigeration [28].

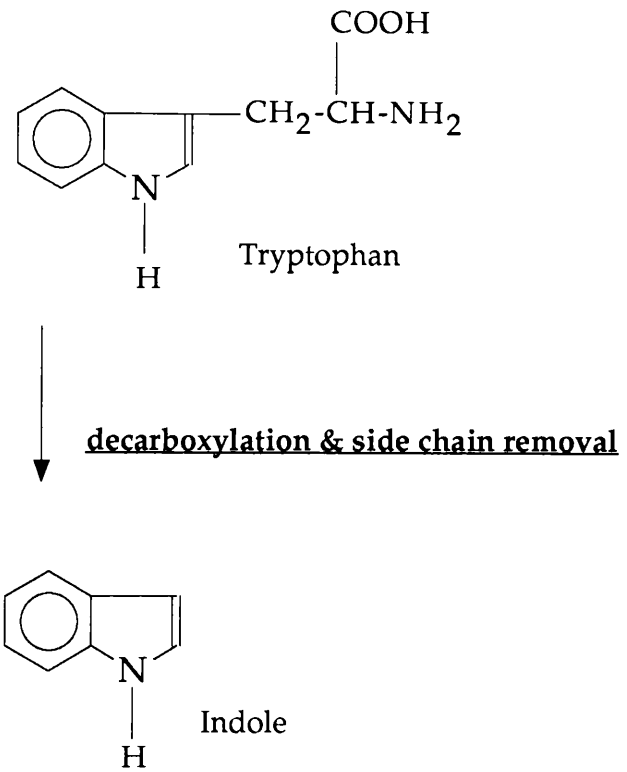


Figure 1. The decomposition of Tryptophan during the Putrefactive process to produce Indole.

Indole has been mentioned as one of the putrefactive amines and the possible interferences with basic drugs determination in blood and urine samples [29].

2.1.3 Non-Acid Interferences

These compounds are known to be present in human tissue as interfering substances from different origins such as putrefaction, artefacts, natural occurrence [20].

This investigation concentrates on the four main putrefactive bases 2-phenethylamine, tyramine, tryptamine and Indole. These compounds are products of the decomposition of phenylalanine tyrosine and tryptophan and are seen to be predominant among the list of interfering substances. The investigation of interfering substances by Kaempe and Oliver represents the most important contribution in this field [20, 30]. Table 2 lists the main interfering non-acids.

2-Phenethylamine

1-amino-2-phenylethane is an endogenous amine related structurally and pharmacologically to amphetamine. Fulton lists sixteen bases that are formed from amino acids [31]. Phenethylamine was mentioned on the list as a product of phenylalanine decarboxylation (decomposition) Figure 2.

TABLE 2.

List of non-acid interfering substances.

COMMON NAME	PREDOMINANT SOURCE
Adenine	Naturally occurring
Amines (simple)	Product of putrefaction
Caffeine	Naturally occurring (dietary)
(+)-1,4-Diphenylbutane-2,3 diol	Naturally occurring (dietary)
Harman	Artefact
Hydroxyharman	Artefact
Nicotinaminde	Naturally occurring
Nicotine	Naturally occurring
Norharman	Artefact
Perloline	Naturally occurring
1-Phenethylamine	Product of putrefaction
2-Phenethylamine	Product of putrefaction
Piperidine	Product of putrefaction
Piperidone	Product of putrefaction
Pyridine	Product of putrefaction
Thymine, Uracil	Naturally occurring
Tryptamine	Product of putrefaction
Tyramine	Product of putrefaction
Xanthine, Hypoxanthine	Naturally occurring

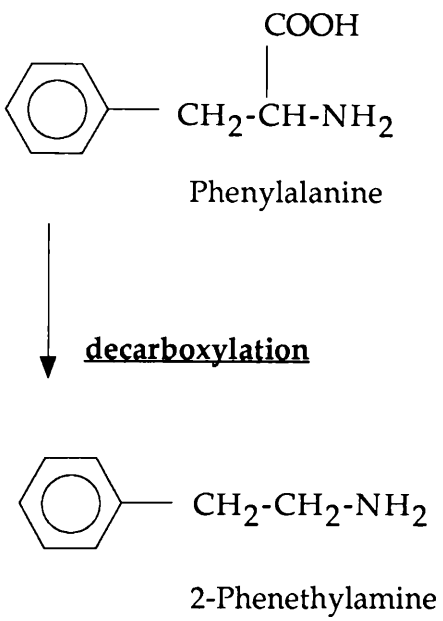


Figure 2. The decomposition of Phenylalanine during the Putrefactive process to produce 2-Phenethylamine.

2-phenethylamine can be regarded as the parent compound of a large group of drugs known as the sympathomimetic amines. The structure is modified by substitution of the aromatic ring, the alpha and beta-carbon atoms and the terminal amino group to yield compounds with a wide range of pharmacological activity [32]. 2-phenethylamine received a great deal of attention in recent years because of its possible involvement in the etiology of a number of psychiatric and neurological disorders [33]. It is present in low levels in tissues and body fluids of living mammals. Patients suffering from paranoid schizophrenia and depression excrete relatively large amounts of 2-phenethylamine in their urine [34, 35].

In forensic toxicology 2-phenethylamine has been encountered as a putrefactive product which interferes with drug determination [36]. Oliver found that 2-phenethylamine was the most frequently encountered putrefactive base and he found that it was impossible to determine the degree to which it occurs with time or predict any pattern of occurrence for the other compounds [37].

Kaempe showed that 2-phenethylamine within the wavelength range of 200-350 nm at both acid and alkaline pH displays ultraviolet absorption curves similar to those of amphetamine and dextropropoxyphene [20]. It can interfere with the separation and detection of amphetamine by gas chromatography [37].

Tyramine

Tyramine [2-p-hydroxyphenylethylamine] is an endogenous putrefactive amine formed by decarboxylation of the amino acid tyrosine, [20], Figure 3. Tyramine is sympathomimetic amine found in cheese, fermented food and red wine [38, 39]. Elevated plasma levels of tyramine have been reported in patients with hepatic encephalopathy and liver cirrhosis [40].

Tyramine was encountered as an endogenous basic compound which appeared at seven days in macerated human liver left in an open jar [41]. Also tyramine has been identified in a one month old blood sample [37]. A high concentration of up to 500ug of tyramine was found in 50g of putrefied liver tissue sample [42]. The concentration of tyramine was shown to be independent of the state of the tissue (whole or minced) and of the place where the organ was kept [in the cadaver or in a glass tube at 4-5^{o}C. Its concentration was not significantly different in individuals dying during the warm season from those dying at other times. Kaempe also noticed that Tyramine occurred more frequently and in higher concentrations after fairly long storage of the specimen [43]. Tyramine concentrations ranging from 3 to 10 ug per 1g of liver have not been noticed until

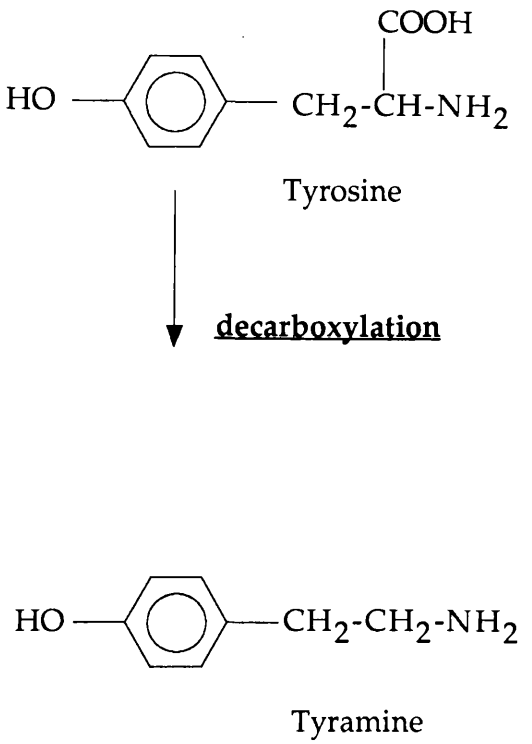


Figure 3. The decomposition of Tyrosine during the Putrefactive process to produce Tyramine.

after storage for 14 days at 4-5^{o}C. Tyramine was found in 25% of the specimens, stored at -18^{o}C for 18 months but at low concentrations of about 0.4 ug per gram of liver. Using polarographic methods for the determination of morphine in a putrefied specimen, the presence of tyramine in the sample at 10 ug/gm gave a false positive morphine concentration of 1.7 ug/gm, [20].

Tryptamine

Tryptamine [3-(2-aminoethyl) indole] is a decarboxylation product of the amino acid tryptophan [31]. Its structural formula can be seen in Figure 4. Tryptamine metabolites in human 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA) and indole acetic acid (IAA) are important in diagnosis of pathological phenomena such as depression, carcinoid syndrome and essential hypertension [44].

Kaempe noticed that tryptamine interfered with the spectrophotometric estimation of ordinarily occurring alkaloids [43]. In a later study he found that tryptamine extracted with non-acid drugs from a strong alkaline solution displayed the ultraviolet absorption characteristics typical of Indole in aqueous acidic extracts [20]. Others [45] found that tryptamine interfered with desipramine determination in blood using gas chromatography. Oliver detected the presence of low concentrations of tryptamine in human tissue after putrefaction for 12 days at 21^{o}C [28]. Stevens found that tryptamine caused serious distortion of the ultraviolet spectra of some drugs and therefore tryptamine required to be separated from the drug [41].

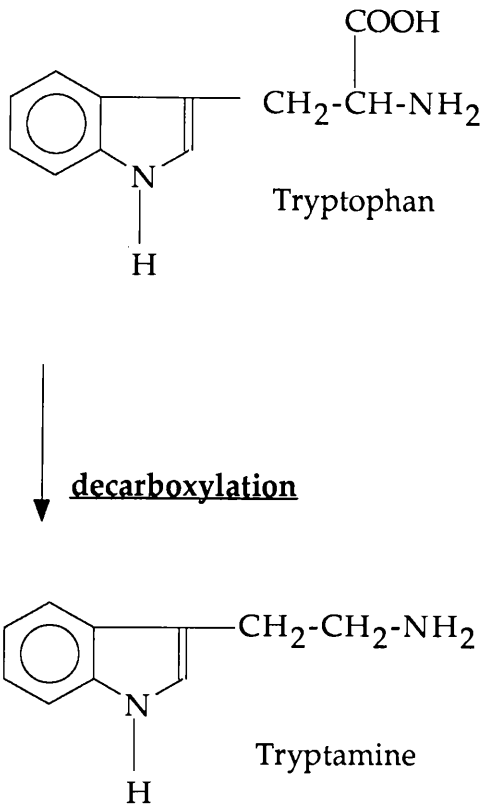


Figure 4. The decomposition of Tryptophan during the putrefactive process to produce Tryptamine.

2.2 PUTREFACTION EFFECT ON DRUGS

2.2.1 Introduction

The effect of various stages of putrefaction on drug concentrations in autopsy specimens is largely unknown. Attempts to study this problem date back to 1942. The early studies are to be considered unreliable because of the poor sensitivity of the methods used. Meaningful quantitative methods were not produced until the mid 1950's.

In one study, liver from humans who had died from barbiturate poisoning was allowed to putrefy in air for three months. Another portion of liver was fixed in formalin embalming fluid. The barbiturates were analysed by ultraviolet and paper chromatography. Unchanged barbiturates were recoverable from the liver after three months of putrefaction and after five-and-a-half months from liver fixed in formalin [23].

In a report of a fatal intoxication with chlorpromazine, putrefaction was shown to influence the toxicological analysis. A non-refrigerated blood sample analysed after six months showed a negative result, while at the time of death it showed a fatal dose concentration [46]. In this work nothing was mentioned about the detection of putrefaction products. Post-mortem changes in drugs of abuse like methamphetamine and amphetamine have been investigated [47]. The study showed a marked increase of amphetamine concentration in the liver samples after one month. This remained high at the end of a two year interval where the samples were stored in a sealed container. Methylamphetamine concentration in the muscle after one month of storage increased by about 4.5 times. In skin the increase was about 2.5 times over the initial level. This rapid increase could be

explained in terms of the weight decrease due to the loss of water in the samples when preserved in open air.

In a study on dogs' blood, the effect of temperature on putrefaction and subsequent barbiturate determination was investigated. A remarkable difference between specimens kept in the refrigerator at $4^{o}C$ and those affected by the onset of putrefaction at room temperature was observed. Also, there was a remarkable difference between specimens preserved under the same ambient conditions but in different containers, [48, 49]. The influence of putrefaction changes on the determination of the herbicide paraquat, showed a decrease in paraquat recovered from the stomach with time after death [50].

The post-mortem stability of some benzodiazepines has been studied over 90 days in blood and tissues at two different temperatures, $4^{o}C$ and $25^{o}C$ [51]. It was found that diazepam, flurazepam and N-1-desalkylflurazepam were stable when stored in blood at room temperature, while chlordiazepoxide, norchlordiazepoxide and nordiazepam were found to be unstable under similar storage conditions for both blood and tissues. No reference was made to the presence of putrefactive products or their interferences with the benzodiazepine determinations.

The most recent study [52] was undertaken to examine the stability of some antiepileptic drugs, benzodiazepines, carbon monoxide and cyanide in formalin-fixed tissues and formalin-blood solutions at toxic concentrations of the drugs. The study showed that the drugs can be measured in the samples up to 30 days after this treatment. The analysis for cyanide and carboxyhaemoglobin was significantly impaired in the presence of formaldehyde.

The behaviour of 56 drugs and drug-related compounds containing various molecular structures in putrefied human liver has been investigated [41]. The samples were placed in an open shed to permit the access of blow-flies. Drugs containing nitro groups, N-oxides and oximes were found to be liable to putrefactive decomposition within 3 to 14 days, while the stability of other drugs indicated that there was a general resistance of carbon-oxygen, carbon-nitrogen, nitrogen-hydrogen and sulphur-oxygen bonds to change. Putrefactive amines showed some interferences with the morphine determination. Tyramine and 2-phenethylamine were encountered as putrefactive amines in the later stages of putrefaction.

By the mid-60's early 70's there was a growing interest on studying the occurrence of putrefactive products in putrefied human tissues. Kaempe listed the main interfering substances with a description of some important characteristics such as infrared and ultraviolet spectra [20]. Oliver and Smith investigated the interference of sixteen putrefactive bases in the analysis of biological materials for drugs. They found that 2-phenethylamine was the most frequently encountered putrefactive base. They could not however, predict the degree to which it would occur with time or predict the occurrence of the other compounds. Analytical data for the identification of sixteen putrefactive bases was presented. This included gas chromatography retention times, thin layer chromatography Rf values and ultraviolet spectral data [37].

Williams studied the interfering substances that are extractable from aqueous biological fluids with organic solvents. In addition to the above he used the technique of infrared spectroscopy and mass spectrometry [22].

Fulton discussed the formation and occurrence of putrefactive bases and pointed out that many were the products of the decarboxylation of amino acids [31]. Most of the decomposition products of amino acids are water soluble. The decomposition products of just three amino acids, tyrosine, phenylalanine and tryptophan seem to be predominant among the lists of interfering substances.

2.2.2 - Acidic Drugs

2.2.2.1 - Anticonvulsants

In the literature, there is little data regarding the influence of putrefaction on the determination of acidic drugs. Most of the information available is focused on barbiturates [23, 48, 53], and the findings are contradictory. Barbiturates were found to be stable in putrefied liver after 3 months of putrefaction [23]. Other studies concluded that putrefaction increases the concentration of barbiturates in blood, and liver when stored at room temperature due to protein-bound drugs being released [53, 54].

Coutselinis and Kiaris found a fluctuation in the barbiturate concentration of the specimens kept in the refrigerator. They observed, however, a reduction in the concentration of barbiturates in putrefied blood specimens kept at room temperature for up to two months [48].

The above contradictory findings with barbiturates was from the use of ultraviolet spectrometry to measure the drugs in aqueous solution following extraction from

putrefied blood or tissue into organic solvents. This technique is no longer in use in the modern forensic toxicology laboratory for either quantitative or qualitative analysis of barbiturates since it is not conclusive [53].

A gas chromatograph was used for qualitative and quantitative determination of barbiturates in putrefied blood and tissues [55] following a multistep extraction of barbiturates from decomposed tissue as a clean up procedure. Still, it was found that the resulting co-extraction of interfering products rendered the quantitation of these drugs by GC impossible. When a slightly more polar solvent was used for the initial extraction, the problem was alleviated. In blood and liver greater than 75% of the drugs were detected at the end of the two-to-three month period when stored at $4^{o}C$ and $25^{o}C$.

In clinical use phenytoin and carbamazepine, as anticonvulsant drugs, are as important as barbiturates. Phenytoin has been studied to determine its stability in stored serum controls and in samples shipped through the mail. It was observed that a 20% decrease in plasma phenytoin concentration occurred when stored at $4^{o}C$ for eight weeks [56]. Schafer reported no decrease in the concentration of phenytoin when stored in serum at $4^{o}C$ for about 12 weeks [57]. Phenytoin and other antiepileptic drugs were found to be stable in plasma when stored at room temperature (without being exposed to light) for at least six months [58].

There is no data available regarding the effect of putrefaction products on the phenytoin determinations or its stability in post-mortem blood.

Carbamazepine has been mentioned in only one study by Stevens. He studied the stability of carbamazepine in putrefied human liver stored for just 13 days at $25^{o}C$. The study showed carbamazepine to be stable in aqueous control solutions and in

the liver macerates after 13 days of storage at 25°C [41]. There is no record in the literature on the effect of putrefactive products on carbamazepine determination or on the stability of the drug for a longer period of time or on the effect of other storage conditions, 5°C - 20°C on its stability. The stability of the drug in post-mortem blood has not been reported.

2.2.3 Non-acid drugs

2.2.3.1 Benzodiazepines

The stability of benzodiazepines in putrefied post-mortem specimens was studied in the 1970's [59]. The investigation of flurazepam, diazepam, desmethyl-diazepam and chlordiazepoxide stored at 4°C for up to 120 days found that all were relatively stable except for chlordiazepoxide. This drug was shown to decompose rapidly in whole blood. It was stable in serum or plasma.

A study on the stability of four benzodiazepines in putrefying human liver macerates left to putrefy in the open air for 14 days with a shedroof to afford some protection against the weather, showed that flurazepam, lorazepam and diazepam were found to be stable while chlordiazepoxide, chlorazepam, nitrazepam and demoxepam were liable to decomposition, [41].

Desoxychlordiazepoxide and desmethyldiazepam were found in liver homogenate spiked with chlordiazepoxide and stored at 4°C for up to 20 days [60]. A study on

diazepam stability showed the plasma diazepam to be stable when stored at -20°C and 5°C up to one year and eight months respectively [61]. In a study of the effect of putrefaction products on the stability of benzodiazepines in blood and tissues at two storage conditions 4°C and 25°C over several months it was claimed that diazepam, flurazepam and N-1-desalkylflurazepam were stable when stored in blood at room temperature, while chlorodiazepoxide, norchlordiazepoxide and nordiazepam were found to be unstable under similar storage conditions in both tissue and blood, [51]. These studies were in agreement regarding the stability of benzodiazepines over the different storage conditions and the specimen types 'blood and tissue'. Studies on the effect of putrefaction on temazepam and its determination have not been recorded. Temazepam is an important drug to study since injectable temazepam has become a street drug of choice, particularly in Scotland [62].

2.2.3.2 Opiates

Aspects of the medical use of the opiate drugs have followed a typical life-cycle. Each new drug is initially hailed as a potent non-addictive analgesic agent which can safely be made available to medical practices and may even be useful in the treatment of pre-existing opiate addiction. Such was the history of morphine and heroin.

The effect of putrefaction on the determination and stability of morphine has not been thoroughly investigated. Morphine may be converted to pseudomorphine in cadavers and be found as such on exhumation, [63]. Morphine was found to be

stable in macerated human liver when stored in sealed containers for a short period of time at ambient temperatures, [41]. A false positive morphine in putrefied tissue due to the presence of putrefactive products has been reported [53], thin layer chromatography (TLC) was used to purify the extract. Morphine was detected in the putrefied liver by GC after this purification. Nothing was mentioned about the storage temperature and the time elapsed before the analysis of the liver specimens. No knowledge could be gained regarding the stability of morphine or qualitative and quantitative of interfering substances in the specimen.

Buprenorphine is often prescribed for the treatment of chronic post-operative pain and for terminal cancer patients, [64] and it has also been used for the treatment of heroin addicts, [65]. Buprenorphine is a recently marketed opiate. Most of the published work focuses on quantitative and qualitative analysis of buprenorphine by radioimmunoassay, [66, 67] or the study of its pharmacokinetics [68, 69]. No data is available regarding the effect of putrefaction on buprenorphine determination or its stability in putrefied post-mortem specimens. Therefore buprenorphine has been selected with the group of drugs chosen to be studied in this work.

2.3 DRUGS UNDER INVESTIGATION

2.3.1 - Anticonvulsant

Introduction

Anticonvulsant drugs are used chiefly in the treatment of various types of epilepsy. Epilepsy has been described as a chronic condition characterised by more or less frequent recurrence of seizures, associated with loss of or disturbance of consciousness and usually with convulsive or other body movement and correlated with an abnormal electro-encephalogram [70].

Over the centuries, many substances and concoctions have been used for the control of epilepsy, but the best early hope was offered by the recognition of bromide's antiepileptic properties in the mid-1800's and by the synthesis and clinical application of phenobarbitone in the 1912. Both substances were generally considered valuable remedies, but were not ideal. The search for better antiepileptic drugs continued into late nineteenth and early twentieth centuries, leading to the discovery of phenytoin, carbamazepine and other drugs.

Phenobarbitone

Phenobarbitone is a 5,5 substituted barbituric acid, 5-ethyl-5-phenyl barbituric acid. The well known structural formula is shown in Figure 5. It was introduced into the treatment of epilepsy in 1912 and was the first effective organic antiepileptic agent. Most other antiepileptic drugs were later developed as structural variations of phenobarbitone.

Most barbiturates have anticonvulsant properties. However, the capacity of some of these agents such as phenobarbitone to exert maximal anticonvulsant action at

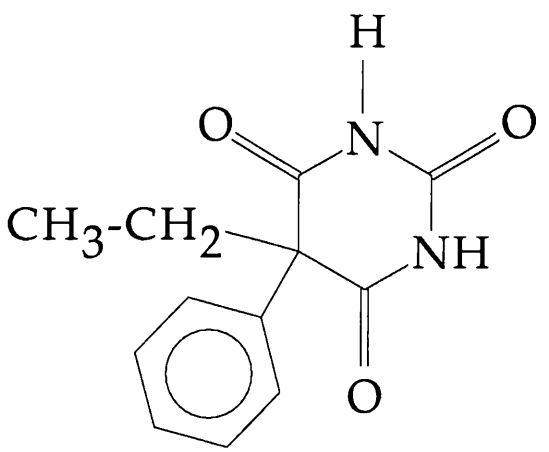


Figure 5. Chemical Structure of Phenobarbitone.

doses below those required for hypnosis, determines their clinical usefulness as antiepileptics.

Phenobarbitone is an effective agent for generalised tonic-clonic (grand mal) and cortical focal seizures. During chronic medication in adults, plasma concentrations of phenobarbitone average 10ug/ml following a daily dose of 1 mg/kg. Plasma concentrations of 10 to 25 ug/ml are usually recommended for the control of epilepsy [71] while the lethal concentration in blood is 75 ug/ml [72]. Phenobarbitone elimination rates are very low compared to other antiepileptic drugs. The major metabolites are N-glucopyranosylphenobarbitone and 4-hydroxyphenobarbitone [73]. The elimination rate is influenced by urine flow and urinary pH.

Carbamazepine

Carbamazepine, 5H-dibenz(b,f)azepine-5-carboxamide, was developed in the laboratories of J.R. Geigy AG (Basel, Switzerland) in the late 1950's. The compound was introduced as an antiepileptic drug in Europe and Australia in the early 1960's [74], and its anticonvulsant properties were demonstrated in 1963 [75]. Carbamazepine behaves as a neutral lipophilic substance. Carbamazepine is related chemically to the tricyclic antidepressants such as imipramine and clomipramine. It is a derivative of iminostilbene with a carbamyl group at the 5 position; this moiety is essential for potent antiepileptic activity. The structural formula of carbamazepine is shown in Figure 6. After oral administration of 600

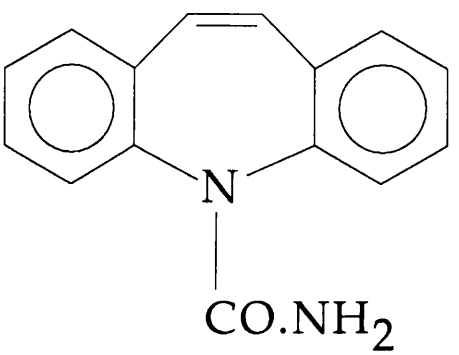


Figure 6. Chemical Structure of Carbamazepine.

to 1,200 mg/day the plasma concentration of carbamazepine and its active metabolite, carbamazepine 10,11, epoxide, may vary from 2 to 20 ug/ml without a clear relationship between the concentration of the parent compound and the metabolite [76]. Other metabolites like trans 10,11,dihydro-10,11-dihydroxy carbamazepine, and glucuronic acid conjugation also occur, [77].

Phenytoin

Phenytoin is a weak organic acid. Its generic name is 5,5 diphenylhydantoin. It has a chemical structure as shown in Figure 7. It is a primary drug for all types of epilepsy except absence seizures [71]. Phenytoin was first synthesized in 1908 by Biltz but its anticonvulsant activity was not discovered until 1938. Phenytoin was the product of a search among non sedative structural relatives of phenobarbitone for agent capable of suppressing electroshock convulsion in laboratory animals. It was first introduced for the treatment of epilepsy in 1938 [78]. Phenytoin is still one of the most widely used anticonvulsants, since it can produce effective anticonvulsant action without significant sedation [79]. Following oral doses of 100 mg three times a day, maximum steady-state plasma concentrations of 3.4 to 30.5 ug/ml (mean 11.8) were reported [80], while fatalities have been associated with blood concentrations greater than 70 ug/ml [81]. Phenytoin is partially metabolized and partially excreted unchanged in the urine. About 4% of the dose is excreted in the urine unchanged and the excretion of unchanged drug is increased when the urine is alkaline [82].

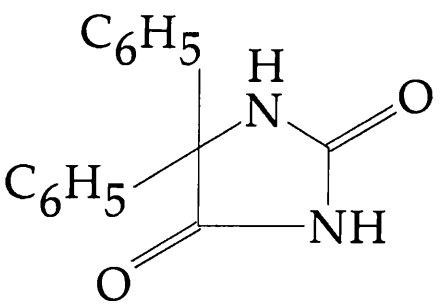


Figure 7. Chemical Structure of Phenytoin.

2.3.2.- Benzodiazepines

Introduction

The term benzodiazepine refers to the position of the structure composed of a benzene ring (A) fused to a seven-membered diazepine ring (B). The structure of benzodiazepines are illustrated in Figure 8 with the structure of temazepam. Benzodiazepines have been widely used as tranquillisers, anti-anxietics, sleep inducers, antiepileptic and hypnotics since the early 1960's. It also can show amnesiac action. More than 300 benzodiazepines have been synthesized and 25 are in clinical use in various parts of the world [83]. Prescribing rates of benzodiazepines increased tenfold from the age of 20 to 70 years and were higher in women than in men, [84].

Temazepam

Temazepam is 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzo-diazepin-2-one. The structural formula is shown in Figure 8.

Temazepam is used as a hypnotic in the short term management of insomnia in doses of 10 to 30 mg and in premedication before minor surgical or investigative procedures [85].

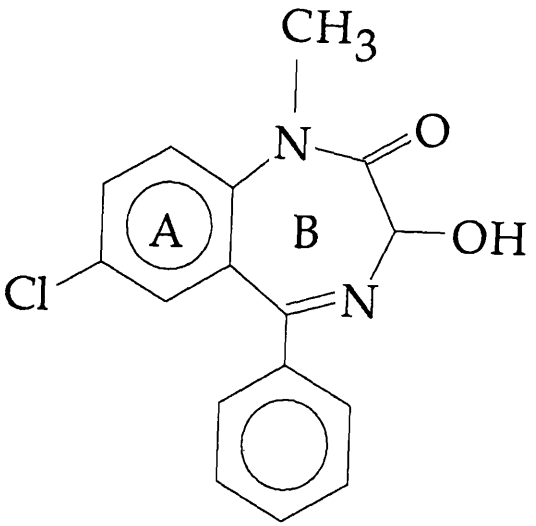


Figure 8. Chemical Structure of Temazepam(A and B represent the core Benzodiazepine).

Therapeutic concentrations 0.22 to 0.75 ug/ml (mean 0.5) were attained in about two hours. After a single oral dose of 30 mg [86], about 97% of Temazepam binds to protein. About 80% of a dose is excreted as the glucuronic acid conjugate. Temazepam has become a street drug of choice [62].

2.3.3 Opiates and Opium

Introduction

Opium, the dried, compressed resin from the seed case of the poppy Papaver somniferum (papaveraceae), has been recognised as a pain-killer for over 4,000 years. The word opium itself derived from the Greek name of juice of the poppy. Opium contains more than 20 distinct alkaloids. In 1803, a German chemist W.W. Serturner isolated from opium, an alkaloid which he named morphine. The discovery of other alkaloids in opium quickly followed that of morphine; codeine by Robiquet in 1832, papaverine by Merck in 1848 [87]. By the middle of the nineteenth century the use of pure alkaloids began to spread throughout the medical world. The problem of addiction to opioids stimulated a search for potent analgesics that would be free of the potential to produce addiction. This led to the discovery of new drugs such as the relatively pure antagonist naloxone and compounds with mixed actions (butophanol and buprenorphine). Such drugs helped in understanding the action of the opioids through the discovery of opioid receptors.

Drug abuse as a significant social problem has only existed in Britain since the early 1960's and drug use in Britain until then was exceptional only for its trivial extent [88].

Morphine

Morphine is the principle alkaloid of opium. Its structure shown in Figure 9. Morphine acts as an agonist with steriospecific and saturable binding sites in the brain and other tissues [87]. These sites are widely but unevenly distributed throughout the central nervous system. It is believed that morphine, its analogs and antagonists occupy the sites normally occupied by B-endorphine and the enkephalins. These are endogenous peptides which act as neurotransmitters or modulators of neurotransmission.

Morphine and related opioids produce their major effect on the CNS and the bowel. Morphine produces analgesia, drowsiness, changes in mood, respiratory depression, nausea, vomiting and alteration of the endocrine and autonomic nervous systems [87]. Other effects are dilatation of the pupils and dilation of the surface capillaries causing flushes. Toxic effects follow from therapeutic effects. An increase in respiratory depression can lead to coma and so to death. An increase in smooth muscle relaxation can then lead to muscle spasm and convulsions.

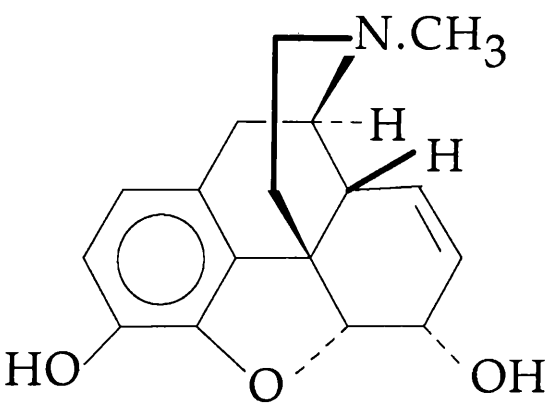


Figure 9. Chemical Structure of Morphine.

Secondary effects from the drug include pneumonia through the anti-tussive effect of morphine coupled with dangers of inhalation of gastric contents. Morphine levels in the body fluids can be used to indicate whether the drug is present in therapeutic or harmful quantities. Peak levels of morphine of 1ug/ml in plasma have been reported following bolus doses prior to surgery [89]. The therapeutic range has been reported as 0.04 to 0.5 ug/ml, [81], and as 0.01 to 0.07 ug/ml. [63]. This wide range (0.01 to 1 ug/ml overall) is due to the fact that the body can develop tolerance to morphine. Tolerance leads to decreased intensity of the pharmacological effects and shortened duration of action, therefore drug addicts require increased doses for the same effect. A habitual morphine user can take 20-30 times the therapeutic dose. This makes the interpretation of blood concentrations of morphine difficult. Morphine 3 and 6-glucuronides are the major metabolites and accounts for about 65 to 70% [63].

Buprenorphine

This is a semi-synthetic, highly lipophilic opioid derived from thebaine. Its structural formula is shown in Figure 10. It is 25 to 50 times more potent than morphine. Buprenorphine is a relatively new drug with mixed agonist and antagonist opiate action. After sublingual administration of 0.4 - 0.6 mg. buprenorphine concentration is in the range - 1 - 4 ng/ml after about two hours [90]. Little is known about the toxicity of buprenorphine in man. The median lethal single doses (LD 50's) for buprenorphine in mice were 24 - 29 mg/kg, 90 - 97 mg/kg and 260 - 261 mg/kg for intravenous, intraperitoneal and oral administration respectively [91]. Buprenorphine shows a much lower physical

dependence liability than morphine [91]. However, buprenorphine does show withdrawal symptoms, albeit milder than those seen in other opiate withdrawal. Recently, buprenorphine was reported to be abused [92].

About 96% of buprenorphine circulating in blood at therapeutic levels is bound to plasma protein [93]. Detoxification of buprenorphine occurs by conversion to water soluble molecules through conjugation with glucuronic acid to buprenorphine 3-glucuronide and N-desalkylbuprenorphine [91].

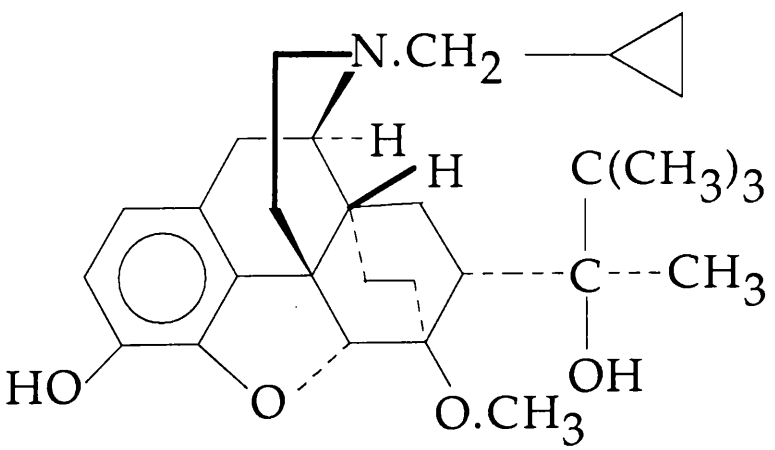


Figure 10. Chemical Structure of Buprenorphine.

2.4 - CHROMATOGRAPHIC ANALYSIS

2.4.1. Introduction

The term chromatography has been adopted universally to cover the 'science of separation.' More accurately the term embraces techniques which enable samples of chemical mixtures to be separated by exploiting the difference in their physical or chemical properties. The separation of compounds is achieved as a result of a partition between two different phases, one mobile phase and the other stationary [94]. Each compound in the mixture partitions to a different degree between the two phases. The longer this process is allowed to continue, the greater the separation achieved until the components emerge from the bed one by one.

There are many forms of chromatography available. Two forms of chromatography will be discussed here; HPLC (high performance liquid chromatography), and GLC (gas liquid chromatography). The chromatogram obtained in both systems shown in Figure 11 contains analytical data for the components of a mixture. Qualitative information appears in the characteristic retention time of each compound.

Quantitative information is contained in the peak height or area. A chromatograph is also a valuable measure of the performance efficiency of the chromatographic system which produced it. The retention time, or retention volume in HPLC is a characteristic of a given sample/chromatographic system combination expressed

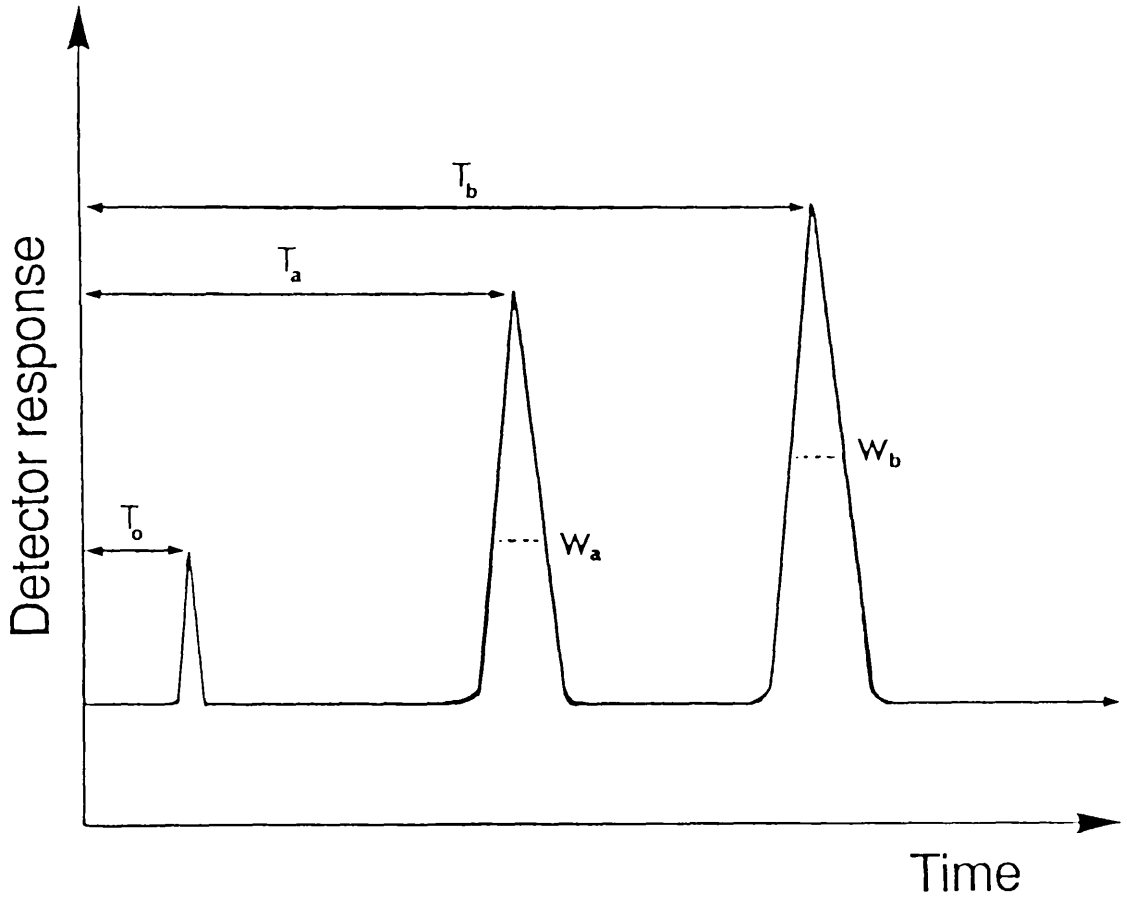


Figure 11. Schematic diagram of GC and HPLC chromatogram showing the measurable parameters.

T_o = elution time of the unretained solute

T_a and T_b = retention time of peaks a and b,

W_a and W_b = peak width at half height of peak a and b

in absolute terms. When it is related to a non-retained sample, usually solvent, it is called the relative partition coefficient or capacity factor (K'):

$$K' = \frac{(T_a) - (T_o)}{(T_o)} \qquad \text{Equation 1}$$

where T_a and T_o are as defined in Figure 11.

If the peaks are sufficiently symmetrical, and if their area ratio is greater than 0.5, then the resolution (R) is considered adequate if:

$$R = \frac{\text{tms (b) - tms (a)}}{\text{b 0.5(b) + b 0.5 (a)}} \geq 1 \qquad \text{Equation 2}$$

tms: total retention time; residence time of sample substance in the mobile and the stationary phase.

The separation factor rab given by:

$$\text{rab} = \frac{K'b}{K'a} = \alpha \qquad \text{Equation 3}$$

Where K'a and K'b are the capacity factor for components a and b respectively and α is the selectivity factor.

The column efficiency is measured by (H), the 'height equivalent to a theoretical plate (HETP)

$$H = \frac{\text{column length}}{N} \qquad \text{Equation 4}$$

Where N is the number of theoretical plates, which measure the column efficiency in producing narrow peaks as is given by:

$$N = 5.54 \left(\frac{T_a}{W_a} \right)^2 \qquad \text{Equation 5}$$

The linear velocity of the mobile phase ($\bar{U}$) is given by:

$$\bar{U} = \frac{\text{column length}}{T_o} \qquad \text{Equation 6}$$

These parameters can be calculated and used as a guide to the operation of the column and for determining the changes that can be made to achieve optimum performance for a given analysis. They are also dependent on the specific mobile phase and stationary phase used and the physical characteristic of the column. The column parameters are described in the Van Deemter equation

$$H = A + B/\bar{U} + C\bar{U} \qquad \text{Equation 7}$$

Where $\bar{U}$ is the flow rate of the mobile phase and A, B and C are constants which are related to the physical processes occurring within the column, a full discussion of the equation is given in the literature [95, 96].

2.4.2. - Gas Chromatography

Gas chromatography is analogous to other forms of chromatography. The separation is performed on a column containing the stationary phase, which is maintained at a defined temperature in an oven and has a constant flow of carrier gas. When a mixture of substances is injected at the inlet on to the start of the column, molecules with the greatest affinity for the stationary phase take more time to reach the detector. The detector produces a signal dependent on the mass of substance passing through it and this signal is processed and fed to a chart recorded or an integrator. Each substance passing through the column will have a characteristic retention time. In order to be able to communicate gas chromatographic 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 Kovats Retention Index ($RI_{\underline{K}}$). In this system, normal alkanes are assigned and $RI_{\underline{K}}$ value of 100 x carbon number. For isothermal operation values are calculated according to the following expression:

$$RI_{\underline{K}} = 100 \left[\frac{\log ti' - \log t'n}{\log t'_{n+1} - \log t'_{n}} \right] + 100\,n \qquad \text{Equation 8}$$

Where $^{t'}i$ = corrected retention time of sample peak [$\underline{ti}$ - $\underline{tair}$], t'_n = corrected retention time of normal alkane with carbon number N, which elutes before the sample 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 Kovats Retention Index values is given in the literature [95, 96].

Gas chromatography may be divided into gas-solid chromatography (GSC) and gas liquid chromatography (GLC). Only the GLC will be discussed.

2.4.2.1 - Gas Liquid Chromatography

The stationary phase in GLC is liquid coated on a support material. The support material should have a very large surface area and uniform particle size, free from fines, and should be mechanically robust and chemically inert.

The most common support particles are formed from diatomites. The stationary phase is selected to suit the analysis required from non-polar, moderately polar and polar liquid phases such as SE-30, OV-17 and OV-225 respectively. Higher efficiency and lower column bleed is obtained with a low loading of the stationary phase (weight percent of the support material), in the range of 1 - 8%

2.4.2.2 - GC Columns

Two main column types are in common use - packed and capillary columns. The packed columns have, in general, low efficiency and separating capabilities, but have a higher capacity for the sample material. Capillary columns are available in glass, stainless steel and fused silica. The first two are not frequently used now due to the fragility of the glass and the presence of active sites on the stainless steel. The coating of the stationary liquid phase inside the capillary column can be in three forms: wall coated open tubular (WCOT), support coated open tubular (SCOT) and porous-layer open tubular (PLOT).

Chemical bonding of the stationary phase to the column (chemically bonded columns) provides stability of the phase and the columns will retain their efficiency longer than non-bonded columns.

Fused silica columns are flexible, and with an external coating of polyimide polymer, are extremely rugged. Both polar and non-polar stationary phases with high efficiency are available. They have superior resolution which can separate complex mixtures with narrow peaks, considerably enhancing the detection limit. Derivatization of polar compounds and non-volatile compounds might be required to convert them to more volatile and easily chromatographed compounds. Alcohols and phenols are reacted to form more inert ester, ether or silyl derivatives.

2.4.3 - Gas Chromatography-Mass spectrometry

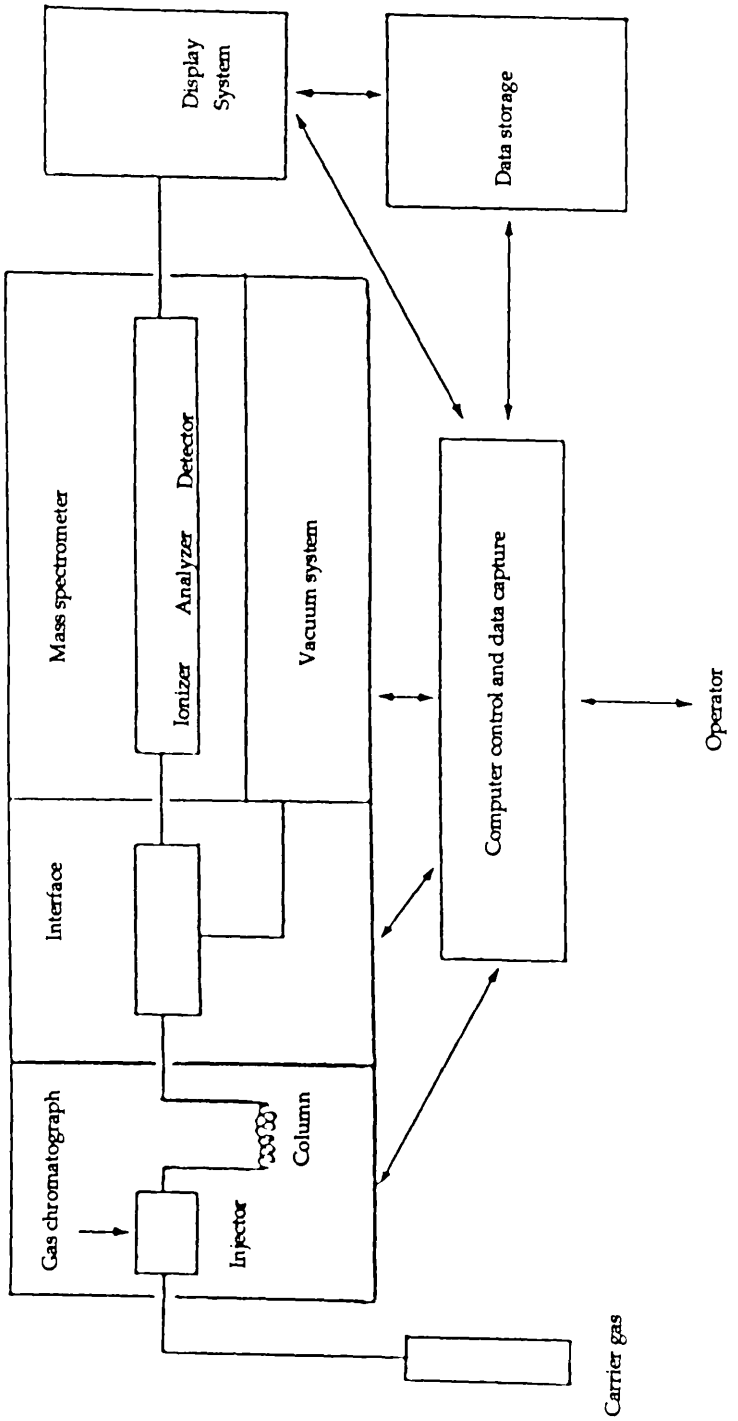
2.4.3.1 - Introduction

The combination of the gas chromatography with mass spectrometry has resulted in an instrument of considerable importance in the field of organic chemistry, pharmacology, biochemistry, pollution studies and toxicology. It is now a well-established routinely-used technique. In this instrument, the gas chromatography provides the separation of compounds and introduces them to mass spectrometry through the interface. MS 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 12 shows the basic component of a typical GC-MS system.

The relative GC-MS techniques applied during the research work will be discused in this thesis. More details of GC-MS are available in references [97, 98, 99, 100].

2.4.3.2 GC-MS Interface

The gas chromatography element leaving the column at atmospheric pressure while 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 the high vacuum condition. The ideal interface device would remove all the chromatographic mobile phase and transfer all the solute to the ion source


Display System
Data storage
Mass spectrometer
Ionizer
Analyzer
Detector
Vacuum system
Interface
Computer control and data capture
Operator
Gas chromatograph
Injector
Column
Carrier gas

without degrading chromatographic elution. The flow rate of a 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.

2.4.3.3 - Ionization Systems

Mass spectrometry requires that molecules for analysis have first to be ionised. The ion source system, Figure 13 incorporates ion lens plates to focus the ion beam as well as extracting and accelerating the ions into the analyser. Two types of ionization systems are available, namely Electron Impact (EI) and Chemical Ionization (CI). Only one type will be discussed (EI).

I - Electron Impact

Electron Impact is the most widely used method of ionization. The vaporized sample molecules are bombarded with a stream of electrons which will ionize and fragment the molecules. The majority of EI work has been done at 70 eV, where the fragmentation pattern at this relatively high electron energy does not vary so much. The excess energy absorbed causes fragmentation of sample

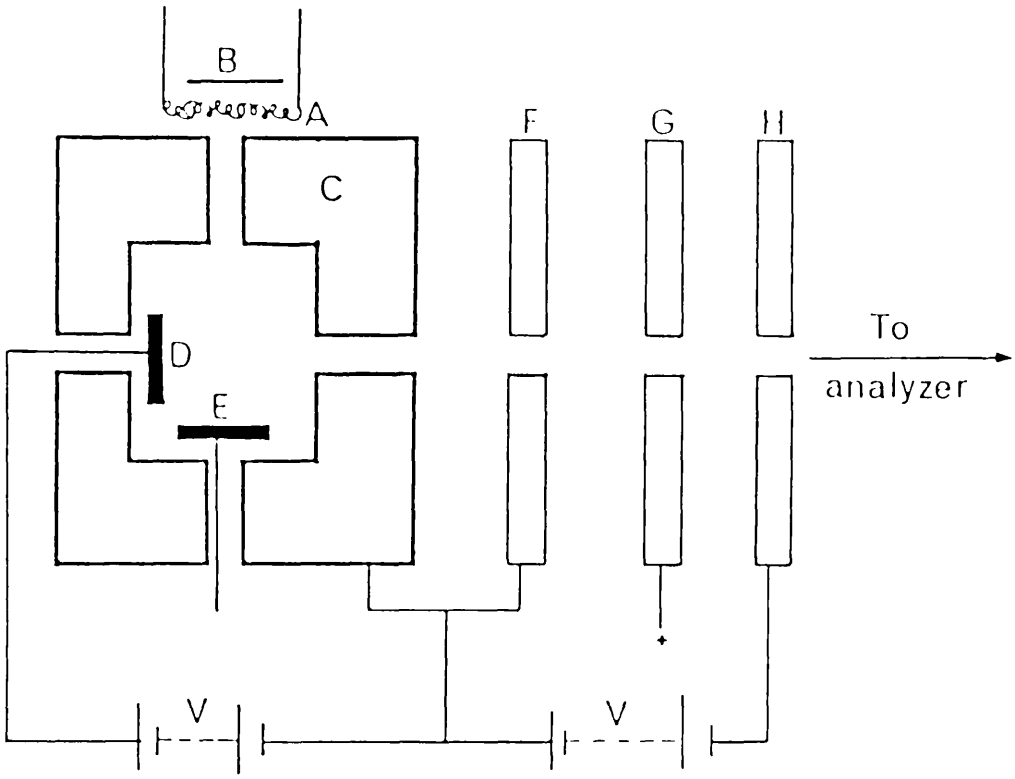


Figure 13. Schematic diagram of an EI ion source

A. Filament	B. Filament shield
C. Source block	D. Repeller
E. Trap	F. Source slit
G. Focussing plates	H. Accelerating slit

molecules to produce both positive and negative ions which can be represented by the following equation:

Positive Ions

Parent molecule ABCD $+e^- \longrightarrow ABCD^{+\cdot} + 2^-e$	Equation 9
$ABCD^+ \longrightarrow A^+ + BCD.$ or $A. + BCD^+$	Equation 10
$\longrightarrow AB^+ CD.$ or $AB. + CD^+$	Equation 11
$\longrightarrow ABC^+ + D.$ or $ABC. + D^+$	Equation 12
$AB^+ \longrightarrow A^+ + B$ or $A + B^+$	Equation 13

Equations 10 - 13 represent fragmentation of the molecular ions where $ABCD^{+\cdot}$ represents a radical ion.

A. etc. represents a free radical.

AB^+ etc. represents an even-electron ion.

Equation 9 represents the formation of the molecular ion.

Negative Ions

$AB + e^- \longrightarrow A^+ + B^- + e^-$ Equation 14

$AB + e^- \longrightarrow A + B^-$ Equation 15

$AB + e^- \longrightarrow AB^-$ Equation 16

Equations 14, 15 and 16 represent production of an ion pair, capture of an electron with dissociation and capture of an electron respectively.

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. The ions of intermediate life time (10^{-5}-10^{-6} sec) are accelerated from the source of the mass spectrometry with mass (M_1) but decompose to a smaller ion of mass (M_2) before reaching the detector.

2.4.3.4 Ion Separation

After leaving the ion chamber in the source, the ions are accelerated with an acceleration potential (V) of about 8Kv into the mass analyser where they are separated according to their m/z ratios by employing a magnetic sector or quadrupole filter. The ions follow a path of radius. Ions of different m/z may be brought to focus at the detector by variation of the magnetic field. Further discussion of the theory of ion separation is given in references 101, 102.

2.4.3.5 - Ion Detection and Amplification

The vast majority of mass spectrometers use electron multipliers for ion detection (EM) which have a fast response and high sensitivity. The other mean of detection is a conversion dynode and photomultiplier. The current generated is amplified typically by a factor of 10^6 the output being recorded by a data handling system.

2.4.3.6 Data analysis

Modern GC-MS systems acquire data by continuous repetitive scanning of the GC column eluate. The rate of scanning usually have values in the range of 0.1 - 1.5 sec per scan. The data system of GC-MS unit is capable of displaying the stored data in a number of ways:

I -Reconstructed gas chromatograph

A chromatogram can be reconstructed as components elute from the column by a plot of the total ion current stored and summed by the computer for each spectral scan. This record is similar to the chromatogram of the GC effluent produced by a flame ionization detector (FID).

II - Mass Chromatogram

When the proper ions are chosen, the use of MC can be very effective for identification when obtaining a peak at the correct mass and retention time for a specific compound is strong evidence for the presence of that compound in the sample. MC is especially useful for rapid surveys of complex GC-MS data.

III - Selected Ion Recording

Selected ion recording [SIR] is used for the detection of very low ion abundance. In this technique the mass spectrometer is only tuned to a few pre-selected ions.

In SIR practice, a few ions are sequentially monitored. Increased sensitivities of 50 - 500 for specific compounds are obtained. Amounts lower than 1pg can be detected on SIR.

2.4.4 - High Performance Liquid Chromatography

2.4.4.1 - Reversed-phase chromatography (RP)

RP Chromatography is so named because it behaves in the opposite way to Normal Phase Chromatography. The stationary phase is silica chemically bonded with organosilanes to give non-polar, hydrophobic surfaces. Solute retention is mainly due to hydrophobic interaction between solutes and the hydrocarbonaceous stationary surface. Polar mobile phases, usually water with miscible organic solvents, are used for elution. Solutes in reversed phase chromatography are eluted in order of decreasing polarity (increasing hydrophobicity).

The most popular RP packing is C18 octadecylsilane (ODS). The retention of solutes usually increases proportionately with the carbon chain length of the bonded group under identical HPLC conditions.

Reversed-phase chromatography is the most versatile and most widely used HPLC mode. Some additives can be used with the mobile phase to give special selectivities to produce a new RP concept like ion pair [IP], ion suppression [IS], etc.

RP columns are stable in the pH range [2 - 7] and at elevated temperatures. The classification of RP chromatography is discussed in references [103, 104].

2.4.4.2 - Normal-phase (NP) chromatography

Adsorption chromatography is also referred to as normal-phase (NP). NP chromatography involves interaction between adsorbent, usually silica, and the solute and solvent molecules in solution. The process considered as competition between the solute and solvent molecules for adsorption sites on the solid surface. The interaction of solute and solvent molecules to the surface of the column packing may be considered as a dynamic equilibrium. The mechanism of adsorption chromatography and the dynamic equilibrium between mobile phase and the adsorptive surface are shown in Figures 14, 15.

The solutes are eluted from normal-phase columns in order of increasing polarity and retention decreases with increasing solvent polarity. NP chromatography is best for the separation of compounds having high solubility in organic solvent, or have low stability or aggregation problems in aqueous mobile phases, (lipid) to separate isomers and to separate compounds are difficult to retain on reversed phase columns. A full discussion of normal phase chromatography theory and its application in forensic science is discussed in reference [105].

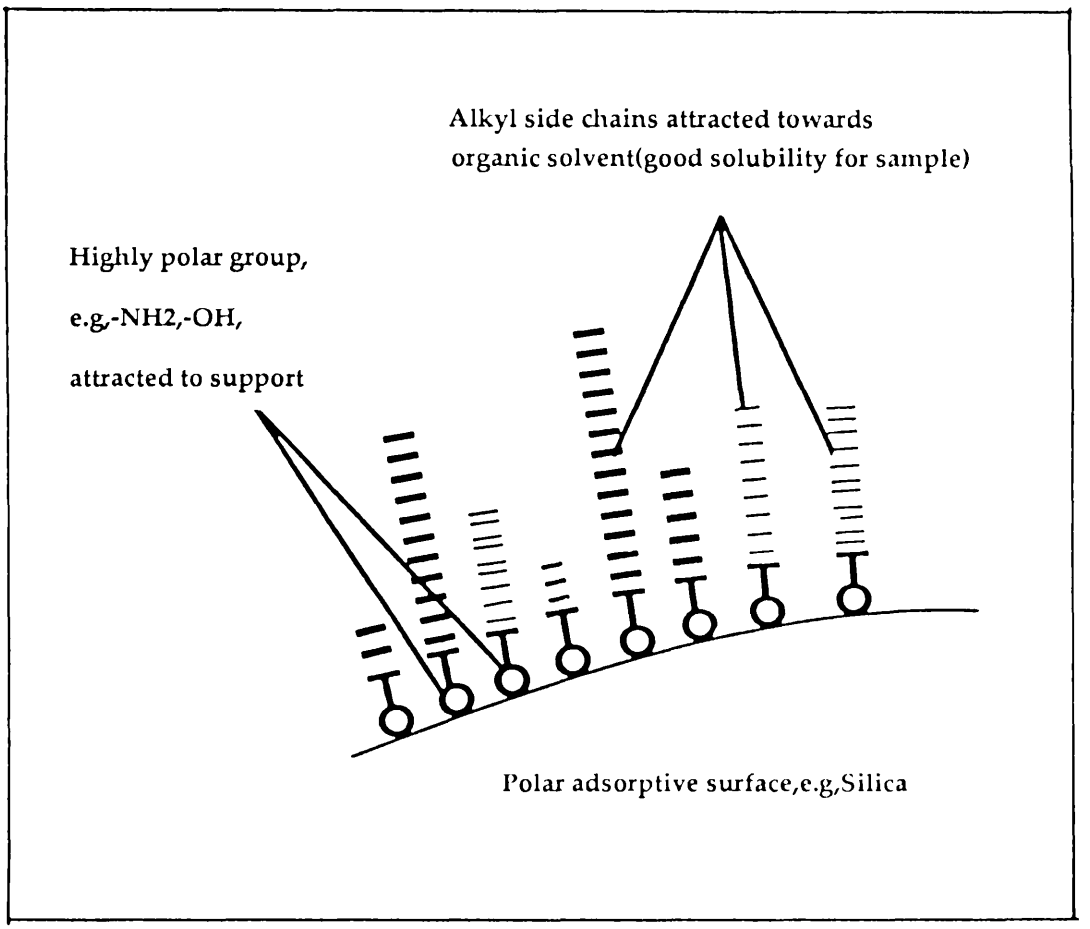


Figure 14. Interaction of compound belonging to homologous series with an adsorptive surface.

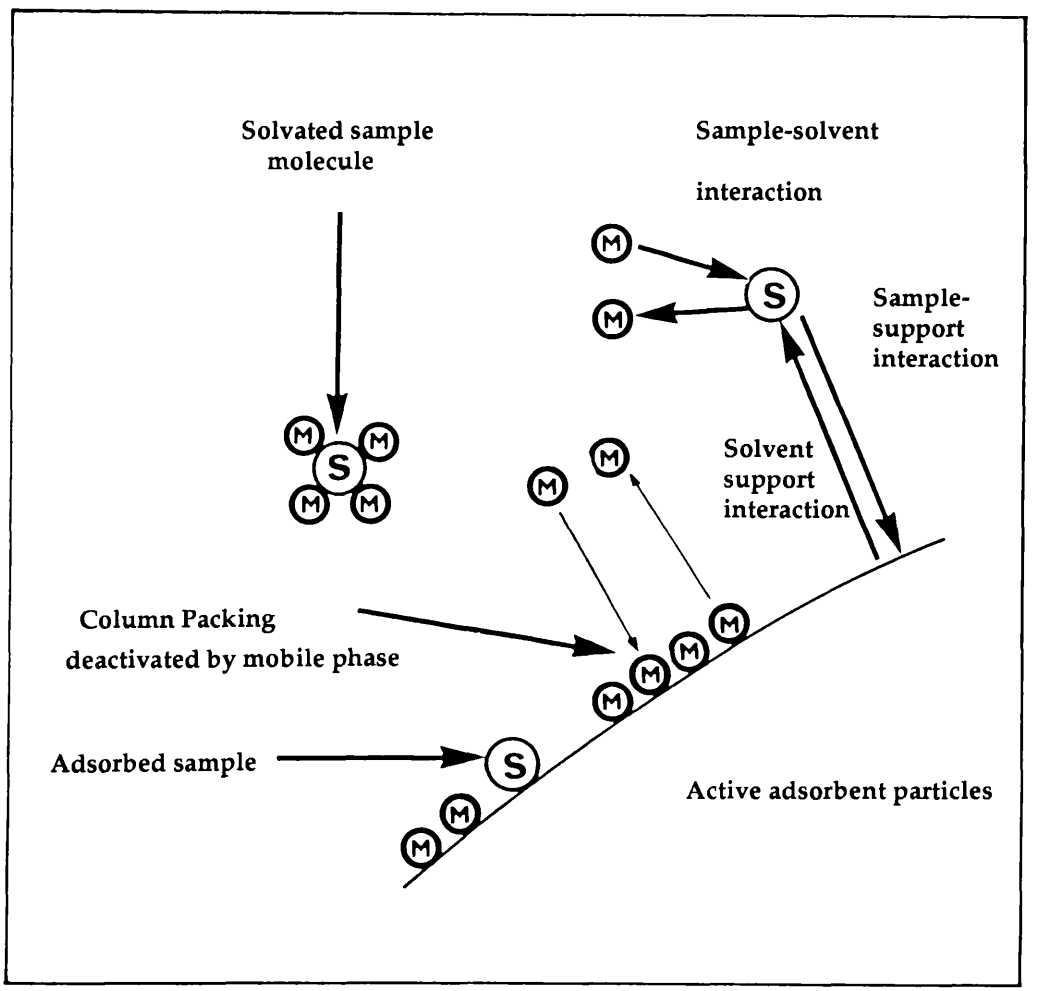


Figure 15. The equilibria at a liquid-solid adsorptive surface.

2.5 EXTRACTION METHODS

2.5.1. - Solid Phase Extraction

2.5.1.1 - Introduction

Over the past decade, sorbent extraction has emerged as a powerful tool for chemical isolation and purification in a broad range of applications: pharmaceutical, fine chemical, biomedical, food analysis, organic synthesis, environmental and many others [106].

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, while 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 other sample components.

Sorbents are formed by 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 product is stable within a pH range of approximately 2 to 7.5. Above pH = 7.5 the silica substrate is susceptible to dissolution in aqueous solutions. Below pH 2.0 the silyl ether linkage is labile and the functional groups on the surface will begin to cleave. In practice bonded silicas may be used for sorbent extractions in pH range 1 to 14 since degradation of the sorbent is a finite process and sorbents are typically exposed to solvents 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 bonded silica sorbents have a particle size distribution of 15 - 100 microns. The nominal porosity of most of the sorbents is 60 angstroms, adequate for compounds with molecular weights up to approximately 15000 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 silicas beneath them 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. Therefore solvation of a sorbent is necessary before the sorbent will interact reproducibly with the isolate. Solvation is a wetting of the sorbent creating an environment suitable to the isolate. Retention is accomplished by passing several bed volumes of a suitable solvent. The adsorptive properties of bonded silica are due principally to the functional groups bonded to the silica substrate. Any unbonded silanols remaining on the surface are also a contributory factor.

Interaction between the substrate and isolate molecules is called secondary interaction. It may be the predominant active property of a sorbent. As a particular sample is passed through a sorbent bed, compounds present in the sample will either pass through the sorbent or be retained on the sorbent due to an attraction which exists between the sorbent and the isolate molecules, causing the isolate to be immobilized; this phenomenon is called "retention" while the process by which an isolate is removed from a sorbent bed in which it has been retained is called "elution."

The capacity of a given sorbent is defined as the total mass of a strongly retained isolate that can be retained by a given mass of the sorbent under optimum conditions. Capacity values range from less than 1% to 5% of sorbent mass that is, 100 mg of sorbent might retain as much as 5 mg of a strongly retained isolate.

The selectivity of the sorbent is the ability of the sorbent to discriminate between the isolate and all other 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.

2.5.1.2 -Non-Polar Interactions

Non-polar interactions occur between the carbon-hydrogen bonds of the sorbent functional groups and the carbon-hydrogen bonds of the isolate. Most of the organic compounds containing alkyl, aromatic alicyclic or other functional groups with significant hydrogen structure will exhibit non-polar interaction.

Non-polar interaction is useful when isolates vary widely in chemical structure. In general, non-polar sorbent are not very selective since most matrix components tend to have some degree of non-polar character. Non-polar interaction can be very strong with non-polar isolates of high molecular weight. In which case, isolates are often difficult to remove.

Buffer or aqueous matrices of high ionic strength may promote isolate elution by reducing the sorbent functional groups interaction with the isolate. The effect of this can be reduced by lowering the ionic strength of the sample through dilution and equilibrating the sorbent with organic buffers only. The non-polar interactions between the isolate and the sorbent are best disrupted by solvents having some degree of non-polar character.

Octadecyl silane 'C18'

Octadecyl silane in the most retentive of all sorbents for isolates being retained by non-polar mechanism. It is the most widely used sorbent for non-polar interactions. It is very effective for isolating groups of compounds that are dissimilar in structure, therefore C18 is regarded as the least selective sorbent. Since it retains almost everything from aqueous matrices, very non-polar compounds are difficult to remove from this sorbent, conversely C18 does not retain certain very polar molecules such as carbohydrates.

2.5.1.3 Polar-interactions

Polar interactions are exhibited by many different sorbents and functional groups on isolates. The most characteristic molecules isolated by this type of sorbent are those containing dipoles. This includes most groups containing hetero-atoms as

well as functional groups with resonance properties, for example, aromatic rings. Polar interactions are very flexible because so many functional groups exhibit polar interactions (dipole/dipole, induced dipole/dipole, pi-bonding 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 polarisations. Retention of isolates by polar interactions is facilitated by non-polar solvents while elution of isolates from polar sorbents is facilitated by polar solvents and high ionic strength.

Polar interactions are very useful for highly selective separation of molecules having similar structures.

Activated Silica (SI)

Unbonded activated silica is fairly acidic and is generally regarded as the most polar sorbent available. SI sorbent in known to have a strong tendency to adsorb water and other polar solvents such as methanol. SI is one of the best sorbent for selectively separating isolates of very similar structure.

Aminopropyl (NH_2)

NH_2 is a very polar sorbent with a capability of exhibiting all possible interactions. NH_2 is a strong hydrogen bonder that also can function as an anion exchanger. 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. NH_2 is excellent for the separation of structural isomers.

2.5.1.4 - Ion-Exchange Interactions

This 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 divide into two classes: cationic (positively charged) include primary, secondary, tertiary amines, etc. Anionic (negatively charged) include carboxylic, sulfonic, etc.

The ionic strength of solvent/matrix and the PKa of isolate and sorbent play an important role in retention and elution of isolates by ion-exchange interactions.

Carboxymethyl (CBA)

One of the most useful characteristics of CBA is its weak cation exchange properties. The most commonly encountered cations in organic isolates are amines. The pka value of CBA is 4.8 at pH above 4.8. CBA carries a negative charge that can be used for retaining cationic isolates, dropping the pH below 4.8 neutralize 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 cationic i.e. high pka cations.

Propylbenzenesulfonyl (SCX)

SCX is a strong cation-exchanger with very low pka due to the presence of the benzene ring on its surface. SCX sorbent show a potential for non-polar interactions. This dual nature is useful with isolates that exhibit both cationic and non-polar character. After retention of the isolate on the column, 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 then be eluted from the sorbent with a solvent that disrupts both ionic and non-polar interactions simultaneously such as methanolic hydrochloric acid.

2.5.2.5 - Bond Elute Certify

This is a new bonded silica solid phase extraction (SPE) column to extract acidic neutral and basic drugs from urine and with special sample preparation in case of blood. The manufacturer of this sorbent did not reveal the chemical structure of this column. Bond elute certify is conditioned with phosphate buffer pH 5.0 for the extraction of basic drugs like amphetamine, while for opiate extraction, the column is conditioned with acetate buffer at pH 4.0.

2.6 ANALYSIS OF INTERFERING SUBSTANCES

2.6.1 Acid Interference

In general these compounds can be extracted from aqueous acid solution or usually co-extracted with acidic drugs such as barbiturates in putrefied tissues or blood. Kaempe published work in which interfering compound were positively identified [43, 20].

An atlas of properties of acidic interference compounds was produced by Williams which included the ultraviolet, infra-red, colour reagent, thin layer chromatography, mass spectra and gas chromatography [22]. Others produced HPLC retention times using two types of mobile phase, one for carboxylic acids and the other for indoles and neutral interfering substances [107].

Indole

The first published work recognizing Indole as an interfering substance with drug determinations, and the quantitative analysis of the compound in putrefied blood and tissue using gas liquid chromatography was described by Oliver [28]. Thelmar and Stevens published data regarding Indole derivatives such as Indole-3-acetic acid (IAA) and Indole-3-aldehyde in putrefied human liver using thin layer chromatography and colour tests [107]. All the clinical works relating to Indole is focussed on neurochemically important Indoles like Indole-3-acetic acid (IAA), and 5-hydroxyindole acetic (5-HIAA) [108, 109]. Different analytical techniques were used to analyse trace amounts of Indole derivatives, e.g. GC-MS [110]. A reversed-phase high performance liquid chromatography using ODS columns with electrochemical [109, 111, 112] and fluorometric detection have been described [113, 114, 108]. Most of the recent work on Indole derivative analysis has been focussed on HPLC systems. The availability of highly efficient HPLC allows the ready analysis of thermally labile, high molecular weight, hydrophilic and hydrophobic compounds. Another advantage of HPLC is the selectivity of retention times which gives a good marker for identification.

A literature search revealed nothing on the analysis of Indole by HPLC. Most of the published work is on Indole derivatives, both qualitative and quantitative, is as discussed earlier. Therefore, a method for Indole analysis using high performance liquid chromatography has been developed using ultraviolet detection for qualitative and quantitative analysis of the substance in putrefied post-mortem blood.

2.6.2 Non-acid Interferences

Non-acid Interferences is a group of compounds which extract from basic aqueous solution into organic solvents. Kaempe and others published data according to the incidence of non-acid interfering substances in liver and visceral material [43, 28]. Their data was obtained using thin layer chromatography, ultra-violet spectrometry and gas chromatography. The gas chromatography column used silicone gum rubber (SE30) as liquid phase for separation and flame ionisation for detection. The retention indices of 296 compounds has been produced on gas-chromatography with SE30 and OV-1 columns which are usually used in toxicological analysis has revealed the presence of 2-phenethylamine, tyramine and tryptamine as interfering substances [115].

The relative retention times of some endogenous putrefactive amines on OV-17 have been measured and shown to be a source of interfering peaks in the gas chromatographic screening of direct chloroform extracts of blood for drugs [45].
There has been an increase in the number of publications on the analysis of trace amines [116, 117, 118]. This has been stimulated in particular by the recognition of the role of 2-phenethylamine, tyramine and tryptamine in neural transmission, particularly where impaired metabolism of these amines is implicated in certain pathological conditions such as migraine, Parkinson's disease, schizophrenia, phenylketonuria and depressive illness [40].
The most specific methods for trace amines analysis include radioenzymatic assay [119], and gas chromatographic-mass spectrometry (GC-MS) [120, 121]. The techniques require expensive instruments, purified enzymes or radioactive compounds. Most of them are not suitable for the simultaneous determination of all trace amines. The most recent analytical method for trace amine analysis is

reversed-phase high performance liquid chromatography with electrochemical detection [122, 123, 124, 125, 126]. Others used fluorometric detection, [127, 128]. These provide good separation and sensitivity with relatively inexpensive equipment. Reversed-phase chromatography has been used to separate the so-called trace amines which include the three putrefactive amines under investigation - 2-phenethylamine, tyramine, tryptamine. It is known that these amines "basic compounds" with nitrogen atoms can show tailing peaks in reversed phase high-performance liquid chromatography when using bonded hydrocarbonaceous packing material. These problems are believed to arise from interaction between the compounds and the adsorption sites on the silica matrix of the packing material, [129, 130, 131, 132, 133, 134]. Most HPLC methods with the ODS-columns use ion-pairing to overcome the tailing and improve the efficiency, [123, 125]. Gradient elution [118], or amine modifiers have been used to reduce peak tailing of 2-phenethylamine drugs in reversed-phase high performance liquid chromatography, [135]. They found large improvements in peak shape by the addition of some amines. Detection was achieved electrochemically or fluorimetrically this latter requiring post-column derivatization. Another limitation of most of the applications in trace amine analysis is that the techniques have been developed for special types of samples like serum, cerebrospinal fluid and urine. As an alternative approach to the separation of the trace amines has been achieved on silica "normal phase" columns.

The use of non-aqueous ionic elements has many advantages in the analysis of basic compounds by HPLC-using silica columns. Greater flexibilities are offered by the ability to control retention and selectivity by the adjustment of pH, ionic strength and the composition of the organic components [136]. Another advantage of silica over ODS columns for basic compound analysis is a sharp peak with a fast elution time without using an ion-pair reagent which requires a few hours to

equilibrate with the ODS-column before the analysis can be started. The use of silica HPLC columns has been widespread in forensic laboratories for the screening and quantitative analysis of basic drugs, [137, 138, 139]. These were introduced for the separation of a wide range of drugs and drugs of abuse. An HPLC system was developed for the analysis of the antiarrhythmic drug, amiodarone based on a silica column [140]. A silica column was used for lysergic acid diethylamide (LSD) separation [141], and an HPLC system with silica has also been used for the separation and quantitative determination of morphine [142] and tricyclic antidepressants, [143].

A comprehensive evaluation of biogenic amine analysis in biological system is given in the literature [144]. The literature search revealed no data regarding the analysis of the three putrefactive amines simultaneously from post-mortem blood. A method has been developed for their analysis using normal phase HPLC-system with ultra-violet detection. The reports include the development of appropriate mobile phases for fast analysis.

2-Phenethylamine

2-Phenethylamine (PEA) can be viewed as the parent compound of a large group of drugs known as sympathomimetic amines [145]. Relatively large amounts of PEA is excreted from the urine of patients suffering from paranoid schizophrenia, [34]. Because of its clinical and pharmacological importance PEA received a great deal of attention in recent years in relation to the development of methods to analyse the normally low levels in tissue and body fluids.

A gas chromatographic procedure for the analysis of 2-phenethylamine in tissues and body fluids has been described involving the use of pentafluorobenzenesulphonyl chloride for extraction and derivatization of 2-phenethylamine [146].

2-phenethylamine with other sympathomimetic drugs was analysed on reversed-phase high performance liquid chromatography with pre-column derivatization and electrochemical detection [147].

Tyramine (TYR)

Tyramine is an indirectly acting sympathomimetic amine found in cheese, fermented foods and red wine, [38, 39]. The involvement of Tyramine in a number of neurological disorders has been postulated. A fluorometric method used for quantitative analysis of Tyramine is based on the reaction of para substituted phenols with 1-nitroso-2-naphthol and $NaN0_2$, [43].

Oliver and Smith successfully analysed Tyramine by gas liquid chromatography using a flame ionisation detector [37]. Data is available in the literature regarding its analysis for clinical purposes in plasma and cerebrospinal fluids. Reversed-phase HPLC with fluorescence detection has been used for Tyramine and other biogenic amines, [127].

A method for plasma Tyramine analysis using reversed-phase ion pair HPLC with electrochemical detection has been described [125] while one other, [118] used derivatization reagent with ultraviolet and fluorescence detection.

Tryptamine (TRY)

Oliver and others analysed Tryptamine by gas liquid chromatography and provided mass and ultraviolet absorption spectra [28]. Marsen and Joseph investigated the analysis of biogenic amines including Tryptamine by reversed-phase ion pair high performance liquid chromatography with electrochemical detection [148]. A gas chromatograph-mass spectrometer equipped with negative ion chemical ionization was developed for the Tryptamine analysis [149]. A simultaneous determination of Tryptamine and its metabolites in mouse brain by high performance liquid chromatography with fluorometric detection has been described [125].

2.7 - DRUGS ANALYSIS

2.7.1 - Acidic Drugs

2.7.1.1 Anticonvulsant (ACD)

The literature survey shows numerous publications concerning analytical methods for anticonvulsant drugs in biological specimens. The methods are designed for the analysis of two or more of the anticonvulsant drugs [150, 151, 152].

Most of the methods are developed towards clinical applications in drug monitoring, either therapeutic or overdose. The samples used are serum or plasma.

Gas chromatography was used for determining phenytoin, phenobarbitone and other anticonvulsants in plasma [153, 151]. Enzyme immunoassay [EIA] has been developed for routine therapeutic monitoring of anticonvulsant drugs [154].

High performance liquid chromatography is an accurate and precise technique for the analysis of drugs. A number of liquid chromatographic methods has been reported in the literature for the determination of anticonvulsant drugs in physiological fluids. Most of the methods used reversed-phase HPLC. Williams and Viola [155] compared the results of reversed-phase HPLC analysis of anticonvulsant drugs with gas chromatography and immunoassay technique. The agreement was excellent with a correlation coefficient of 0.95. A very fast HPLC analysis was achieved with a C8-column (100 mm x 4.6 mm, 3 um particle size). A reversed-phase HPLC method is described [156] for the analysis of eight common

anticonvulsant drugs and metabolites in serum. High performance liquid chromatography has the advantage that the analysis of anticonvulsant drugs requires no derivatization.

HPLC has another advantage. It is a useful method to separate the parent drug from its metabolites [157]. The method used in this work for anticonvulsant drugs is specially developed for post-mortem blood using reversed-phase high performance liquid chromatography [158]. The effect of interfering substances present in putrefied post-mortem specimens on anticonvulsant drugs analysed by this method has never been previously investigated.

2.7.2 - Non-Acidic Drugs

2.7.2.1 Benzodiazepines

Numerous methods are currently available for the analysis of the benzodiazepine drugs in biological samples. Immunochemical techniques generally employed as a screening test are able to identify the presence of benzodiazepines at nanogram levels but they do not discriminate within the group. Moreover, quantitative analysis is difficult because of the responses of the different benzodiazepines and their metabolites towards the antigen-antibody reaction is not the same. Most of the techniques used for benzodiazepine analysis are focused on high performance liquid chromatography and gas chromatography.

Methods for toxicological analysis of the common benzodiazepines in biological fluids and tissues either using gas chromatography with nitrogen-phosphorus or electron capture detection to measure benzodiazepines and metabolites have been described [159, 160]. Underivatized benzodiazepines were successfully separated using gas chromatography by Ferguson and Curi [161]. Some benzodiazepines such as chlorodiazepoxide are thermolabile and therefore cannot be subjected to the high temperatures needed for GC.

High performance liquid chromatography (HPLC) has been used to analyse a single benzodiazepine [162], or a wide group of benzodiazepines and their metabolites, [163, 164]. Reversed-phase HPLC with a photodiode array spectrophotometric detector has been described [165] which makes the HPLC detection more reliable, informative and non-destructive.

2.7.2.2 Opiates

Analyses of opiates by chromatographic techniques are the methods of choice. High performance liquid chromatography with ultra-violet detection has been described [166, 167], but showed a lack of sensitivity due to the low extinction coefficient of morphine. It utilizes the ability of the phenol moiety of morphine and structurally-related drugs to absorb UV light.

Analysis by HPLC with electrochemical detection has been described for morphine and buprenorphine in urine and blood [168, 169]. They achieved a sensitivity of 0.5 - 10ng/ml. The major problem with electrochemical detection is with the

contamination of the electrode surface during operation. HPLC with chemiluminescence (CL) detection has been used for opiate analysis, [170]. In this technique morphine is oxidized to pseudomorphine by potassium permanganate solution.

The use of the native fluorescence of morphine and its metabolites by using fluorescence detection following HPLC separation has been described [171, 172]. The tedious optimization of the operating conditions for fluorometric derivatization and detection, the frequent contamination of the electrodes in electrochemical detector, the interferences from matrix and the huge influence of the mobile phase composition have decreased the extensive application in routine analysis of these techniques. Gas chromatography is employed as a screening procedure for morphine and other narcotics as well as confirming the positive results of the preliminary screening methods [TLC, UV, RIA]. Both packed and capillary columns have been used. The capillary column provides better resolution and sensitivity. Since morphine is a polar drug the extracts for morphine analysis have to be derivatized before application to the GC column.

^{63}Ni electron-capture gas chromatography with pentafluoropropionic acid anhydride [PEPA] as derivatization reagent has been used to analyse morphine, [173], buprenorphine and its metabolites in urine and blood with sensitivity down to 1 ng/ml. [174]

Other derivatization reagents have been used such as acetic anhydride [175] and Trimethylsilylimidazole, [176]. Any of the above derivatization procedures are suitable for detection of morphine to low nanogram levels.

Analysis of opiates by mass spectrometry coupled to gas chromatography [GC-MS] provides sufficient sensitivity and offers the best available technique for the unequivocal identification of opiates in biological material. Mass spectrometry in the SIR mode has been applied to the detection of opiate drugs in both electron impact, [177, 178] and chemical ionization [179, 180]. Selective ion recording (SIR) enhances the sensitivity of detection by a factor of 50-500 or more when monitoring the effluent of a GC column [181].

From the literature review above, it can be seen that the analysis for morphine and buprenorphine with the different techniques are mostly dealing with each drug separately. A method has been developed by Battah to analyse both morphine and buprenorphine from post-mortem blood with gas chromatography-mass spectrometry [182] using the same extraction procedure and derivatization reagent has been adopted in this work for analysis of morphine and buprenorphine.

2.8 EXTRACTION OF INTERFERING SUBSTANCES

2.8.1 Acid Interferences

Indole

The NH group of Indole is relatively acidic [25]. Indole has been shown by Williams to be extractable from both acidic and basic solutions [22]. Therefore Indole could interfere with both acid and basic drug determinations. Oliver demonstrated the extraction of Indole from putrefied biological materials with about a 100% efficiency using diethylether from the alkalinized sample [28]. Indole derivatives Indole-3-acetic acid (IAA) 5-hydroxyIndole acetic acid (5-HIAA) have

been shown to have considerable clinical and biochemical importance [108]. Indole derivatives have been analysed following extraction from biological fluids using organic solvent [112]. The use of solid-phase sorbent, C18-column, to extract Indoles from serum [111] and Amberlite ion-exchange column to extract Indoles derivatives from urine have been described [183]. The derivatives have been analysed directly by injection of the supernatant after protein precipitation of biological materials with mainly 0.1M perchloric acid or trichloroacetic acid [113, 114, 184]. A literature review provides no reports on the extraction of Indole from post-mortem blood employing solid phase sorbent systems. A method has been developed to extract Indole from putrefied post-mortem blood using a solid phase sorbent described in this thesis, 3.6.1.

2.8.2 - Non Acid Interferences

Of the group of compounds known as biogenic amines, 2-phenethylamine, Tyramine and Tryptamine are the most frequent non acid interference substances encountered. The three biogenic amines are primary amines and can be extracted from biological material at pH 8-10 with high efficiency using chloroform as extracting solvent. Tyramine extracted with the solvent mixture chloroform/Isopropanol (4:1 v/v) at pH = 9.5 and 2-phenethylamine extracted at pH above 12 with chloroform, gave high extraction efficiencies 95-100% [43]. 2-phenethylamine was extracted from aqueous solution using ethyl acetate at a pH range of 4-9, [126] reflecting the high lipophilicity of 2-phenethylamine. Tryptamine was found to be extracted well from strongly basic aqueous homogenates in high yield, using polar solvents such as butanol and methylene

chloride. A further extraction and purification step were needed since a large amount of lipids were co-extracted with Tryptamine. Most of the extraction methods focus on extracting one biogenic amine [185, 125, 128, 186, 146]. There is less published work describing the extraction of two or more of the three biogenic amines from biological material [4, 118]. All the procedures available in the literature are based on solvent extraction which require long analysis times, large volumes of solvent and centrifugation steps, the latter to overcome emulsion formation. Amberlite was used to extract 2-phenethylamine and tyramine from biological material with an efficiency of 63% and 83% for 2-phenethylamine and tyramine respectively. This method was used as a cleaning step before further extraction, [4]. The extraction of 2-phenethylamine, Tyramine and Tryptamine from post-mortem blood using bonded phase has not yet been reported. Post-mortem blood requires special treatment prior to application to solid-phase sorbent. A method has been developed to extract the three putrefactive amines using Extrelut^{R} and solid phase sorbent.

2.9 DRUGS EXTRACTION

2.9.1 Acidic Drugs

2.9.1.1 Anticonvulsant Drugs [ACD]

Of the various sample preparation methods, liquid-liquid extraction with non-polar, water-insoluble organic solvents has been most widely used. A single organic solvent for extraction was used by Scheal et al [187], Thomas et al [188] and Kabra et al [189] while others used a mixture of different solvents [190]. The methods can extract drugs present in low concentrations.

Extraction of biological materials with organic solvents is a common means of obtaining solutions suitable for instrumental analysis. Therefore a clean-up procedure of extracts is needed to minimise the interfering substance. Barbiturates from blood analysed by GC needed three extraction-purification steps, to produce a 1000-fold reduction of the interfering lipid level [191]. This makes liquid-liquid extraction a long and tedious method compared with solid-liquid (solid phase) extraction.

The solid phase extraction has advantages which include the simultaneous extraction of a number of samples, the prevention of emulsion formation, the short sample preparation time, the removal of evaporation steps and the potential for automation.

Solid material such as activated charcoal has been used for anticonvulsant drug extraction, [192] where drugs are first adsorbed onto charcoal and eluted from it with organic solvent. The extract is clean, but the procedure is time-consuming and gives extraction efficiencies ranging from only 41 - 73%. Extrelut^{R}, "Diatomaceous earth," has been described for extracting AD from blood [193, 194, 195]. The use of bonded silica to extract ACD from serum has been described [196]. Post-mortem blood is not directly applicable to bonded silica, therefore, a protein precipitation step is needed before applying the sample onto the bonded silica column. The use of diatomaceous earth for extraction is more versatile since post-mortem blood can be applied directly to sorbent. A method for extraction of acidic drugs and anticonvulsants has been described [158] which demonstrate a detection limit of 0.25 ug/ml for each drug. This method has been adopted in this work to extract and analyse the drugs under investigation.

2.9.2 Non-Acidic Drugs Extraction

2.9.2.1 Benzodiazepines Extraction

Until recently the majority of methods used for extracting benzodiazepines from biological fluids have been based on liquid-liquid extraction [197, 198, 199]. Recoveries of drugs with liquid-liquid extraction are dependent on the number of extractions and back extractions performed. A high recovery >90%, of some benzodiazepine from plasma with liquid-liquid extraction has been obtained [200]. Enzymic digestion can be used to release the benzodiazepines and their metabolites from human tissues to yield higher recoveries [201]. Eppel described a method for some benzodiazepine analysis including the one under investigation "Temazepam" [202]

In forensic science work, as elsewhere, the application of solid-phase extraction has been promoted by the commercial availability of prepacked columns and cartridges. An extraction procedure for one of a group of benzodiazepines and metabolites using bonded-phase extraction columns from serum with a recovery of >80% has been reported by Good and Andrews [203] and Carlucci [204]. The limitation of this procedure is that it is not applicable to post-mortem blood or tissue. Moore and Oliver developed a rapid solid-phase extraction procedure for benzodiazepines from greyhound urine following enzyme hydrolysis [205]. A method for the extraction procedure for most encountered benzodiazepines in forensic and clinical toxicology has been described [206]. It is claimed by Suzuki et al that an excellent recovery for all benzodiazepines in urine has been achieved.

The recovery of medazepam in plasma was, however, only 10 to 20%. Improved recoveries were achieved by deproteinization of the plasma samples.

The post-mortem blood routinely encountered in forensic toxicology casework is varying and usually extensive haemolysis and is often putrified. Large amounts of solidified and coagulated material may be present and therefore may cause serious contamination if applied directly to the solid-sorbent. It is possible, therefore, that protein precipitation followed by filtration of the extract would be necessary prior to pH adjustment before extracting the sample through the solid-phase sorbent. In general the recoveries by SPE of benzodiazepines after protein precipitation have been shown to be low [206]. However, others [198] obtained a recovery >75% from a small sample volume of 200 ul of blood for sixteen benzodiazepines. A method for benzodiazepines extraction from post-mortem blood based on liquid-liquid extraction has been adopted in this work [202]. The presence of interfering substances in benzodiazepine analysis by this method have been investigated.

2.9.2.2 - Opiate Extraction

Morphine and related drugs need a rigourous extraction procedure to isolate them from biological media with sufficient purity and efficiency. Opiate-type drugs are polar and hydrophilic. At physiological pH, morphine and buprenorphine molecules have a low lipophilicity due to protonation of the amine group. Therefore their extraction efficiency at the physiological pH, is poor. The pKa values of morphine are 8.0 for the amine group and 9.9 for the phenol group (log P (octanol/pH 7.4) - 0.1) [63]. For buprenorphine the pKa values are 8.24 and 10.0 for

the amine and phenol groups respectively [207]. To increase the solubility of the opiates in organic solvent, the lipophilicity of morphine and buprenorphine should be increased. This can be achieved by adjusting the pH of the specimens to the range of 8.5 - 10. This pH range gives the optimum extractability, [182]. Extraction of morphine and related compounds with one solvent [177, 208] or solvent mixtures [180, 175, 209] have been described. Butanol or isopropanol are usually used in solvent mixtures to increase the polarity of the extracting solvent. In liquid extraction, the solvent used is usually three to four times the volume of the aqueous layer. After centrifuging, the extracting solvent is usually back extracted into 0.1M hydrochloric acid or 0.05M sulphuric acid to purify the extract from co-extracted endogenous material co-extracted from the biological sample. The acid aqueous portion is then made alkaline to pH 8-10 and extracted again twice with organic solvent which is separated and evaporated to dryness. The extract is then reconstituted with an appropriate solvent for chromatographic analysis (TLC, GC, HPLC). Acid hydrolysis with hydrochloric acid or enzymatic hydrolysis (glucuronidase) is usually used for estimating the total morphine in biological specimens. In general liquid extraction procedures of opiates require long analysis times, large volumes of solvent and are complicated by potential emulsion formation and the need for several centrifugation steps. Adoption of solid-phase extraction for opiate analysis is known to be efficient, require short analysis times and minimal solvent consumption. Various materials have been used in SPE for the analysis of opiates such as XAD-2, a neutral styrene-divinylbenzene which has the capacity to adsorb drugs (organic material) from aqueous solution [210].

Diatomaceous earth (Extrelut^R) adsorbs the sample when applied to prepacked columns The aqueous material adsorbs into the Extrelut^R leaving the drug available for elution with organic solvent. High recoveries (98%) are achieved even when the eluate from the Extrelut^R column is back extracted into acid and re-

extracted using Extrelut^{R} for further purification [211]. The types of chemically bonded silica and their selective properties for extraction procedure has been described earlier (2.5).

Silica bonded to octadecyl (18-carbon straight-chain hydrocarbon) is the most commonly used reversed-phase packing material to extract morphine or its metabolites [212, 167, 170] A range of solid-phase extraction columns have been used, including C2, C8, C18 and phenyl to extract morphine and its metabolites, the morphine 3 and 6 glucoronides [172].

Until recently, no method has been described for the extraction and analysis of morphine and buprenorphine together. Battah developed an extraction procedure for morphine and buprenorphine in post-mortem fluids using a combination of Extrelut and the ion exchange column (SCX). This was followed by derivatization of morphine and buprenorphine with diethyl tetramethyldisilazane (DETMDS) prior to analysis [182]. The method showed an extraction efficiency 95 $\pm$ 4% at a concentration of 560 ng/ml blood, for morphine and 87 $\pm$ 5% at 8 ng/ml blood for buprenorphine.

CHAPTER 3

EXPERIMENTS

3.1 Drug Spiking

Blank blood supplied from the Scottish Blood Transfusion Service was tested for the presence of all of the drugs under investigation and found to be negative. A portion was transferred into clean vials and sealed, designated as blank blood and was stored with spiked samples under the same conditions. Other portions were used for the preparation of drug solutions in blood. They, and aqueous solutions of the drugs under investigation were prepared and stored for the same period of time and at the same temperature. This enabled a comparison to be made between the amount of chemical decomposition and of putrefactive degradation for each drug. The concentration of drug in blood and the aqueous solution was the same and was equivalent to the toxic level of each of the drugs under investigation. At the day of spiking a sample of spiked blood and of aqueous solution was analysed to measure the concentration of the drug on that day. This was designated as day zero concentration.

3.1.1 - Acid Drugs "Blood and Water Spiking"

3.1.1.1. Anti-Convulsants

Phenobarbitone:

20 ml of 100 ug/ml of phenobarbitone in methanol were added into a clean conical flask. The methanol was evaporated to dryness under a steam of nitrogen on hot plate at 60^{o}C. The phenobarbitone was reconstituted with 200 ml of the blank blood and then mixed for two hours using a magnetic stirrer to ensure both the solubility and the proper distribution of the drug in the blood. The same procedure and quantity of drug was used to produce an aqueous solution of the drug. The resulting concentration of phenobarbitone in blood and control water was 10 ug/ml. The water used was double deionized water. The pH of the water was adjusted to physiological pH 7.4 by adding dilute sodium hydroxide. 5 ml of each (blood/aqueous solution) was individually transferred into clean 6 ml capacity hypo-vials which were then sealed with butyl rubber septa. The vial was stored at different selected temperatures and times.

Carbamazepine

20 ml of stock standard solution of 30 ug/ml carbamazepine in methanol was evaporated to dryness. The residue was reconstituted with 200 ml of blank blood added as above to produce a spiked blood of 3 ug carbamazepine per ml. The

procedure was repeated with water to produce an aqueous solution with concentration of 3 ug carbamazepine per ml. Aliquots were transferred into hypo-vials and sealed for further investigation as above.

Phenytoin

20 ml of stock standard solution of 100 ug/ml of phenytoin in methanol was processed as above to produce a concentration of 10 ug phenytoin per ml of blood. The procedure was repeated to prepare the aqueous solution. Aliquots were transferred into hypo-vials and sealed for further investigation as above.

3.1.2 - Non-acidic drugs

3.1.2.1 - Benzodiazepine

Temazepam

A stock standard solution of Temazepam was made up in methanol (10 ug/ml). 60 ml of the above stock was transferred into a clean conical flask and evaporated to dryness under nitrogen steam on a hot block 60^{o}C. The Temazepam was redissolved in 600 ml of the blood as above to produce a concentration of 1 ug drug per ml of blood. 15 ml aliquots were transferred into clean hypo-vials of 25 ml capacity and then sealed with butyl rubber septa. The procedure was repeated with water to produce an aqueous solution of Temazepam.

3.1.2.2 Opiates

A standard solution of morphine (0.5 ug/ml) and buprenorphine (0.25 ug/ml) in blood and aqueous solution were prepared as above. Five ml aliquots were transferred to pre-silanised hypo-vials as above. All the glass vials were silanised with dimethylchlorosilane [$H(CH_3)_2Si\ Cl$] and then washed with methanol to react with the excess DMCS and left to dry before use.

3.2 DRUGS STORAGE

Two vials for each measurement of spiked blood, aqueous solution and blank blood for each drug were stored at different temperatures and periods of time before re-analysis to measure the effect of temperature and time interval on the drug concentration. The interval of times between each analysis was designated as two, four, six, eight weeks and three, six, nine and twelve months. The drugs were stored at $5^{o}C$, $25^{o}C$ and $-20^{o}C$. Two samples each of spiked blood, blank blood and aqueous solution of each drug were removed from storage, opened and analysed at each designated period of time.

3.3 - TEMPERATURE MONITORING OF SPIKED SAMPLES

The spiked blood and aqueous solution with drugs under study was monitored from the day of spiking until the end of the experiment at the three storage temperatures (5, 25, -20^{o}C).

3.4 - ANALYSIS OF INTERFERING SUBSTANCES

3.4.1 - Acid Interferences "Indole"

Equipment

The method used for Indole analysis was high performance liquid chromatography based on a Pye Unicam PU 4015 pump (Philips) with dual piston heads. The detector used was a variable wavelength ultra-violet detector (Pye Unicam PU 4025) with an 8 ul flowcell with a path length 10 mm. The column was 25cm x 4.6 mm I.D. (internal diameter) cartridge pre-packed with Hypersil 5 um ODS C18 (Capital HPLC Specialists) fitted with 5 cm 4.6 mm I.D. ODS C18. guard column. The injector port was a Rheodyne 7125 with 20 and 100 ul loop. Indole was detected at 210 or 273 nm. The chromatograms were recorded on a BBC Goerz Metrawatt SE120 recorder, operated at 1 cm/min and at 10mV full-scale deflection.

Mobile Phase

The mobile phase used to separate Indole consists of 0.02M sodium acetate/acetonitrile/methanol (400:180:180 v/v/v). The pH of the mixture was adjusted to 5.5 using glacial acetic acid. Sodium acetate buffer of 0.02M was prepared by dissolving 2.72g of sodium acetate (M.wt.136) in deionised water and made up to 1 litre.

Choice of Internal Standard

Indolin has the same Indole structure with only one difference, there is no double bond on position (2-3). A standard solution of Indolin was prepared in methanol to give a concentration of 1 ug/ml.

Indomethacin is an analgesic and one of the Indole derivatives. A standard solution of Indomethacin was prepared by dissolving 2 mg in 100 ml methanol to produce a concentration of 20 ug/ml.

Fenoprofen Standard solution of Fenoprofen was prepared by dissolving 10 mg in 100 ml methanol to produce a standard concentration of 100 ug/ml.

Butalbital is used as an internal standard for acidic drugs determination. Standard solution of butalbital prepared in methanol with a concentration of 10 ug/ml. by dissolving 1 mg of butalbital in 100 ml methanol.

Butobarbitone

A standard butobarbitone solution with a concentration of 16 ug/ml was prepared by dissolving 1.6 mg in 100 ml methanol.

5-ChloroIndole

5-ChloroIndole is also one of the Indole derivatives, a stock standard solution of 5-ChloroIndole was prepared by dissolving 5 mg of 5-ChloroIndole in 100 ml methanol to produce a stock standard solution of 50 ug/ml. This solution was diluted with methanol or deionized water to obtain standard solution of 5 ug/ml.

The Maximum Absorptivity of Indole:

The UV spectrum of Indole and the chosen internal standard in the above mobile phase was obtained using a diode array spectrophotometer (Hewlett Packard 8751A), the cell volume was 3 ml with a 10 mm path length.

Linearity and Minimum Detectability of the HPLC System

A series of Indole standards was made up in the mobile phase. The concentration range was 0.5, 1.0, 2.0, 3.0, 4.0 and 5 ug/ml. Six analyses at each concentration were carried out at 0.02 and 0.08 AUFS. The average peak heights were used to construct the calibration curve for the Indole analysis.

A serial dilution of the Indole standard of 500 ng/ml in mobile phase was used to determine the minimum detectability.

Reproducibility of the HPLC System

A standard solution of Indole at a concentration of 1 ug/ml was prepared in the mobile phase. The reproducibility of the HPLC was determined by injecting the standard Indole (1 ug/ml) twenty times each day over three successive days under the same conditions. Flow Rate = 2 ml/min. : Range (AUFS) = 0.02 : Chart Speed = 1 cm/min. using the described HPLC [3.4.1].

3.4.2 Non Acid Interferences

The three putrefactive amines, 2-phenethylamine, tyramine and tryptamine were analysed with the developed high performance liquid chromatograph system described below.

Equipment

The chromatographic system was based on a Pye Unicam PU 4015 pump incorporating a two piston head. The eluent was monitored at 210 nm using a Pye Unicam PU 4025 variable wavelength ultra-violet detector incorporating an analytical flow cell assembly of volume 8 ul and path length 10 mm. Spectra were recorded on a BBC Georz Metrawatt SE 120 recorder operated at 1 cm/min and 10mV full-scale deflection. The column used for separation of the three putrefactive amines was 5 cm x 4.6 mm internal diameter pre-packed with silica and fitted with guard column 1 cm. x 4.6 ID supplied from Jones Chromatography fitted with a 20, 100, 500 ul loop in Rheodyne 7125 injection valve.

Preparation of Standard Solutions

A stock standard solution of the three putrefactive amines was made up in 100 ml methanol to produce the following concentration 15 ug/ml Tryptamine hydrochloride, 5.4 ug/ml 2-phenethylamine hydrochloride and 52 ug/ml Tyramine hydrochloride. These solutions were diluted with the mobile phase to obtain the desired concentrations.

Mobile Phase Development

It is known that the separation of basic compounds can be achieved by normal phase chromatograph (silica column) with a non-aqueous mobile phase, [136, 137]. A mobile phase has been developed for fast and better separation of the three putrefactive amines by using different ratios of acetonitrile/methanol/ammonia.

Choice of Internal Standard

The internal standard should not be expected to be found in putrefied blood, therefore different chemicals and drugs have been tested for their suitability to working as internal standard in the analysis of the three putrefactive amines. 5-chloroIndole, amitriptyline, phenylethylacetylurea, Flupenthixol, nalorphine,

morphine and dihydrocodeine, have been tested. A standard solution of each drug was prepared in mobile phase. 20 ul was injected in the normal phase HPLC system to investigate their separation by HPLC.

Linearity of the HPLC-system

Standard solutions of 0.5, 4, 8, 16 ug/ml of 2-phenethylamine hydrochloride and 0.5, 2, 4, 6, 8, 10 ug/ml both tyramine and tryptamine hydrochlorides were individually prepared in methanol. Five analyses were performed at each concentration within the same day.

Minimum Detectability and Maximum Absorptivity

Standard solutions of 0.4 ug/ml 2-phenethylamine 0.2 ug/ml Tryptamine and 0.2 ug/ml tyramine was made up in mobile phase. A serial dilution of the three putrefactive amines standard solutions in mobile phase was used to determine the minimum detectability of the three amines on the HPLC system described in 3.4.2. The wavelength of maximum absorption of the three putrefactive amines was determined by scanning a standard solution of 20 ug/ml of each in the mobile phase. A Hewlett Packard 8451 A diode array spectrophotometer was used to scan the solutions over the spectrum range 200-350 nm.

Reproducibility of the HPLC-system

Standard solutions of 2 ug/ml of 2-phenethylamine, Tyramine, Tryptamine hydrochloride were prepared in methanol. Five injections of each amine were performed to determine the day variation and day-to-day variation.

3.5 DRUGS ANALYSIS

3.5.1 Acid Drugs

3.5.1.1. - Anticonvulsants

Preparation of Standard Solutions

Stock standard solutions of 100 ug/ml phenobarbitone, 30 ug/ml carbamazepine, 100 ug/ml phenytoin and 100 ug/ml butalbital (internal standard) were made up in methanol.

Mobile Phase Equipment and Column

The equipment and column used to analyse acid drugs is as described in 3.4.1.

The mobile phase used for separation of acid drugs consists of 0.02M sodium acetate/methanol/acetonitrile (400:180:180 v/v/v) Battah [158]. The pH of the mobile phase was adjusted to 5.5 using glacial acetic acid and was degassed for 10 minutes before use.

3.5.2 Non-acid Drugs

3.5.2.1 Benzodiazepine - HPLC System

Mobile Phase

The separation of Temazepam and the internal standard prazepam was achieved by preparing a mobile phase of 0.01M Na_2HPO_4/methanol (25:75 v/v) as described by Eppel [202]. The pH of the mixture was 8.8.

Equipment and Column

The pumping system, the detector and the column is used as described in 3.4.1. The eluent of the column was monitored at 230 nm. and a flow rate of 1.5 ml/min. 20 ul of Temazepam was injected into the HPLC system.

Preparation of Standard Solutions

A stock standard solution of Temazepam 10 ug/ml and Prazepam (internal standard) 30 ug/ml were made up in methanol and used to prepare a working standard temazepam of 1 ug/ml and 3 ug/ml for prazepam (I.S) in blood and aqueous solution.

3.5.2.2 Opiates

Equipment

The separation of derivatized morphine, buprenorphine and their internal standard D_3-morphine and D_2-buprenorphine were achieved using Hewlett Packard model HP5980 gas chromatograph fitted with a capillary column (Chrompack CP-Sil 5, 25m-x-0.32mm internal diameter with 0.4 um film thickness). The injector temperature was 300^{O}C. The initial oven temperature was 180^{O}C. This was maintained for one minute and then increased at 10^{O}C/min to 300^{O}C. This final temperature was maintained for ten minutes. The capillary interface to the mass spectrometer was kept at 250^{O}C. The mass spectrometer used was a VG model 70-250 S operated at 8kv. High resolution selective ion recording was used to reduce interference in the analysis. The SIR channels were calibrated and tuned using perfluorokerosene (PFK) reference mass at m/z 454 at a resolution of 1000.

Then the peak height was reduced to 10% by the exit slit. The peak was reduced by a further 50% by closing the collector slit to give a resolution of 10,000. The collector was then opened slightly to obtain a flat peak top. Electron Impact (EI) was used as ionization method.

Gas Flow Rate on the GC-MS

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 of 5 psi.

Preparation of Standard Solutions

A stock standard solution of morphine hydrochloride and buprenorphine hydrochloride were made up in methanol at a concentration of 5 ug/ml and 2.5 ug/ml respectively. The internal standards were also made up in methanol at concentrations of 5 ug/ml for D_3-morphine hydrochloride and 2.5 ug/ml D_2-buprenorphine hydrochloride.

Synthesis of the derivatization Reagent (DETMDS)

A 250ml three neck round bottom flask was used for the reaction. The middle neck was connected to a venting tube containing calcium oxide granules. A thermometer was fitted through the second neck to monitor the reaction temperature and a gas leak was fitted to the third neck permitting small bubbles of ammonia to pass through the reaction mixture. The flask was placed in a liquid paraffin bath on a stirrer hot plate. Stirring of the mixture was accomplished with magnetic stirrer bars in both flask and the paraffin bath. Liquid was placed in a 500 ml round bottom flask which was cooled in a bath of acetone and dry ice. Ammonia gas effluent was passed through two Drischel baths, the first containing a layer of liquid paraffin to monitor the flow rate and the other (empty) bottle was to prevent the solvent from the reaction mixture sucking back. 150 ml of dry redistilled benzene was added to the reaction flask and the flask was purged with ammonia gas for a few minutes at 50-60^{o}C before the addition of 30 grams of dimethylethylchlorosilane with continuous stirring for 45 min. The reaction was stopped by disconnecting the ammonia supply. The following procedures were conducted to isolate the product diethyltetramethyldisilazane (DETMDS). The bulk of the solvent containing the product was filtered through a filter paper placed on a Buchner funnel. Vacuum was generated by a water pump, so this step was conducted very quickly to minimise exposing the product to hydrolysis. The collected clear solvent was placed in a distillation apparatus and the bulk of benzene was removed by distillation. The remaining fluid (25 ml) was transferred to a smaller distillation apparatus where the benzene residue was removed by distillation at 78^{o}C. The product was recovered at 172^{o}C and stored at 4^{o}C.

Derivatization with DETMDS

The derivatization reagent was prepared from 40% of DETMDS in solvent composed of acetonitrile and toluene (7:3 v/v) stored at 4°C in a screw capped readi-vial. This mixture was used for the derivatization of morphine and buprenorphine in extracts reviously evaporated to dryness, then heating the samples at 60°C for 15 minutes with 100 ul of the derivatization reagent in a screw capped readi-vial.

3.6 EXTRACTION OF INTERFERING SUBSTANCES

3.6.1 - Acid Interferences

The following experiments were conducted to select a quick and efficient protein precipitation reagent for the Indole extraction procedure to provide an extract suitable for application to solid-phase extraction.

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. The ratios of protein precipitation reagent to whole blood to give precipitation >95% protein has been discussed in the literature [213].

a) Methanol

0.1 ml of blood spiked with Indole at a concentration of 50 ug/ml (blood) was added 0.3 ml methanol. The mixture was vortexed for one minute and then centrifuged for five minutes. Aliquots of the supernatant was injected onto the HPLC system.

b) Isopropanol

0.3 ml Isopropanol was added to 0.1 ml blood and the sample processed as above.

c) Zinc Sulphate/Methanol

This protein precipitation reagent was prepared by dissolving 5gm of ZnSO4 in 100 ml distilled water. 70 ml of this solution was mixed with 30 ml Methanol and stored in a sealed reagent bottle. 0.2 ml of the ZnSO4/Methanol reagent was mixed with 0.1 ml blood and processed as above.

d) Perchloric Acid

Stock solution (3.0M) of perchloric acid was diluted with water to prepare an 0.4M solution. 0.2 ml of the reagent was added to 0.1 ml blood and processed as above.

e) Sulphosalicylic Acid

To produce a 10% w/v of sulphosalicylic acid reagent, 10g of sulphosalicylic acid was dissolved in 100 ml of water. 0.2 ml of the reagent was mixed with 0.1 ml blood and processed as above.

f) Protein Precipitation with Methanol/Isopropanol

0.1 ml each of stock Indole standards and internal standard 5-ChloroIndole in concentrations of 2.5, 10 and 50 ug/ml were added to 0.8 ml of blank blood to produce spiked blood Indole and 5-ChloroIndole at concentrations of 0.25, 1 and 5 ug/ml. To each of five 1 ml samples of spiked blood in a screw-capped vial 2 ml Methanol/Isopropanol (5:1 v/v) was added. The vial was sealed, vortexed for one minute and then centrifuged at 5000 rpm on an angle head centrifuge for three minutes. The supernatant was transferred to a clear vial for analysis by HPLC.

Recovery of Indole with Methanol/Isopropanol

Five samples of spiked blood Indole and 5-chloroIndole (I.S) at three different concentrations (0.25, 1, 5 ug/ml) were treated as in 'f' above. The supernatant was analysed to estimate the Indole and 5-chloroIndole recoveries over the above concentration range. A set of Indole standards and 5-chloroIndole standards were prepared in Methanol/Isopropanol (5:1v/v) at the above concentrations to estimate Indole and 5-chloroIndole recoveries.

Solid Phase Extraction of Indole

Standard Solution Preparation

A stock standard Indole solution was prepared by dissolving 5 mg of Indole in 100 ml deionized water to produce a stock solution of 50 ug/ml. Further dilutions were made up in methanol to produce working standard solution of 0.5 and 5 ug/ml. The same procedure was repeated to produce standard solutions of 5-ChloroIndole (internal standard).

Bond Elut Certify

This type of sorbent was recently introduced by Analytichem in columns with 5 ml capacity. The column was conditioned with 2 x 2 ml of methanol followed by 2 x 2 ml of 0.1M phosphate buffer pH 6.0. One ml of Indole standard stock solution 5 ug/ml was prepared in deionized water and mixed with 5 ml phosphate buffer in a screw-capped vial. The specimen was added to the column. The specimen was drawn slowly through the column. The effluent of the column was collected and analysed for the presence of Indole (un-retained Indole). The column was rinsed with 1 ml 0.1M phosphate buffer pH6/Methanol (80:20 v/v) and then washed with 1 ml 1.0M acetic acid followed by 1 ml hexane. The effluent of the column from the three washing steps were collected and analysed for the presence of Indole to measure the losses during the washing steps. Indole was eluted with 2 x 1 ml dichloromethane. The 0.1M phosphate buffer was prepared by dissolving 13.61g of KH2P04 (M.wt.136.09) in deionized water and made up to 1 litre. The pH was adjusted to 6 with 1.0M potassium hydroxide. The 1.0M acetic acid was prepared by pipetting 5.75 ml glacial acetic acid into a 100 ml volumetric flask half filled with deionised water. This was mixed and brought to volume with deionised water.

Non-Polar Sorbent C18

Bond Elut C18 columns of 3, 6 ml capacity were pre-conditioned with two bed volumes of methanol followed by two bed volumes of deionised water.

Buffers Preparation

Different buffers were tested for their suitability for conditioning and extraction of Indole by solid phase sorbent. 0.5M disodium hydrogen phosphate (M.wt. 141.96) was prepared by dissolving 70.98g in deionised water and made up to 1 litre. The resulting pH was 9.5. A low ionic strength of disodium hydrogen phosphate 0.05 M was prepared by dissolving 7.1g of Na2HPO4 deionised water and made up to in 1 litre. The third buffer was sodium acetate with 0.1M prepared by dissolving 1.36g of sodium acetate (M.wt. 136) in deionised water and made up to 100 ml. The pH was adjusted to 12 by adding a few drops of 0.1M sodium hydroxide.

Extraction

The Indole standard was mixed with each buffering system individually. The mixture was slowly drawn through a pre-conditioned C18-column under vacuum and then washed with 2 x 1 ml of deionized water. Indole was eluted from the column with 2 x 0.5 ml methanol and analysed by HPLC.

Recoveries of Indole on Bond Elut C18 with Sodium Acetate Buffering

Indole and 5-chloroIndole standards (20 samples) were extracted using C18-columns. The recovery was calculated by comparing the peak height of extracted Indole with non-extracted Indole standards analysed at the same time by HPLC.

Day-to-day Recoveries of Indole on Bond Elut C18

A set of standard Indole samples were extracted with C18-columns on a weekly basis for three weeks. The Indole standard concentrations were 0.5 and 5 ug/ml.

Re-using Bond Elut C18-Columns

Ten samples of Indole standard was extracted through the same column. The eluent of the column was collected and analysed by HPLC. The experiment was conducted in duplicate and with different Bond Elut C18-lot numbers.

Indole Extraction Calibration Curve

A set of Indole standards were prepared from a stock solution of 50 ug/ml to produce different concentrations of Indole. The concentration of the internal standard 5-chloroIndole was 5 ug/ml while Indole concentrations in whole blood were 0.1, 0.25, 0.5, 2.5, 5 ug/ml. Methanol/Isopropanol (5:1) was used to precipitate the protein as described earlier. The supernatant was mixed with 2 ml of 0.1M sodium acetate buffer and extracted through the C18 columns. Six samples were extracted on C18 for each concentration and analysed by HPLC.

The Effect of Column Washing on Extract Cleanliness

The effect of washing C18-column with 12% Methanol/water was investigated by applying a washing step before eluting the Indole from the column, and others left without washing. The eluent of both groups were analysed by HPLC.

Indole Losses With Storage

A standard Indole concentration of 0.6 ug/ml was prepared in deionised water. Aliquots of 1 ml were placed in a screw-capped vial and left at room temperature. The concentration of Indole was measured after five days. A fresh standard Indole was prepared to calculate the losses of Indole with storage.

3.6.1.1 Acid Interferences in Blood Samples Spiked With Drugs

The whole blood samples spiked with anticonvulsants, benzodiazepine and opiates were stored at three difference storage conditions (5, 25, -20^{o}C) for different time intervals were analysed for Indole concentration. The drugs concentrations are given in 3.1.

3.6.2 - Non-acid Interferences

Standard Putrefactive Amines Preparation

2-phenethylamine stock solution (10mg/ml) was prepared by dissolving 1.047 ml of 2-phenethylamine (parity 99%, density 0.965) in deionozed water to a total volume of 100 ml.

A standard solution of 2-phenethylamine (100 ug/ml) was prepared by adding 1 ml of aqueous stock 2-phenethylamine solution (10 mg/ml) into 100 ml volumetric flask. The volume was made up to 100 ml by adding deionized water. A standard solution mixture of 2-phenethylamine, tyramine and tryptamine were made up by dissolving 1 mg of tyramine and tryptamine free base in 100 ml deionised water followed by adding 0.1 ml of stock 2-phenethylamine free base solution (10 mg/ml). The resulting concentration for the three putrefactive amines was 10 ug/ml.

Protein Precipitation

Whole blood cannot be extracted directly through solid phase sorbent therefore treating the whole blood with a protein precipitation reagent is necessary to obtain a supernatant which is easily applicable to the solid phase sorbent.

a. Methanol

Spiked blood with 2-phenethylamine was prepared by adding 0.1 ml of a stock solution (100 ug/ml) of 2-phenethylamine free base in water to 0.9 ml blank blood, producing sample concentration of 10 ug/ml. Three mls of methanol was added to a screw-capped vial containing the spiked blood. The sample was then vortex-mixed for one minute, centrifuged for five minutes using an angle head centrifuge at 5000 rpm. The supernatant was transferred into a clean vial. 20 ul aliquots of the supernatant was injected onto the HPLC system in three successive injections.

b. Zinc Sulphate

Preparing zinc sulphate as a protein precipitation reagent was described in 3.6.1. Sheep serum was supplied by the Scottish Antibody Production (Lanarkshire, Scotland). The serum was spiked with 2-phenethylamine free base to produce a concentration of 10 ug/ml. 2 mls of zinc sulphate reagent were added to vials containing the serum spiked with 2-phenethylamine. The sample was vortex

mixed for 30 seconds then left to stand for five minutes at room temperature before being centrifuged on an angle head centrifuge for five minutes. The supernatant extracted through C18-column as described below and analysed for 2-phenethylamine by HPLC.

c. Acetonitrile/Isopropanol (5:1 v/v)

Five samples of sheep serum was spiked with the three putrefactive amines, 2-phenethylamine, tyramine and tryptamine, in concentrations of 10 ug/ml. The samples were processed as above and 20 ul of the supernatant was injected onto the HPLC system.

Diatomaceous Earths (Extrelut^{R})

Column Preparation

A Merck Extrelut^{R} was washed with dichloromethane before use and left to dry at room temperature. A plug of glass wool treated with dimethyldichlorosilane was introduced into a 10 ml plastic syringe. On the top of the glass wool plug the syringe was filled with 1.5 gm of Exrelut^{R}.

Preparing of Standard Solution

A mixed standard solution containing 100, 100, 50 ug/ml of 2-phenethylamine, Tyramine and Tryptamine mixture in deionized water, (0.1 ml) was added to 0.9 ml of blank blood to produce a working standard concentration of 10, 10, 5 ug/ml of 2-phenethylamine, Tyramine and Tryptamine respectively.

Sample Preparation

Two buffering systems were investigated for sample extraction from Extrelut. The two buffers were 0.5M Na_2HPO_4 and 5% w/v sodium bicarbonate ($NaHCO_3$). 1 ml of spiked blood was mixed with 2 ml of buffer and added to the column and allowed to stand for 10 minutes prior to elution.

Elution

The three putrefactive amines were eluted from the diatomaceous earth column with about 12 ml of diethylether/isopropanol (7:3 v/v) to produce a total volume of 8 ml. 20 ul was injected into the HPLC system as described in 34.3.2. The effluent was monitored at 230 nm.

Solid Phase Extraction

Non-Polar sorbent C18

A working standard of 2-phenethylamine free base (10 ug/ml) was prepared from a stock solution of 10 mg/ml in methanol by adding 0.1 ml of stock to 100 ml methanol, the same procedure repeated to prepared a standard 2-phenethylamine solution of 10 ug/ml in deionized water.

Condition of the Column

Bond Elut C18 columns of 3 ml capacity were used. The columns were conditioned with two bed volumes of methanol followed by two bed volumes of water followed by 2 and 3 ml 0.1M sodium acetate buffer pH = 12.

Addition of Sample and Elution

1 ml samples of the standard 2-phenethylamine solution (10 ug/ml) were mixed with 2 ml 0.1M sodium acetate buffer pH = 12, then added to pre-conditioned columns and drawn through under vacuum. The columns were then washed with deionized water (2 x 0.5 ml). The elution of 2-phenethylamine was achieved

0.5 ml of elution mixture (82:25:0.3 v/v/v) acetonitrile/methanol/ammonia or 2 x 0.5 ml methanol. The eluent was analysed by HPLC.

Bond Elut Certify Column

Bond Elut certify is a newly marketed solid phase sorbent from Analytichem International. They claim that the certify column is capable of extracting acidic, neutral and basic compounds from urine and with special sample preparations in the case of blood [214].

Bond Elut Certify Conditioning

The column was conditioned by passing 2 ml methanol followed by 2 ml 0.1M sodium acetate buffer, pH 6.

Sample Preparation

Spiked sheep serum containing 2-phenethylamine, Tyramine and Tryptamine free base at a concentration of 10 ug/ml were prepared by adding 0.1 ml of each stock

standard (100 ug/ml) solution of each drug to 0.7 ml sheep serum. 2 ml of zinc sulphate was used to precipitate the proteins [214]. The mixture was vortexed for 30 seconds, let stand for five minutes at room temperature, vortexed again for 30 seconds and centrifuged for 10 minutes at 3000 rpm. The supernatant was transferred to each column. The column was positioned in a 10 column capacity VaC Elut^{R} system. 2 ml 0.1M sodium acetate buffer, pH 6, was added and allowed to stand for 10 seconds to allow mixing. The vacuum was applied and the sample was drawn slowly through the column. It took at least two minutes for the specimen to pass through the Bond Elut Certify column. The column was washed with 1 ml 1.0M acetic acid, dried under full vacuum for five minutes and then washed with 6 ml methanol. The column was dried under full vacuum for two minutes and the putrefactive amines eluted with 2 x 1.0 ml acetonitrile/methanol/ammonia (82:25:0.3 v/v/v).

Cationic Exchange Sorbent

Conditioning of the SCX Column

The SCX column of 1 ml capacity were conditioned with 2 x 1 ml methanol followed by 2 x 1 ml diethylether/Isopropanol (7:3 v/v).

Preparation of the Standard Solution

A standard solution mixture containing 1, 1, 0.5 ug/ml of 2-phenethylamine, Tyramine and Tryptamine respectively as their free bases were made up in diethyl ether/isopropanol (7:3 v/v).

Addition of the Sample

1 ml of the above standard putrefactive amine mixture was added to the SCX column drawn slowly through by controlling the vacuum. The effluent of the columns was collected and analysed for unretained putrefactive amines.

Elution

The putrefactive amines were eluted in turn with 2 x 0.5 ml of:

1) 15% ammonia in diethyl ether/Isopropanol (7:3 v/v).
2) 20% ammonia in methanol.
3) Acid methanol (25 ul 10% H_2S0_4 in 5 ml methanol).

Conditioning of CBA Column

The CBA columns of 1 ml capacity were conditioned with two column volumes of methanol followed by one column volume of water and two column volumes of diethylether/isopropanol (7:3 v/v).

Standard Putrefactive Amine Preparation

The three putrefactive amines were prepared in diethylether/isopropanol (7:3 v/v) at different concentrations of 1, 1, 0.5 ug/ml and 10, 10, 5 ug/ml for 2-phenethylamine, Tyramine and Tryptamine free base respectively.

Specimen Application

1 ml of the standard solution mixture was added to the pre-conditioned CBA-column via an adaptor. The specimen was drawn slowly through the column with vacuum pressure of 5 mm Hg.

Elution of Putrefactive Amine

Acid methanol was prepared by adding 10 ul of 10% H_2S0_4 to 5 ml methanol. The molarity of acid in methanol was 3.6 x 10^{-3}M. 2 x 0.5 ml of acid methanol was used for elution.

Re-Using CBA-Columns

1 ml capacity CBA-columns of different lot numbers were tested for their ability to be used several times. Six samples of the three putrefactive amines standard mixture at concentrations of 1 ug/ml 2-phenethylamine, Tyramine and Tryptamine as their free bases were prepared in diethylether/isopropanol (7:3 v/v). The CBA-columns were positioned in a 10 column capacity Vac Elut^R system. The pressure was adjusted to 5 mm Hg and then each column was conditioned with 2 x 1 ml methanol followed by 2 x 1 ml deionized water. One ml of the standard solution was applied to the columns and drawn through.

The adsorbed putrefactive amines were eluted from the column with 2 x 0.5 ml acid methanol. 100 ul of the effluent was analysed by HPLC. The same procedure was repeated six times using the different lot number CBA-columns.

Anion Exchange Column

Amino propyl NH_2 column is a weak anion exchange sorbent. It has been used for the purification of C18-extracts of some steroids [215]. Amino propyl columns of 3 ml capacity were conditioned with 2 x 3 ml methanol followed by 2 x 3 ml deionized water. The ability of the column to give a clean extract was tested by passing acidic methanol (3.6×10^{-3}M H_2S0_4 containing the three putrefactive amines mixture in a concentration of 1, 1, 0.5 ug/ml for 2-phenetheylamine, Tyramine and Tryptamine respectively. The effluent was collected and analysed by HPLC. The un-extracted standard mixture was used to calculate the recoveries.

Recoveries from Combined Extrelut-CBA-NH_2 Column

Extrelut is used as an alternative to the protein precipitation in sample preparation for solid phase extraction. The reproducibility of the combined Extrelut-CBA and NH_2 were investigated. A set of standard solutions of the three putrefactive amines mixture were prepared in blank blood to produce the following concentrations of putrefactive amines:

1. 0.1 ug/ml 2-phenethylamine, Tyramine, Tryptamine
2. 1 ug/ml 2-phenethylamine, Tyramine, Tryptamine
3. 5 ug/ml 2-phenethylamine, Tyramine, Tryptamine
4. 10 ug/ml 2-phenethylamine, Tyramine, Tryptamine

Six samples of each concentration set were extracted through Extrelut^R and eluted to produce a total volume of 8 ml diethylether/Isopropanol (7:3 v/v). The Extrelut eluent was passed through the pre-conditioned CBA-column. The three amines

were eluted from the 1 ml capacity CBA-column with acidic methanol (2 x 0.5 ml) then the acidic methanol was passed through a pre-conditioned NH_2 column of 3 ml capacity. The effluent of the NH_2 column was analysed by HPLC.

Calibration Curve

A set of the three putrefactive amines standards mixture were prepared in whole blood in concentrations of 0.1, 1, 5, 10 ug/ml. The samples were extracted through the combined Extrelut-CBA-NH_2-Column. The internal standard concentration was 2 ug/ml. The peak height ratio in duplicate, against concentration were plotted to construct a calibration curve for each amine.

3.6.2.1 Non-Acid Interferences in Samples Spiked with Drugs

Samples of whole blood spiked with anticonvulsants, benzodiazepines and opiates which were stored at different temperatures and times as described earlier, were extracted by the appropriate methods described earlier to investigate the presence of the three putrefactive amines in blood stored in sealed vials over the different storage intervals and times.

Standard and Internal Standard Solutions

A standard solution mixture of the putrefactive amines containing 20 ug/ml of 2-phenethylamine, tyramine, tryptamine respectively as their free base were prepared in deionized water. The internal standard (dihydrocodeine) stock solution was made up in deionized water at a concentration of 20 ug/ml.

Preparation of the Samples for Extraction

In a 6 ml screw-capped vial containing 1 ml of whole blood sample (spiked, post-mortem) was mixed with 2 ml 5% w/v $NaHC0_3$ and 0.1 ml of 20 ug/ml internal standard dihydrocodeine.

Standard and Blank Preparation

0.1 ml of the above standard solution (20 ug/ml) of the three putrefactive amines mixed with 0.9 ml deionized water in a 6 ml capacity screw-capped vials. 0.1 ml (I.S.) and 2 ml 5% w/v $NaHC0_3$ were added. The blank sample was prepared by adding 1 ml deionized water to a vial containing 2 ml of 5% w/v$NaHC0_3$ and 0.1 ml (I.S.). The total volume in all samples (blood, standard, blank), was 3.2 ml. The prepared samples were extracted using the procedure mentioned above.

3.7 DRUGS EXTRACTION

3.7.1 - Acid Drugs

3.7.1.1. Anticonvulsants

Preparation of Extrelut^{R} Column

150g of Extrelut^{R} was washed with 500 ml of dichloromethane, then the Extrelut was collected on filter paper and allowed to dry out completely at room temperature.

A plug of silanised glass wool was introduced into small Pasteur pipettes then filled with the washed Extrelut^{R} to 1 cm from the top (approximately 0.6g of Extrelut^{R}).

Standard Preparation

A standard solution containing each of 100 ug/ml phenobarbitone and phenytoin and 30 ug/ml carbamazepine was made up in methanol. A solution of 100 ug/ml butalbital (internal standard) was made up in methanol.

Extraction Procedure

Extraction Buffer Preparation

70.98 grams of disodium hydrogen phosphate (Na_2HP0_4) were dissolved in deionized water and made up to 1 litre to produce a buffering solution of 0.5M Na_2HP0_4 with a pH of 9.0.

Specimen Preparation

Standard

0.1 ml of the acid drug standard was added to 0.9 ml of blank blood or deionized water in a screw-capped vial and mixed well.

Sample - "Blood Spiked with Anticonvulsant Drugs"

In a glass screw-capped vial, 0.4 ml of sample or spiked standard was mixed with 0.4 ml of extraction buffer and 0.1 ml of internal standard (butalbital 100 ug/ml), and allowed to stand for two minutes.

Blank

0.4 ml of blank blood or deionized water was mixed with 0.4 ml of extraction buffer and 0.1 ml internal standard (butalbital 100 ug/ml) in a glass screw-capped vial and allowed to stand for two minutes.

Extraction Procedure Using Extrelut^{R} Column

0.65 ml of the prepared specimen "standard, sample, blank" was applied to the Extrelut column and allowed to absorb for 10 minutes. It was then eluted with 3 ml dichloromethane, 0.5 ml at a time using an automatic pipette. The eluent was collected in a screw-capped glass vial. A little pressure was applied on the top of the column using rubber teat to assist recovery of the eluting solvent. This was evaporated to dryness by placing the vial on a hot plate ($60^{o}C$) under a stream of nitrogen gas, then the extract was redissolved in 100 ul of mobile phase, ultrasonicated for five minutes and 20 ul injected onto the HPLC system.

3.7.2 - Non-Acid Drugs

3.7.2.1 - Benzodiazepine "Temazepam"

Extraction Buffer Preparation

Disodium hydrogen phosphate dihydrate (Na_2HP0_4. $2H_20$ M.wt. 177.99) buffer was made up by dissolving 1.78 grams of Na_2HP0_4 $2H_20$ in deionized water and made up to 1 litre, producing a buffer concentration of 0.01M with a pH of 7.4.

Standard Solution Preparation

Temazepam standard solution was made up in methanol by dissolving 1 mg of Temazepam in 100 ml methanol. The resulting concentration was 10 ug/ml. The internal standard Prazepam was prepared by dissolving 3 mg in 100 ml methanol to produce a Prazepam standard concentration of 30 ug/ml.

Extraction Procedure

1 ml of the phosphate buffer (0.01M) and 0.1 ml of the internal standard prazepam (30 ug/ml) were added into a ground-glass stoppered test tube. 1 ml of sample

(blood/aqueous solution/blank blood) were added into the tube. In the case of standard preparation 0.9 ml of blank blood or deionised water followed by 0.1 ml of standard Temazepam (10 ug/ml) were added then the above solutions were extracted with 5 ml dichloromethane in ground glass stoppered test tubes placed on a (rock and roll) machine for 20 minutes. The supernatant layer was removed by Pasteur pipette. The dichloromethane was filtered through phase separation paper into a clean screw-capped glass vial. The dichloromethane evaporated to dryness by placing the vial on a hot plate 60^{o}C under nitrogen gas stream. The benzodiazepine was redissolved by adding 100 ul of HPLC mobile phase. 20 ul was injected onto the HPLC system.

3.7.2.2 Opiates

Specimen Preparation

Spiked Standard

In a 6 ml screw-capped vial 1 ml of sample (blood/aqueous solution/blank blood) was mixed with 0.1 ml of opiates internal standard (5 ug/ml D_3-morphine, 2.5 ug/ml D_2-buprenorphine), then the samples were buffered with 1 ml of 0.1M ammonia solution mixed and allowed to stand for 2 minutes before being applied to the Extrelut^{R} column.

Extrelut Column Preparation

Extrelut^{R} (Merck) was placed in conical flask and washed with copious amounts of methanol/ethanol (1:1). The Extrelut was collected in filter paper and allowed to dry out completely. A plug of silanised glass wool was introduced into a plastic syringe of 10 ml capacity. This was filled with 2 gm of Extrelut^{R}.

Conditioning of SCX Column

SCX columns of 1 ml capacity were positioned in a 10 column capacity Vac Elut^{R} system. Pressure was adjusted to 10-15 mm Hg. SCX column were conditioned by passing 2 x 1 ml methanol followed by 2 x 1 ml deinonized water and followed by 2 x 1 ml ethylacetate/Isopropanol (9:1 v/v).

Extrelut-SCX Extraction of Opiates

The buffered specimens (blood spiked with morphine and buprenorphine) were applied to the Extrelut column and allowed to stand for 5-10 minutes. The columns were washed with 5 ml hexane. The Extrelut columns were then attached to the pre-conditioned SCX column via an adaptor. The SCX column was put on the Vac-Elut^{R} system. Morphine and buprenorphine were then eluted from the

Extrelut column with 12 ml of ethylacetate/Isopropanol (9:1) V/V under vacuum of $\simeq$ 5 mm Hg. Discard the Extrelut column and wash the SCX column with 5 ml acetonitrile/methanol (1:1 v/v). Morphine/buprenorphine was eluted from SCX column with 3 x 1 ml of 10% ammonia in acetonitrile/methanol (1:1 v/v) into a screw-capped glass vial. The elute was evaporated to dryness on a hot plate 60^{o}C under nitrogen gas stream then derivatized with 100 ul diethyltetramethyldisilazane (DETMDS). 1 ul was injected into the GC/MS system described in 3.5.2.2.

3.8 - APPLICATIONS

3.8.1 - Interfering Substances in Post-mortem Samples

As it is known that the putrefaction process begins in the cadaver immediately after death, post-mortem on the cadaver usually takes place within the first 24-48 hours after death. Blood specimens are taken from different sites of several cadavers according to availability.

The specimens from the cadaver, in most cases, are delivered for toxicological analysis within a few hours of the post-mortem examination. A full toxicological investigation on the specimens usually takes three to four days from the time of receiving the specimens. No preservatives such as sodium fluoride are normally used in preserving the post-mortem specimens. Samples under toxicological investigation are usually kept at 5^{o}C. When the investigation is complete the samples are stored at -20^{o}C for six months for any further enquiry. The samples are then destroyed.

Under the above procedure of handling and storing the post-mortem blood, some degree of putrefaction is expected to occur.

Eleven samples of post-mortem blood representing different storage periods have been analysed for the presence of the putrefactive amines. Two samples of blank blood which were left uncovered for two months were analysed for the presence of acid and non-acid interferences.

Analysis for Acid Interferences

Post-mortem blood samples were analysed for Indole by the method developed for Indole determination in post-mortem blood 3.4.1. Samples were extracted in duplicate as described in 3.6.1 on the same day. Deionised water was used as a blank and to prepared standard Indole solutions.

Analysis for Non-Acid Interferences

The same samples as above were analysed as described in 3.4.2 for the presence of 2-phenethylamine, tyramine and tryptamine by buffering 1 ml of blood with 2 mls of 5% w/v $NaHCO_3$. Then 0.1 ml of the internal standard solution of dihydrocodeine (2 ug/ml) was added. The mixture was then poured into a 10 ml

syringe filled with diatomaceous earth (Extrelut^{R} Merck). The three amines were eluted to a CBA-column, as described in 3.6.2.

3.8.2 Analysis of Drug Positive Post-mortem Samples

Samples of post-mortem blood which had been shown to be positive for drugs were stored at 5^{o}C and -20^{o}C for different time periods. The samples were re-analysed for the presence of putrefactive amines and the changes in drug concentrations.

3.8.2.1 Acid Drugs

Anticonvulsants

Post-mortem samples containing anti-convulsant drugs were analysed after different storage periods. The method of extraction was described in 3.7.1 and the HPLC system used for anti-convulsant drug analysis was described in 3.5.1.

3.8.2.2 Non-acid Drugs

Benzodiazepine

Post-mortem samples shown to be positive for Temazepam were re-analysed after different storage periods. The method of extraction and analysis used was as described earlier in 3.7.2.1 and 3.5.2.1 respectively.

Opiates

Five samples of the post-mortem blood were re-analysed after different storage periods. These samples had been analysed and found to be positive for morphine. A method of analysis used for opiate analysis was based on HPLC [211] described below.

Extraction of Morphine from Post-mortem Blood

Extrelut Column

The Extrelut was firstly washed with ethanol/methanol (1:1) and dried thoroughly in an oven at 100^{o}C before use. 1.2g of Extrelut^{R} (Merck) was added to a 5 ml capacity syringe plugged with silanized glass wool as an outlet filter.

Preparation of Extraction Buffers

1M ammonia solution was titrated with 2M hydrochloric acid using a pH meter with glass electrode until pH 9 was obtained.

Preparation of Standard Solutions

A standard solution was prepared by dissolving 2 mg of morphine hydrochloride in 100 ml methanol to produce a standard concentration of 20 ug/ml.

1 mg. of nalorphine hydrochloride was dissolved in 100 ml of methanol to produce an internal standard of 10 ug/ml

Sample Preparation for Extraction

One ml of post-mortem blood was mixed with 1 ml of buffer in a screw-capped vial and then 100 ul of internal standard (Nalorphine) was added followed by 100 ul of deionised water to make the total volumes 2.2 ml. Standard morphine extract was prepared using 1 ml of blank blood, 1 ml buffer, 100 ul standard morphine and 100 ul of internal standard (Nalorphine). The solution was mixed together in a screw-capped vial.

Blank samples were prepared by mixing 1 ml blank with 1 ml buffer, 100 ul internal standard (nalorphine) and 100 ul deionized water.

Extraction

The standard morphine, morphine samples and blank were transferred into a separate Extrelut^{R} columns and left for 5-10 minutes.

Elution of Morphine

After 5 - 10 minutes the Extrelut^{R} columns were washed with 5% v/v solution of n-butanol in dichloromethane until 8 ml were eluted into 20 ml capacity tubes. The elution solutions were evaporated under nitrogen until there was sufficient solution (2 ml) to transfer into a small screw-capped vial (3 ml capacity). It was then evaporated to dryness.

Analysis of Opiates

Mobile Phase

Potassium dihydrogen phosphate of 0.01M was prepared by dissolving 1.3g of KH_2PO_4 in one litre of deionised water. 400 ml of the buffer was mixed with 100 ml acetonitrile, then degassed for five minutes. 1.1g of octane sulphonic acid sodium salt was added to the mixture as an ion pairing agent. The pH of the mixture was adjusted to pH3 by adding a few drops of orthophosphoric acid and then degassed.

Equipment and Column

The HPLC system described in 3.4.1 was used to analyse morphine. The mobile phase used consisted of 0.01M potassium dihydrogen phosphate/acetonitrile (400:100 v/v). The mobile phase preparation is described above. The column eluent was monitored at 210 nm.

3.9 - THE EFFECT OF INTERFERING SUBSTANCES ON DRUG ANALYSIS

The interferences from the four putrefactive amines on qualitative and quantitative determination of acidic drugs and benzodiazepines were investigated by analysing the four putrefactive amines through the HPLC systems usually used for acidic drugs and benzodiazepines analysis.

3.9.1 - Acid Drugs

Anticonvulsant

The HPLC system used to analyse the acid drug studied in this thesis, phenobarbitone, carbamazepine and phenytoin were also being used to analyse other acid drugs like primidone, butobarbitone, amylobarbitone, methaqualone

and quinal barbitone. Therefore, the possible interferences from the presence of the four putrefactive amines with this procedure was investigated.

Equipment and Column

The HPLC system consisted of a Pye Unicam pump (4015) double two-head piston used to deliver the mobile phase with flow rate of 1.5 ml/min, incorporating a Pye 4025 variable wavelength ultra-violet detector set at 210 nm to monitor the column eluent.

The column used was 25 cm x 4.6 mm internal diameter (I.D) and guard column 5 cm x 4.6 mm (I.D) both pre-packed with Hypersil 5 um octadecylsilane C18 and fitted with a Rheodyne 7125 injection port with a 20 ul loop. The spectra were recorded on a BBC Goerz Metrawatt SE120 chart recorder operated at 1 cm/minute and 10mV full scale deflection.

Mobile Phase

The elution system was composed of 0.02M sodium acetate/acetonitrile/methanol (400:180:180 v/v). The pH of the mobile phase was adjusted with glacial acetic acid to 5.5.

Preparation of Drugs Standard Solutions

A stock standard solution mixture of all acid drugs was prepared in methanol. The drugs are primidone, phenobarbitone, butobarbitone, butalbital "internal standard," phenytoin, carbamazepine, amylobarbitone, methaqualone and quinalbarbitone. Their concentrations were 12.42, 10.98, 10.2, 5.76, 11.63, 5.54, 13.39, 11.68, 11.15 mg/100 ml respectively. 20 ul of this standard mixture was injected onto the acidic drugs HPLC system.

Preparation of Putrefactive Amines Standard Solution

A stock solution of each of the putrefactive amines in free base form were prepared by dissolving 5 mg of 2-phenethylamine, tyramine and tryptamine and Indole in 100 ml deionised water individually to produce a standard concentration of 50 ug/ml. 20 ul of each amine was injected onto the acid drug and benzodiazepine drug HPLC systems to determine their retention times.

3.9.2 - Non-Acid Drugs

Benzodiazepine

The method described in 3.7.2.1 is used routinely for the screening and quantitative analysis for Temazepam and other benzodiazepines such as Triazolam, chlorodiazepoxide, desmethyl diazepam and diazepam in blood samples.

Equipment Column and Mobile Phase

The HPLC system consists of Pye Unicam PU 4015 pump with two-head piston used to deliver the mobile phase incorporating with Pye Unicam PU 4025 variable wavelength ultra-violet detector set at 230 nm to monitor the column eluent. The column used was 25 cm x 4.6 mm (I.D.) and guard column 5 cm x 4.6 mm (I.D.) both pre-packed with Hypersil 5 um Octadecylsilane C18 and fitted with Rheodyne 7125 injection port with 20 ul loop. The elution system composed of 0.01M Na_2HPO_4/methanol 30:70 v/v. The spectra were recorded on BBC Goerz Matrawatt SE120 chart recorder operated at 1 cm/minute and 10 mV full scale deflection.

Standard Solution Preparation

A stock standard benzodiazepines solution of 2 ug/ml for the six benzodiazepines, Triazolam, Temazepam, chlorodiazepoxide, desmethyl-diazepam, diazepam and prazepam (Internal Standard) were prepared in methanol. 2ul of this standard mixture was injected onto benzodiazepines HPLC system.

CHAPTER FOUR

RESULTS AND DISCUSSION

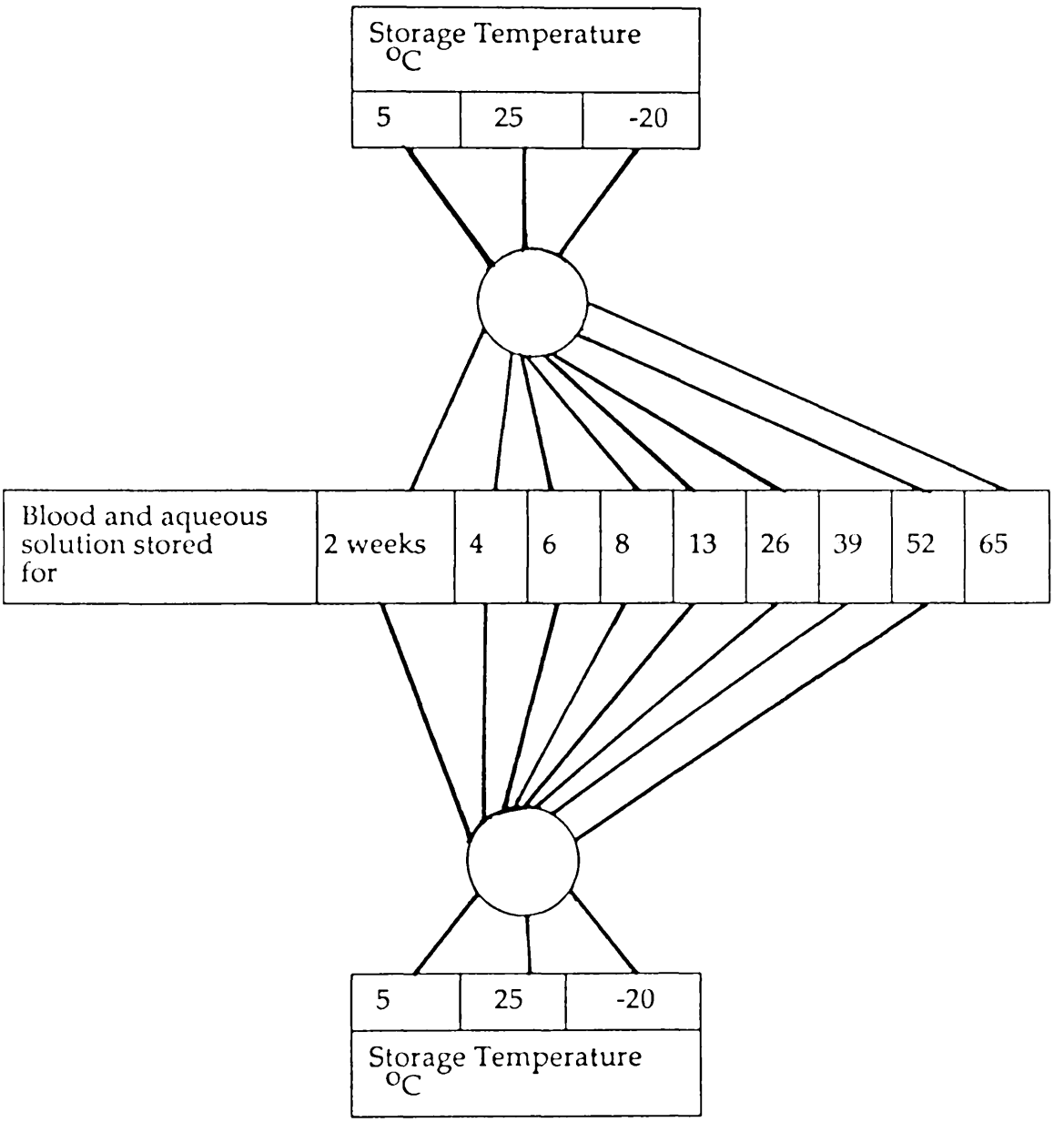
4.1 SPIKED SAMPLES, STORAGE, EXTRACTION AND ANALYSIS PROTOCOL

Blood and aqueous solutions of the drugs under study were prepared at a toxic level since most of the forensic toxicological cases involve measuring the drugs in specimens following overdose. Therefore in an investigation of the stability of drugs of forensic interest in post-mortem blood, it is of paramount importance to the toxicologist interpreting results that the study should comply with actual casework.

The storage temperatures were chosen to reflect common laboratory practise. The 5^{o}C 'refrigeration' represents 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 pass between acquisition and drug quantitation. Then the samples were kept at -20^{o}C prior to analysis. Usually they are destroyed after six months from receipt of sample. The 25^{o}C represents the upper realistic temperature which the corpse may have been exposed to for a few days after death. Therefore, it is necessary to ensure that the drugs are sufficiently stable at different storage temperatures and time intervals. The blood and aqueous solutions of drugs were kept under the same storage conditions and then analysed at the designated time. This enabled a comparison to be made between the amount of chemical decomposition and the degree of putrefactive

degradation for each drug. The concentrations of each drug in blood and aqueous solution was analysed at the designated time. Freshly prepared standard drug samples were used to calculate the concentration of the drug in the spiked samples. The results were compiled in a table. In calculating the recovered drug the initial quantitation (day zero) was assigned a value of 100%. Successive quantitations were made and the '% of original present' was calculated. Figure 16 shows the protocol of storage, extraction and analysis of the specimens for both drug stability and the formation of putrefactive amines in blood. 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 and aqueous solution samples. The obtained decrease rates for acid drugs and non-acid drugs are presented in Tables 34 and 38.

ACIDIC DRUGS



NON-ACIDIC DRUGS

Figure 16. The spiked samples, storage, extraction and analysis protocol.

4.1.1 - Temperature Monitoring of Spiked Samples

Samples of whole blood and water were spiked with anticonvulsant, benzodiazepine and opiate drugs and stored in a refrigerator (5°C), an 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 two weeks from spiking date (day zero) and ended at twelve months in the case of non-acid drugs and 15 months in the case of acid drug samples.

Results and Discussion

Figures (17, 18, 19) demonstrate the fluctuation of the set storage temperatures 5°C, 25°C, -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 temperature are summarised in Table 3. It shows that there was a ± 2.6% variation at 5°C, a ± 0.9% variation at 25°C and a ± 7.6% variation at -20°C over a 24 hour period. Over 7 days the fluctuation was ± 5.2% variation at 5°C, ± 1% variation at 25°C and a ± 8.1% variation at -20°C. Over the 12 months there was a ± 5.8% variation at 5°C, a ± 1.2% variation at 25°C and a ± 4.8% 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.

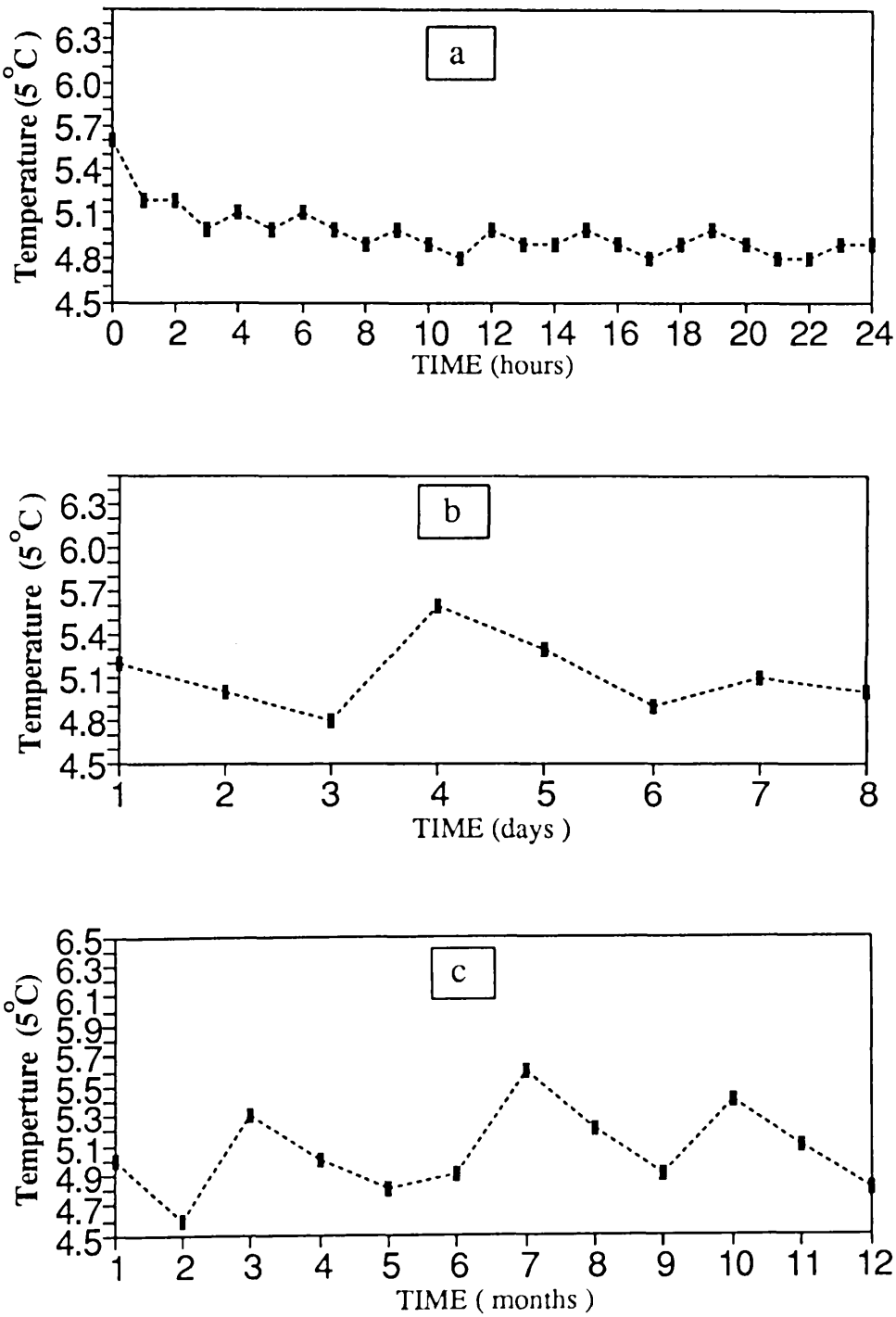


Figure 17. Fluctuation of storage temperature 5°C monitored over: a) 24 hours: b) seven days: c) twelve months.

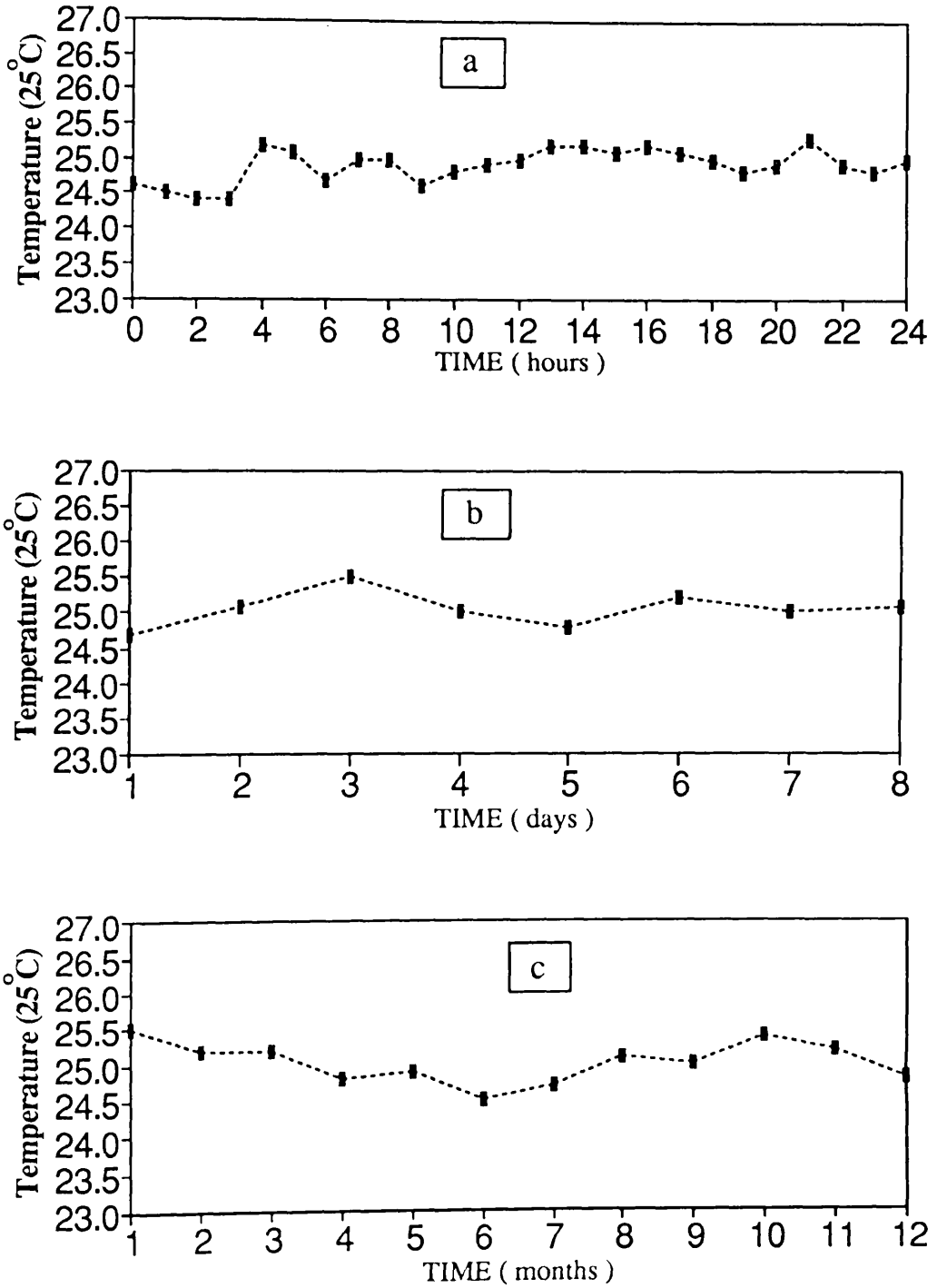


Figure 18. **Fluctuation of storage temperature 25°C monitored over: a) 24 hours: b) seven days: c) twelve months.**

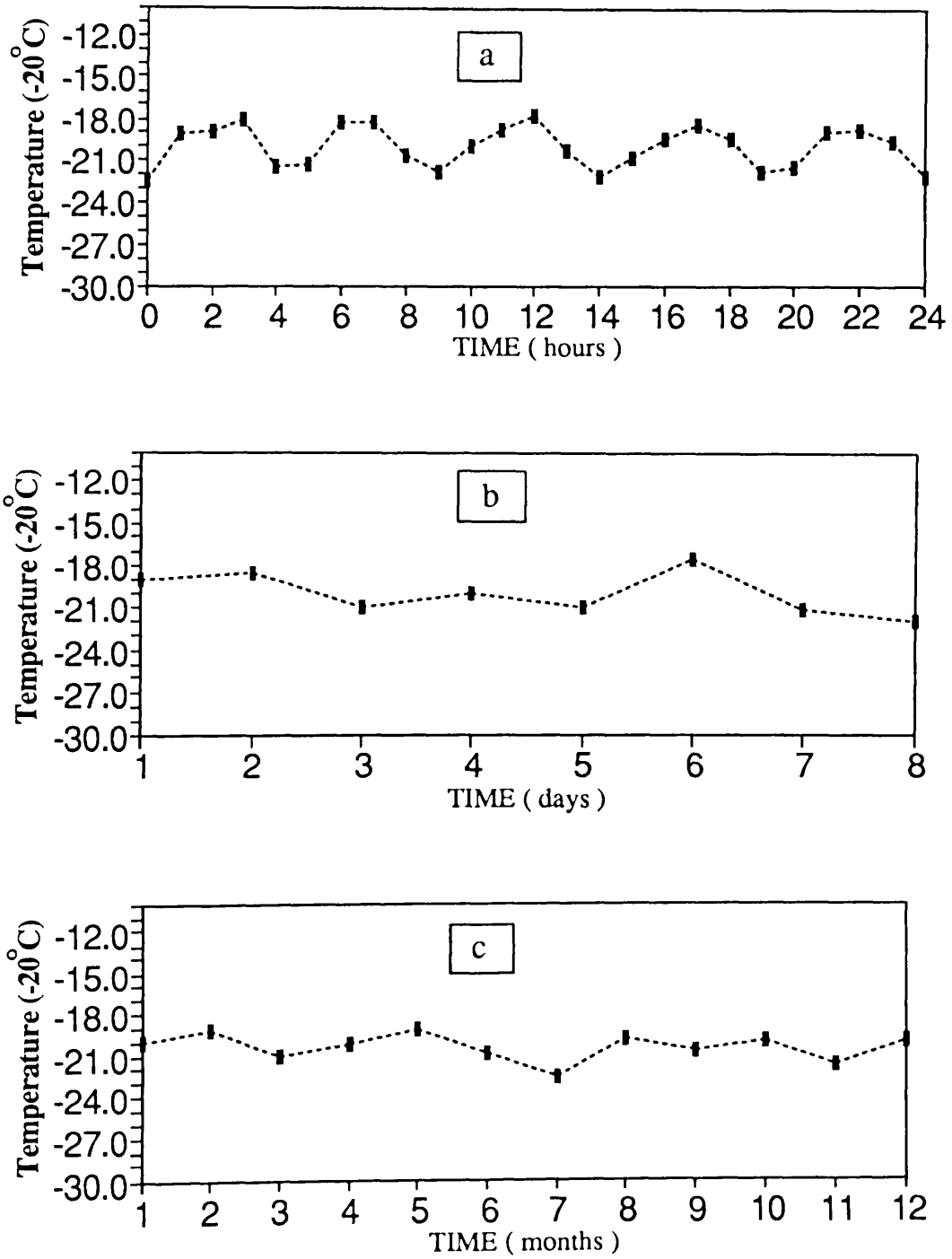


Figure 19. Fluctuation of storage temperature -20°C monitored over: a) 24 hours: b) seven days: c) twelve months.

TABLE 3.

The average measured temperature and the standard deviation of the three storage conditions monitored over different time intervals (24 hours, 7 days, 12 months).

Monitoring Intervals	Refrigerator Temperature set at 5°C	Incubator Temperature set at 25°C	Deep Freeze Temperature set at-20°C
24 hours	5.14 ± 0.13	24.7 ± 0.23	-19.8 ± 1.52
7 days	5.16 ± 0.27	24.9 ± 0.26	-19.0 ± 1.54
12 months	4.95 ± 0.29	25.1 ± 0.3	-20.0 ± 0.96

Conclusion

The temperature fluctuation of the three storage conditions are shown to be minimal and not significant, and within the normal temperature fluctuation of refrigerators, incubators and freezers, with less than 5% differences between set temperature and actual temperature of the three storage media.

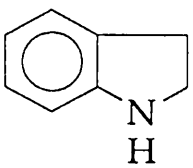
4.2. - ANALYSIS OF INTERFERING SUBSTANCES

4.2.1 - Acid Interferences "Indole"

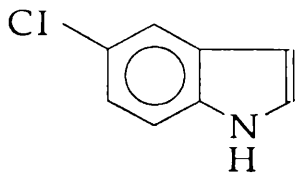
Indole was analysed on the HPLC system described in 3.4.1. fitted with 100 ul loop; the flow rate was 1.5 ml/min. The filter of the detector was set at 0.5 seconds and the column eluant was monitored at 273 nm.

Choice of Internal Standard

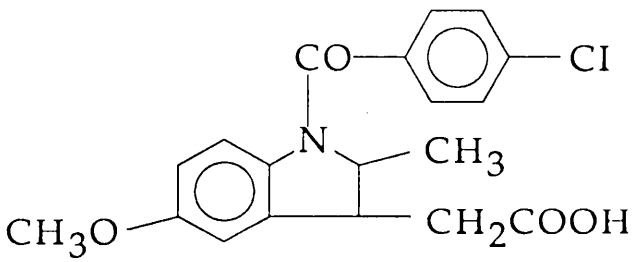
Six chemical compounds and drugs were tested for potential use as an Internal Standard for Indole analysis by HPLC. The structures of the compounds are shown in Figure 20. The similarity to that of Indole can be seen in some of the compounds. The others were selected because of their use with acidic drug analysis in the routine laboratory.



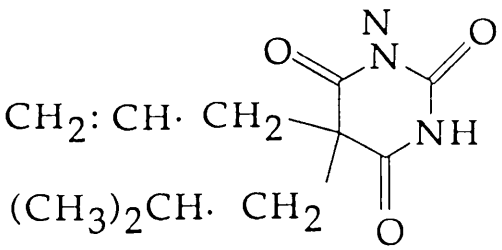
Indolin



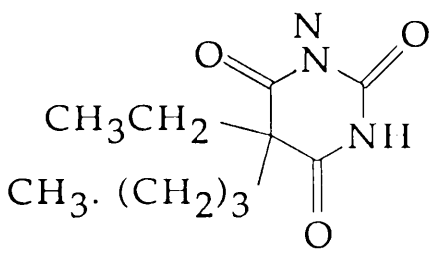
5-ChloroIndole



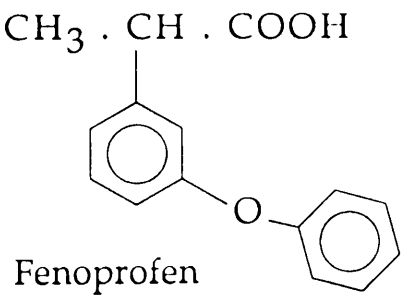
Indomethacin



Butalbital



Butobarbitone



Fenoprofen

Figure 20. Chemical structure of compounds chosen as internal standard for Indole analysis.

Result and Discussion

Indolin showed a very good response on the HPLC. The Indolin standard solution (concentration of 0.1 ug/ml) gave a peak height of 16 cm at range (AUFS) = 0.16 compared to 6.9 cm for Indole (concentration of 5 ug/ml) when detected at 210 nm. Since Indole and Indolin co-eluted from the column, Indolin can not be used as an internal standard. Indomethacin eluted from the column at 20.3 minutes compared to 6.2 minutes for Indole at flow rate of 1.5 ml/min. Indomethacin had a poor response, a broad peak and a long retention time. It would therefore be time-consuming to use Indomethacin as an internal standard for the Indole analysis.

Fenoprofen has a more reasonable retention time of 14 minutes (Indole has 6.2 minutes) for the conditions used. It has, however, a poor response at the system working wavelength which would result in the use of a high concentration of Fenoprofen.

Barbiturates were tested as possible internal standards because they are known to separate extremely well using the system [158]. Butalbital and butobarbitone have sharp peaks with good retention times 3.8 and 4.2 respectively compared with Indole 6.2 minutes. Both were shown to have poor recovery with the method used for Indole extraction. 5-chloroIndole, an Indole derivative, was tested for its suitability as an internal standard for the Indole analysis. 5-chloroIndole would be expected to behave in the same manner as Indole on the HPLC and the extraction procedure. 5-chloroIndole was shown to have a symmetric peak shape with a retention time of 12.3 minutes compared to 6.2 for Indole. 5-chloroIndole also demonstrated a good absorptivity within the working wavelength of the HPLC

system. 5-chloroIndole was not mentioned as a putrefactive product in the literature, therefore it can be used as an internal standard.

The Maximum Absorptivity of Indole and 5-ChloroIndole (I.S)

The maximum absorptivity of Indole in the mobile phase was at 218 nm and 272 nm, and for 5-chloroIndole was at 234 and 278 nm. The detector was set at 273 nm for Indole and 5-chloroIndole for analysis by HPLC. A very stable baseline was obtained at this wavelength and little interference was encountered from other co-extractable materials.

Linearity and Minimum Detectability of the HPLC

A series of standard Indole solutions was analysed by HPLC. The peak height to the Indole concentration response on the above system was linear over the concentration range 0.5 - 5 ug/ml. The regression equation (y = A + BX) and correlation coefficient (r) are as follows:

A	B	r
-0.14	2.37	0.998

Where 'y' is the peak height and 'x' is the concentration of Indole. Table 4 shows the relation between Indole concentration and the peak height using HPLC system at AUFS = 0.02 and 0.08. The calibration curve produced between the peak height to concentration shown in Figure 21. A 50 ng/ml of Indole standard solution was found to be easily detected at range (AUFS) = 0.02 and filter set at - 0.5 secs. The response to background noise ratio was above five, Figure 22.

TABLE 4.

Relation between Indole concentration and the peak height using HPLC system.

Indole Concentration ug/ml	Average Peak Height (cm) n = 2
0.5	2.4
1.0	7.0
2.0	10.6
3.0	15.7
4.0	19.4
5.0	24.0

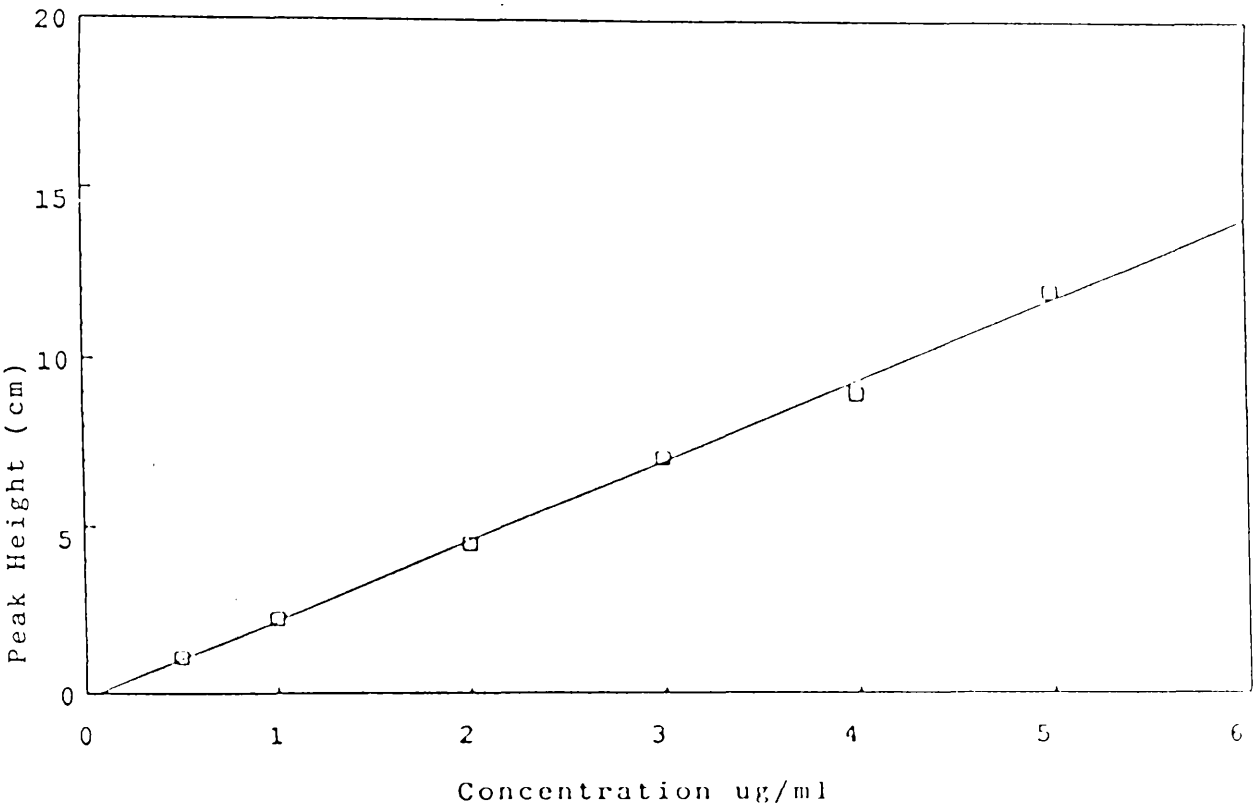


Figure 21. Indole measurement by HPLC (linearity of response).

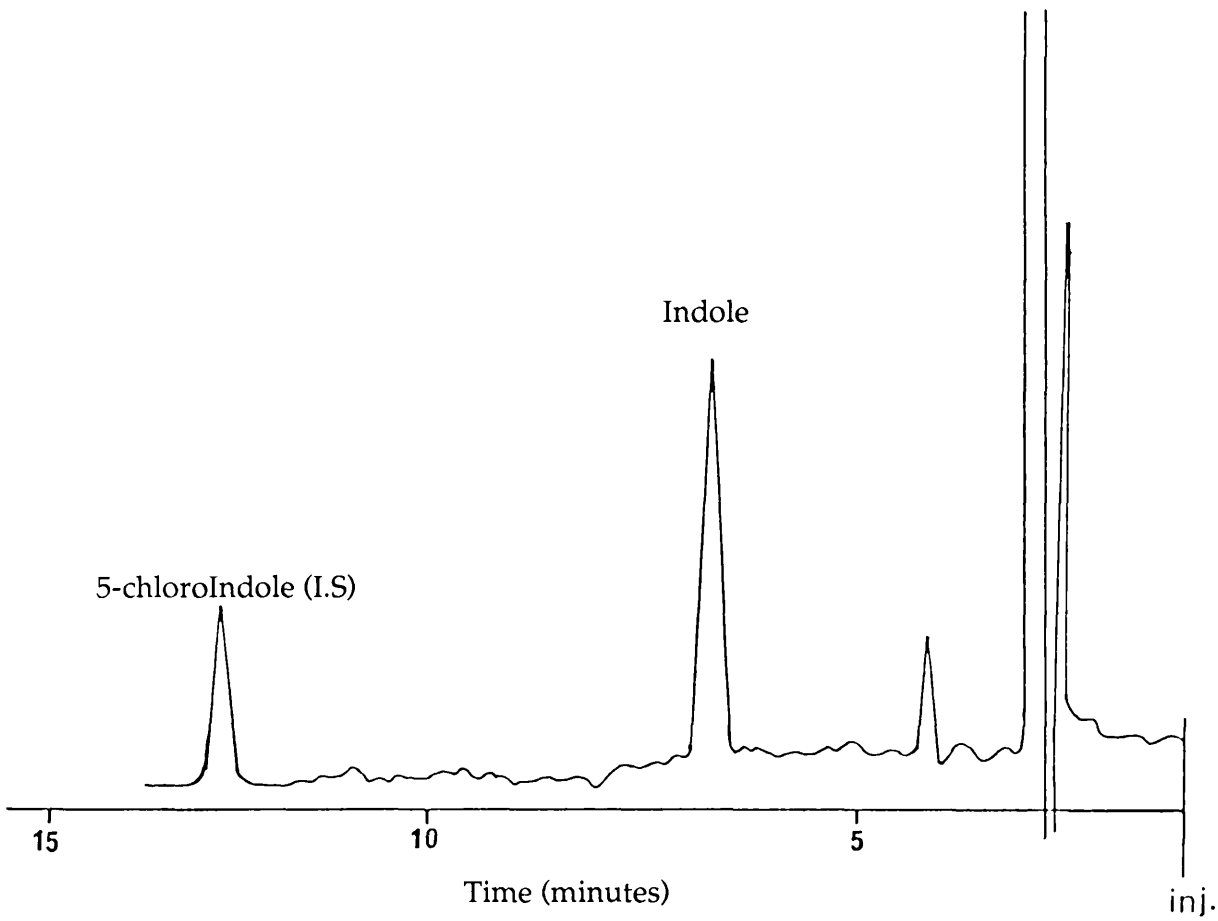


Figure 22. Separation of Indole and 5-chloroIndole (I.S) by HPLC.

Reproducibility of the HPLC

The reproducibility of the HPLC system for Indole analysis was shown to be 2.7% (n = 5) for the within-day variation, and in the range of 1.7% to 3.9% (n = 15) for the day-to-day variation at 0.02 absorbance unit full scale (AUFS).

Conclusion

The developed method for Indole analysis offered a very good sensitivity when the detector was set at 273 nm with a very stable baseline and little interference was encountered from other co-extractable material compared with the detection at 210 nm. The reproducibility was excellent. From the materials tested, 5-chloroIndole was found to be suitable for use as an internal standard. The system also offered a reasonable analysis time of less than 15 minutes at a flow rate of 1.5 ml/minute.

4.2.2 - Non-Acid Interferences

Mobile Phase Development

In normal phase chromatography a non-aqueous mobile phase is usually used. Therefore, different organic solvent ratios have been investigated to obtain the best mobile phase to separate 2-phenethylamine, tyramine and tryptamine. The HPLC equipment used in developing the mobile phase was described earlier **(3.4.2).**

Result and Discussion

Table 5 shows the different ratios of organic solvents (v/v/v) used to achieve separation of the three putrefactive amines. 2-phenethylamine and Tryptamine are soluble in ethanol while Tyramine is soluble 1 to 10 in boiling ethanol. The three compounds are primary amines. This group is responsible for the basic properties of these compounds. Separation achieved by 'adsorption' chromatography is usually based on the competition for sites on the active adsorbent surface of silica between the molecules of samples and the molecules of mobile phase "acetonitrile/methanol and the modifier ammonia." On this mobile phase ammonia and the ($-NH_2$) group of the putrefactive amines are competing for the adsorptive sites of the column. Increasing the amount of ammonia leads to earlier elution of the putrefactive amines. If the affinity of the column packing for the sample molecules is greater than its affinity for the mobile phase, then the sample will be retained and the previously adsorbed solvent molecules displaced. Conversely, a stronger affinity for the mobile phase will lead to rapid elution of the sample. The chosen mobile phase gave an intermediate set of conditions with a

degree of retention rather than complete or zero retention of the putrefactive amines.

The initial solvent mixture chosen as mobile phase was acetonitrile/ methanol/ ammonia (60:40:0.3 v/v/v). The obtained chromatogram is shown in Figure 23a. A poor separation of the three putrefactive amines was noted. By increasing the amount of acetonitrile and ammonia and decreasing the methanol content, a better separation was achieved. The shorter retention times could be explained by the increased amount of ammonia added, which reduced the availability of adsorptive sites for ($-NH_2$) group of the putrefactive amines. This leads to faster elution of the three putrefactive amines Figure 23b. On the other hand, decreasing the amount of ammonia and acetonitrile gave a better separation of the three putrefactive amines Figure 23c but with poor baseline separation. Making a mobile phase with equal amounts of both acetonitrile and methanol gave a better separation betweeen Tyramine and Tryptamine at the base line but with a poor separation between Tyramine and 2-phenethylamine. There was no significant effect on the retention times of the three putrefactive amines when the amount of modifier (ammonia) was kept constant, Figure 23d.

TABLE 5.

Different organic solvent ratios tested for putrefactive amines separation on a normal phase HPLC system.

	Solvent Ratios (V/V/V)	Solvents
a	60: 40: 0.3	Acetonitrile/Methanol/Ammonia
b	70: 30: 0.4	Acetonitrile/Methanol/Ammonia
c	35: 65: 0.3	Acetonitrile/Methanol/Ammonia
d	50: 50: 0.3	Acetonitrile/Methanol/Ammonia
e	83: 22: 0.2	Acetonitrile/Methanol/Ammonia
f	82: 25: 0.3	Acetonitrile/Methanol/Ammonia
g	82: 10: 15: 0.3	Acetonitrile/Methanol/ Isopropanol/Ammonia

The best separation of the three putrefactive amines was observed with a mobile phase containing 80% acetonitrile and 20% methanol. An obvious effect of the modifier "ammonia" concentration on the retention times of the three putrefactive amines is shown in Figure 23e and 23f. As the amount of ammonia increases the retention time decreases.

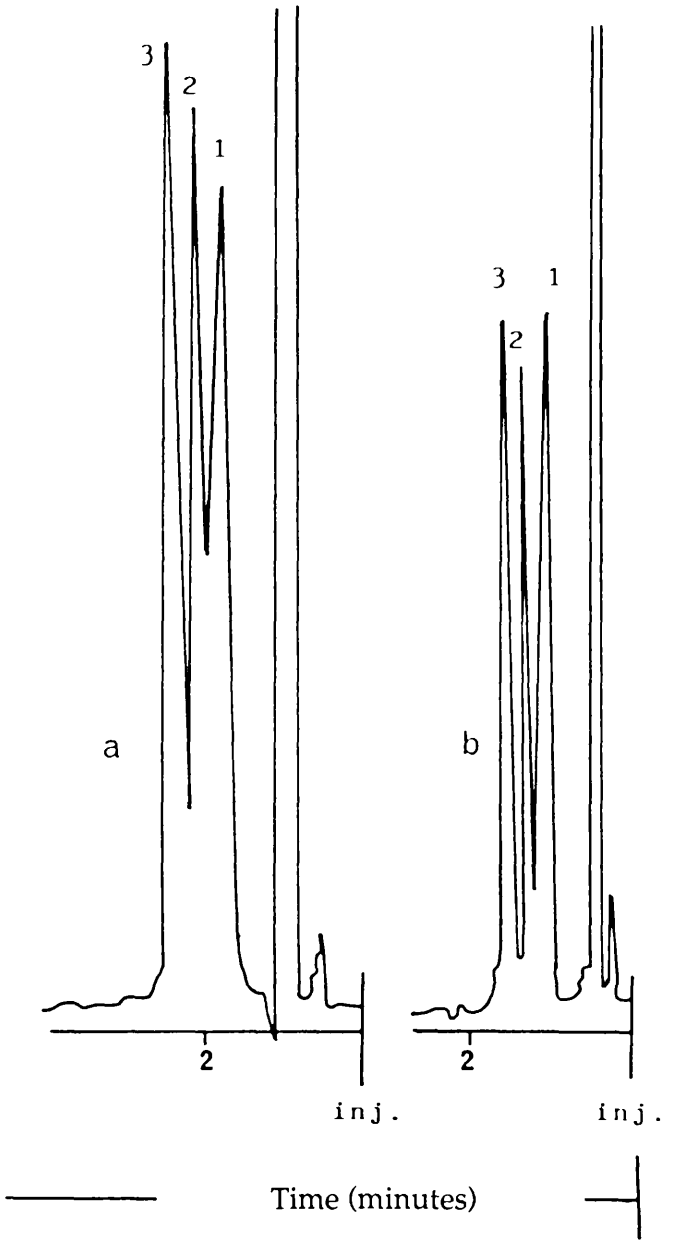


Figure 23. **The effect of different ratios of acetonitrile/methanol/ammonia on the separation of the three putrefactive amines on a normal phase HPLC.**
1. 2-phenethylamine. 2. Tyramine.
3. Tryptamine.

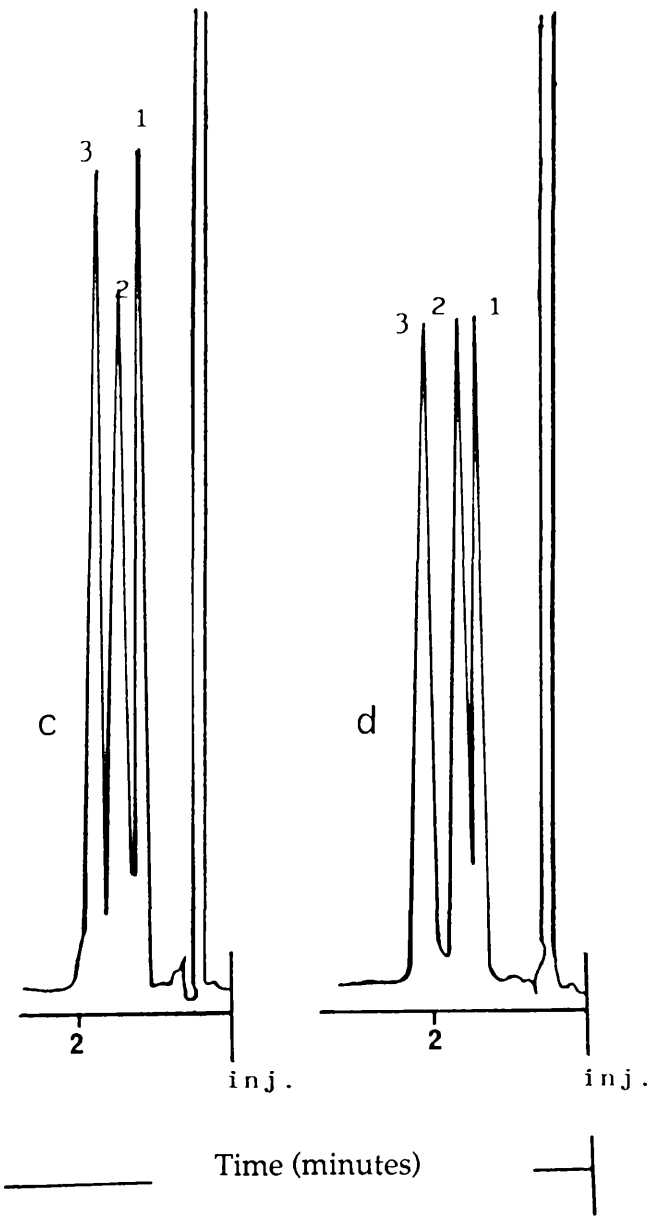


Figure 23 (Continued)

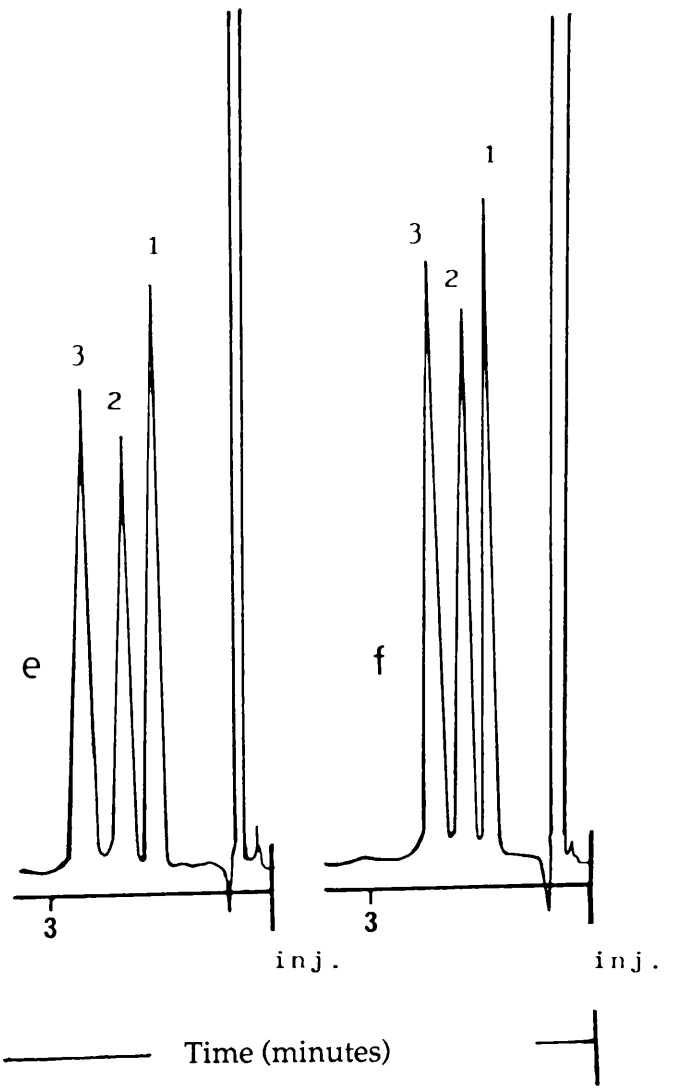


Figure 23 (Continued)

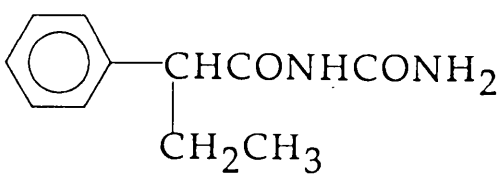
Choice of Internal Standard

Different compounds were tested for suitability of use as an internal standard for putrefactive amine analysis. All of them satisfy an important criteria in that they are not encountered as an interfering substance such as artefacts, naturally occurring substance or products of putrefaction. Their chemical structure is shown in Figure 24.

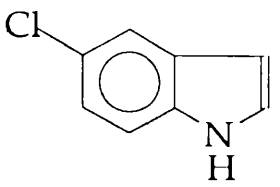
Result and Discussion

The behaviour of the tested internal standards on the normal phase HPLC system in mobile phase (f) are demonstrated in Table 6. It shows that most of the compounds are co-eluted with the first amine (2-phenethylamine) or eluted with solvent front. Only "Dihydrocodeine" appeared to be a candidate for use as an internal standard since it eluted from the HPLC system after the three putrefactive amines. It was, however, poorly separated from Tryptamine. Therefore different amounts of Isopropanol were added to the mobile phase (f) to improve the separation. The new mobile phase (g) resulted in a very good separation between Tryptamine and dihydrocodeine (I.S) and maintained a good separation between the other putrefactive amines, Figure 25, with an analysis time of less than five minutes.

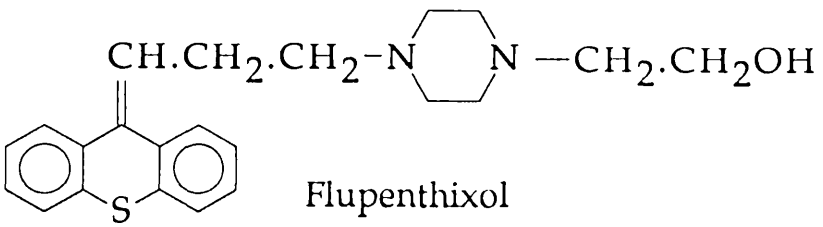
The developed mobile phase (g) which consisted of acetonitrile/methanol/ isopropanol/ammonia (82:10:15:0.3 v/v/v/v) was adopted for the analysis of the three putrefactive amines in putrefied blood.



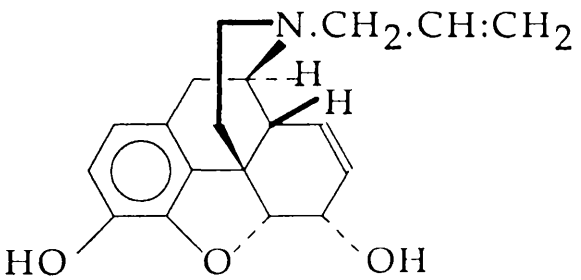
Phenylethylacetylurea



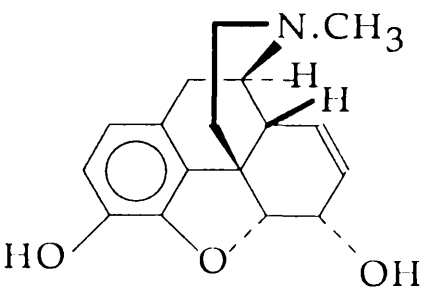
5-Chloroindole



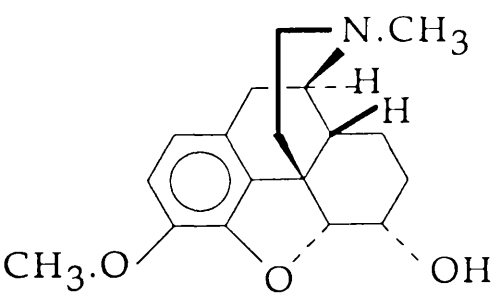
Flupenthixol



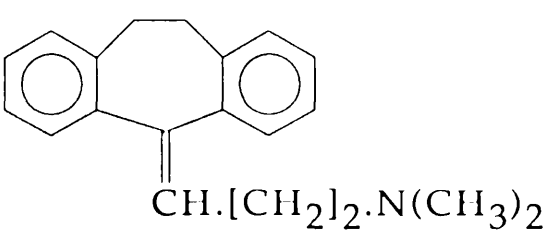
Nalorphine



Morphine



Dihydrocodeine



Amitriptyline

Figure 24. The chemical structure of compound tested for suitability of use as an internal standard in putrefactive amines analysis using normal phase HPLC system.

TABLE 6.

The separation of chosen internal standards on the developed HPLC system for non-acid interfering substances analysis using mobile phase (f).

Compound	Elution order of chosen internal standard
5-chloroIndole	Failed to chromatogram
Amitriptyline	Eluted with 2-phenethylamine
Phenylethylacetylurea	Eluted with 2-phenethylamine
Flupenthixol	Eluted with Tyramine
Nalorphine	Eluted with solvent front
Morphine	Eluted with 2-phenethylamine
Dihydrocodeine	Eluted after Tryptamine with poor separation

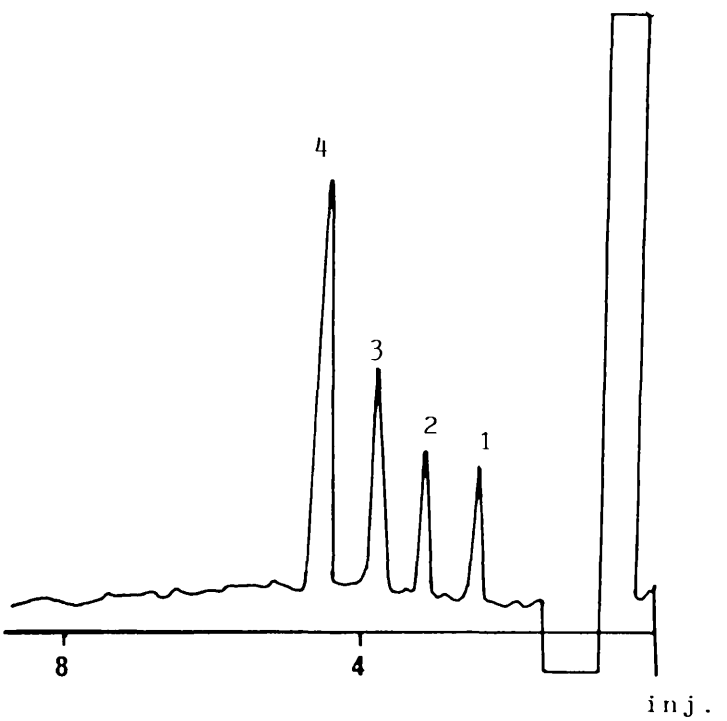


Figure 25. Separation of the three putrefactive amines and the internal standard on normal-phase HPLC system.

1. 2-phenethylamine.
2. Tyramine.
3. Tryptamine.
4. Dihydrocodeine.

Linearity of HPLC-System

The peak height of each putrefactive amine was plotted against its concentration over the concentration range 0.5 to 10 ug/ml for tyramine and tryptamine and 0.5 - 16 ug/ml for 2-phenethylamine and were found to be linear, Figure 26. The regression equations (Y = A + BX). Where 'y' is the peak height, 'x' is the concentration of putrefactive amine and (r) the correlation coefficient for the three putrefactive amines. These are listed in Table 7 and the relation between the peak height of each putrefactive amine and concentration is shown in Table 8.

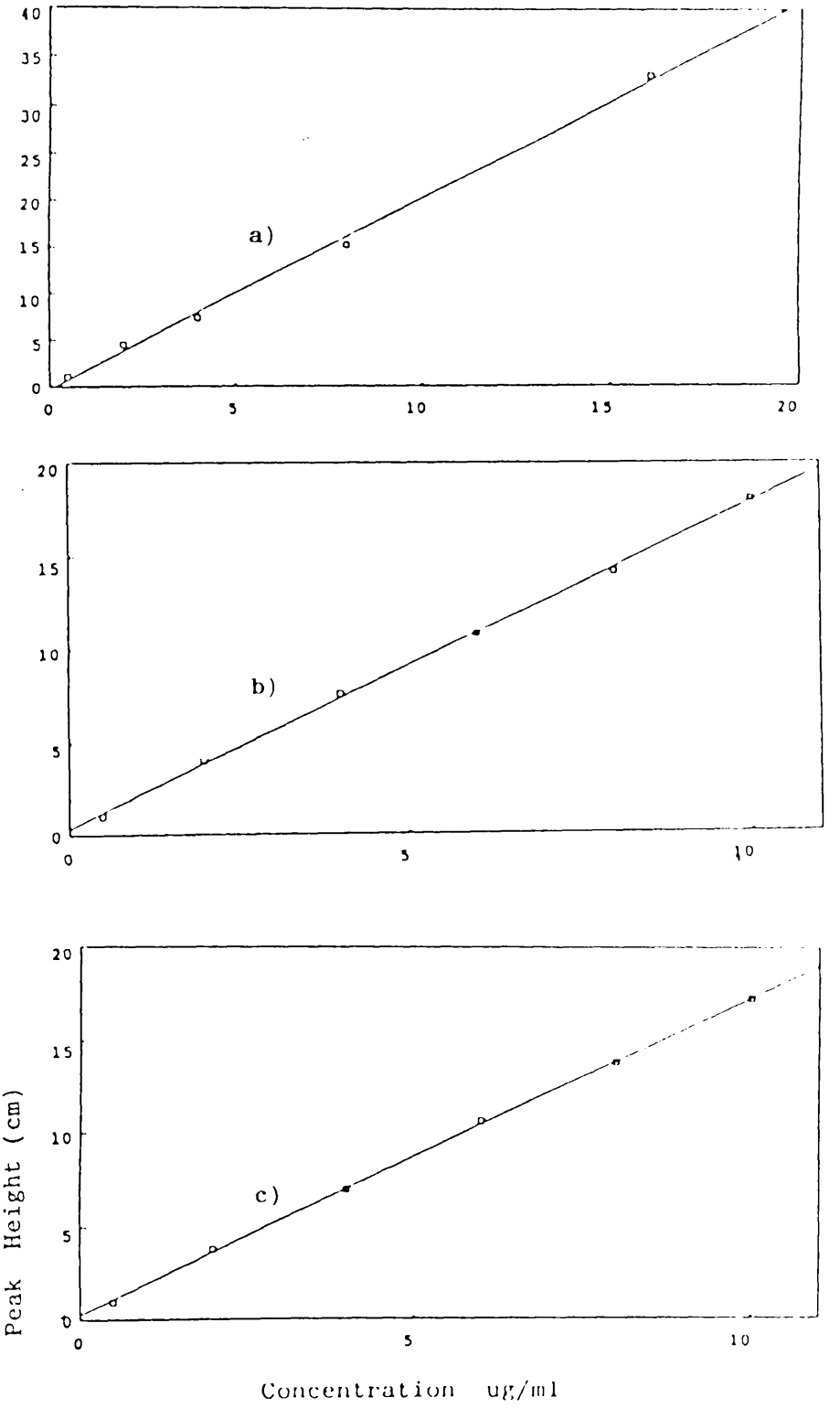


Figure 26. Three putrefactive amines measurement by HPLC (linearity of response) a) 2-phenethylamine: b) Tyramine: c) Tryptamine

TABLE 7.

The correlation coefficient (r), A and B values for the three putrefactive amines obtained from they regression equation (Y = B + BX).

	A	B	r
2-phenethylamine	-0.26	2.04	0.998
Tyramine	0.32	1.75	0.999
Tryptamine	0.24	1.70	0.999

TABLE 8.

Relation between putrefactive amines concentration and peak height using normal phase HPLC system at range AUFS = 0.02 and 0.08.

	Concentration ug/ml	Average peak height (cm) n = 5
Phenethylamine	0.5 2 4 8 16	1.1 4.5 7.3 15.2 33
Tyramine	0.5 2 6 8 10	1 4 10.8 14.2 18
Tryptamine	0.5 2 4 6 8 10	0.95 3.8 7 10.6 13.8 17.2

Minimum Detectability and Maximum Response

In the developed normal phase HPLC system with the mobile phase (g), a standard solution of 50 ng, 50 ng, 50 ng/ml for 2-phenethylamine, tyramine and tryptamine were found to be detected easily with signal to background noise above three at a range (AUFS) = 0.02, filter setting 2 sec. Figure 27. The standard solution of the three putrefactive amines in the mobile phase at a concentration of 20 ug/ml showed a maximum absorbance at 216 nm for 2-phenethylamine, 225 nm for tyramine and 226 nm for tryptamine. Tryptamine showed the best absorption over 2-phenethylamine and tyramine, Figure 28.

Reproducibility of the HPLC-System

The reproducibility of the developed HPLC system for the three putrefactive amines were tested over three days. A summary of the reproducibility for the three putrefactive amines shown in Table 9.

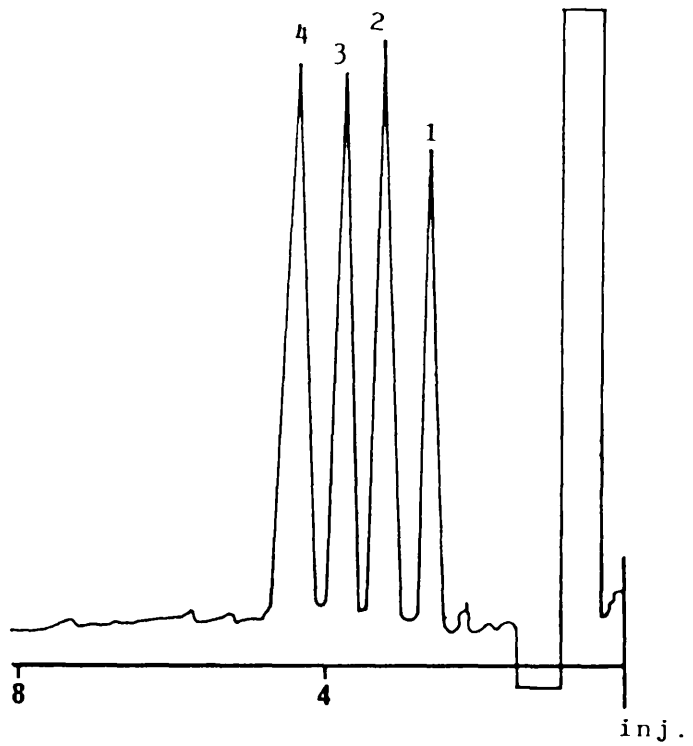


Figure 27. Separation of the three putrefactive amines and dihydrocodeine (I.S) on normal phase HPLC at low concentrations. The HPLC condition:
Range (AUFS) = 0.02
Detection Wavelength = 210 nm
Sample Volume = 100 ul
Flow Rate = 1 ml/min
Detector Filter = 2 sec.
Standard solution concentration - 50 ng/ml for each putrefactive amine.
Internal standard concentration 100 ng/ml

1. 2-phenethylamine
2. Tyramine
3. Tryptamine
4. Dihydrocodeine (I.S)

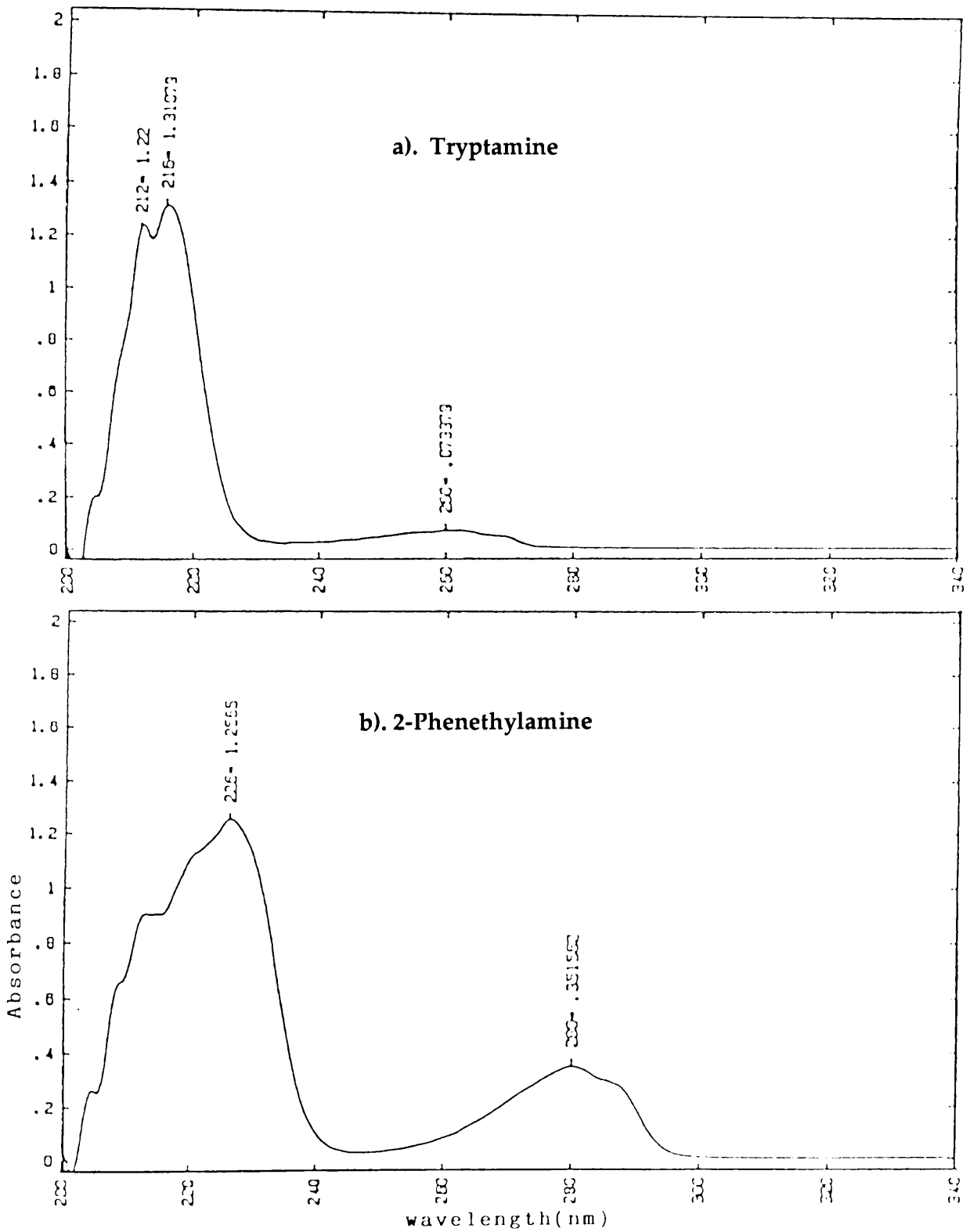


Figure 28. The ultra-violet spectra of the three putrefactive amines in mobile phase (f).

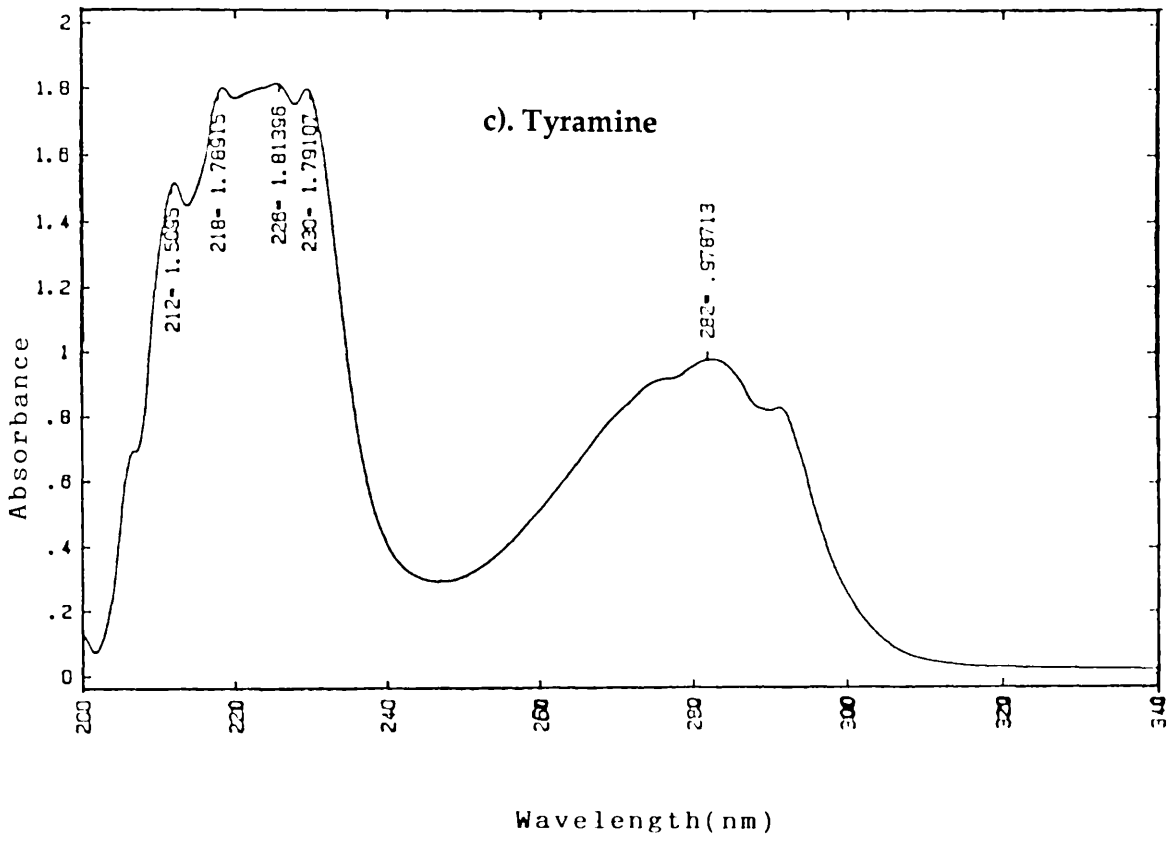


Figure 28 (continued)

TABLE 9.

Reproducibility of normal phase HPLC system for the analysis of the three putrefactive amines.

Within Day Variation COV (Six Samples Analysed)	Day-to-day Variation COV Over three days (18 Samples Analysed)
2-phenethylamine 6.1%	3.3 - 7.01%
Tyramine 4.2%	2.9 - 5.1%
Tryptamine 4.9%	2.8 - 6.6%

Conclusion

The developed method for 2-phenethylamine, Tyramine and Tryptamine offered a good senstitivity of 50 ng/ml for each amine. The reproducibility is good. The system also offered a short analysis time for the three putrefactive amines simultaneously with analysis time of less than five miutes at a flow rate of 1.0 ml/min.

4.3 DRUGS ANALYSIS

The sample of anticonvulsants, benzodiazepines and opiates spiked in whole blood and water and the blank blood which were stored at the three different temperatures (5, 25, -20^{o}C) were analysed at the designated time according to protocol (4.1). Standard blood and aqueous solutions of each drug were prepared freshly for each analysis.

4.3.1 - Acid Drugs

4.3.1.1 - Anticonvulsants

Whole blood and water spiked with the anticonvulsant drugs phenobarbitone, carbamazepine and phenytoin were analysed using the chromatographic system described (3.5.1) and extracted using the described method in 3.7.1.1.

Results and Discussion

The retention times of the three anticonvulsant drugs and their internal standard (Butalbital) are shown on Table 10. The day-to-day and within-day variation for the entire method of extraction and analysis of the anticonvulsant drugs by HPLC are shown in Table 11 and the chromatograms of phenobarbitone, carbamazepine, phenytoin and (I.S) butalbital analysed on this system is shown in Figures (29, 30, 31).

TABLE 10.

Retention times of anti-convulsant drugs on acidic drug HPLC system.

Drug	Retention Time
Phenobarbitone	6.2 mins
Carbamazepine	11 mins
Phenytoin	11.4 mins
Butalbital (6.5)	8.5 mins

TABLE 11.

Variation of anti-convulsant drugs analysis within a day and from day-to-day.

	Within the Day Variation		Day-to-Day Variation	
Drugs	COV* in blood sample	COV* in aqueous solution	COV* in blood sample	COV* in aqueous solution
Phenobarbitone	4.7% n = 6	6.5% n = 5	8.5% n = 8	9.1% n = 8
Carbamazepine	8.2% n = 6	7.6% n = 6	9.5% n = 8	9.5% n = 8
Phenytoin	6.8% n = 6	8.1% n = 6	8.6% n = 8	9.3% n = 8

*** COV = Coefficient of variation = (standard deviation/mean) X 100**

Column: Hypersil 5 um C18 = 25 cm x 4.6 mm I.D
Mobile phase: (0.02M) sodium acetate/acetonitrile/methanol (400:180:180) pH = 5.5
Detection wavelength 210 nm
Flow Rate: 1.5 ml/minute
Drugs concentration 10 ug/ml phenobarbitone
3 ug/ml carbamazepine, 10 ug/ml phenytoin and 10 ug/ml butalbital (6.5)

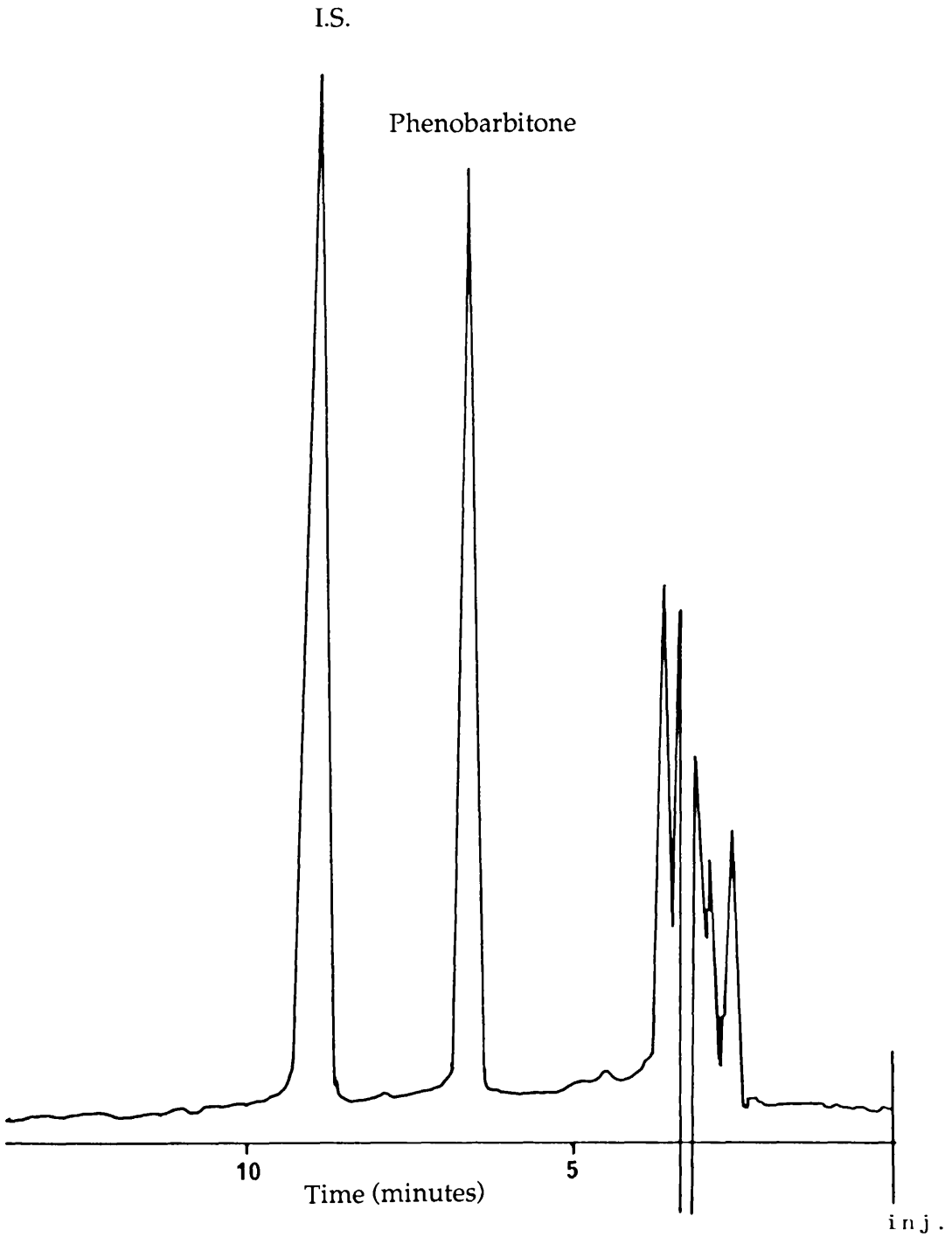


Figure 29. **The chromatogram of phenobarbitone and butalbital (I.S) on acid drugs HPLC system.**

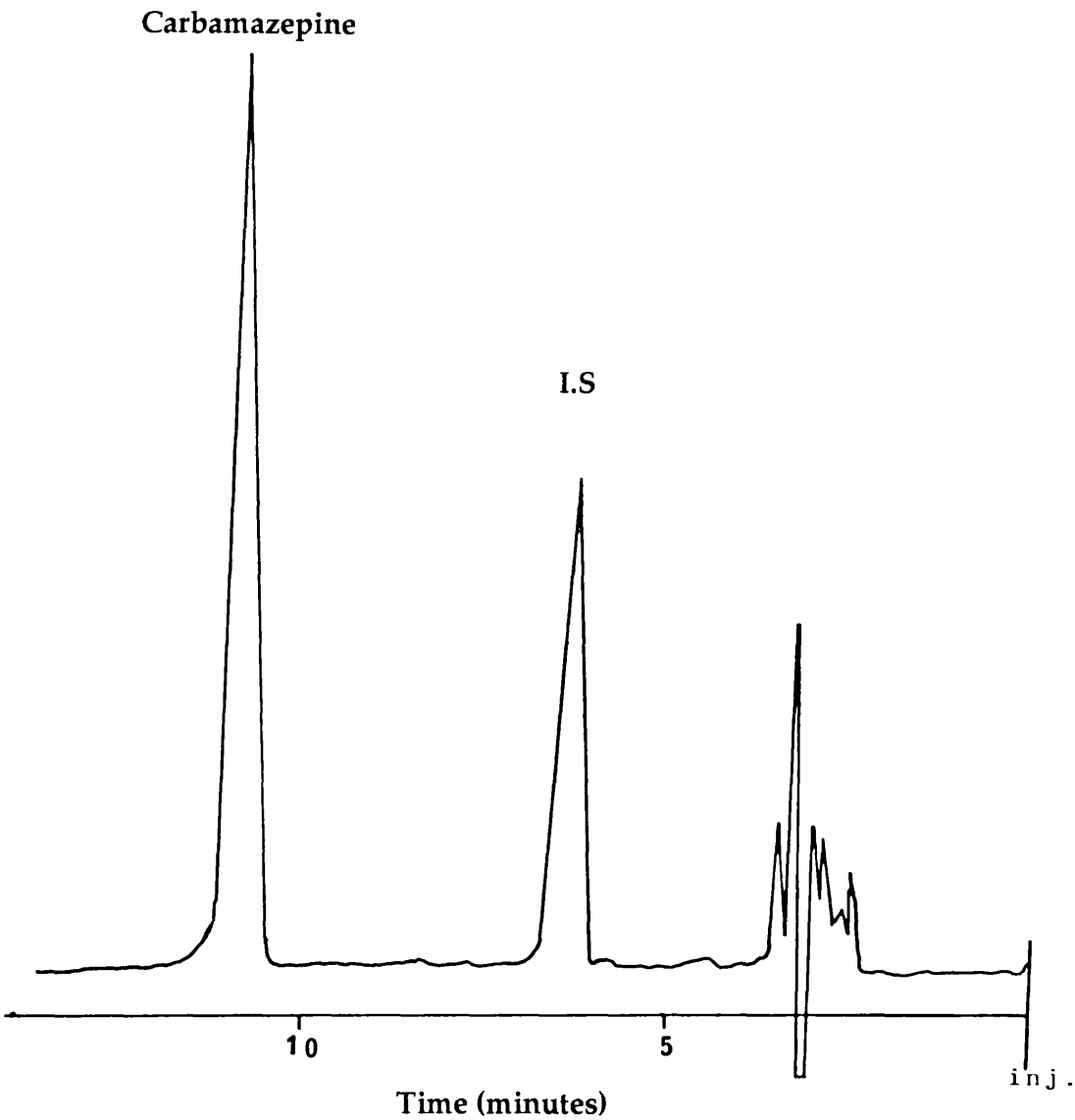


Figure 30. The chromatogram of Carbamazepine and butalbital (I.S) on acid drugs HPLC system.

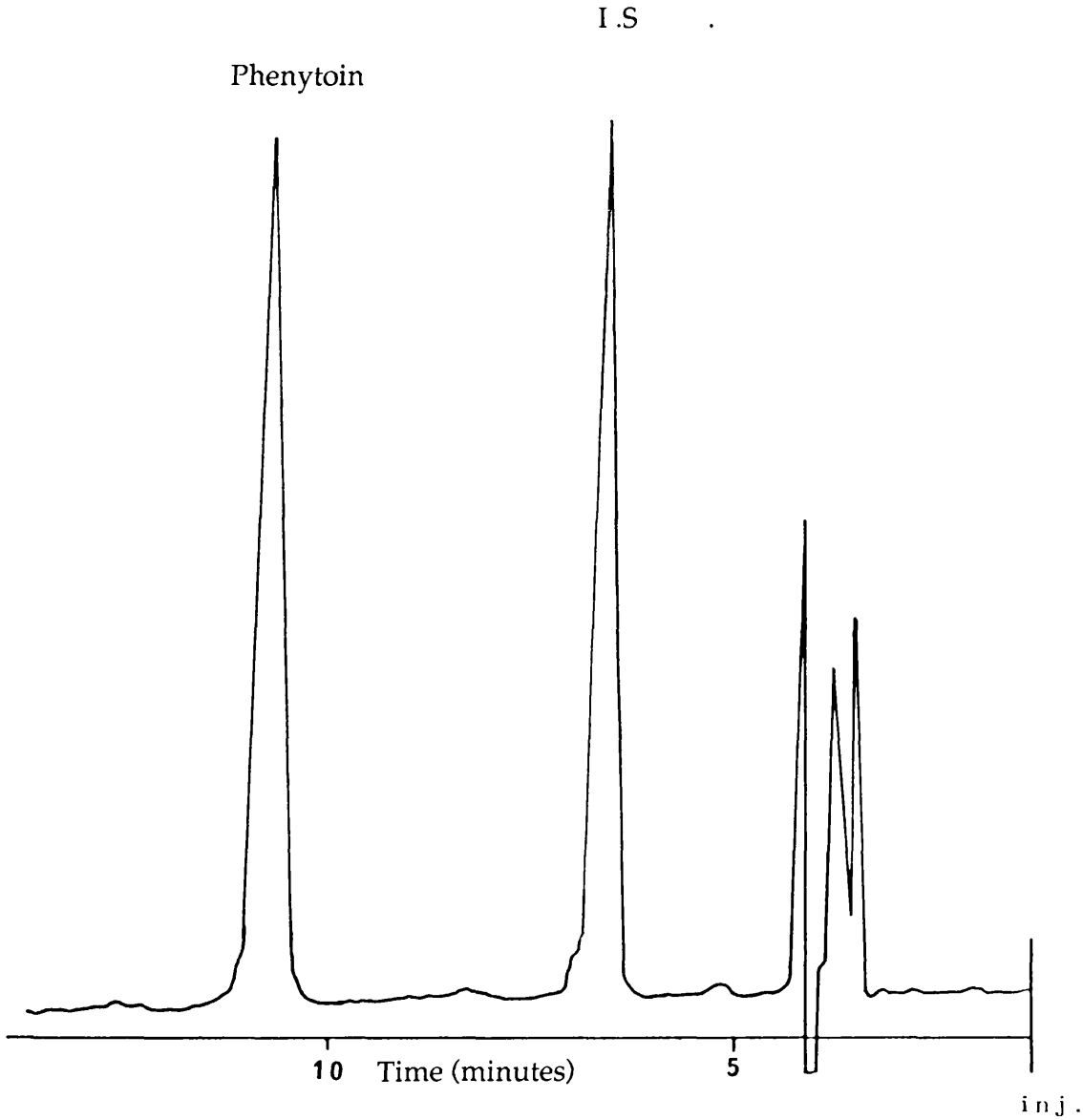


Figure 31. The chromatogram of Phenytoin and butalbital (I.S) on acid drugs HPLC system.

4.3.2 Non-Acid Drugs

4.3.2.1 - Benzodiazepine

Temazepam separation was achieved using the described HPLC system, 3.5.2.1 and extracted using the described method in 3.7.2.1.. Fresh standards and blanks were used for each analysis.

Result and Discussion

The retention time of Temazepam and the internal standard prazepam were 5.4 and 11.0 minutes respectively, Figure 32. The method of benzodiazepine analysis showed coefficients of variation of 4.1% and 4.8% for the within a day and day-to-day variations for temazepam in blood samples respectively, while the coefficients of variation for aqueous solutions were 5.2% and 5.4% repsectively.

4.3.2.2 Opiates

Gas chromatography-mass spectrometry was used to analyse morphine and buprenorphine as described, 3.5.2.2, and extracted using the method described in 3.7.2.2. The derivatization reagent used was 40% DETMDS -(diethyltetramethyl-disilazane) in acetonitrile/toluene (7:3 v/v).

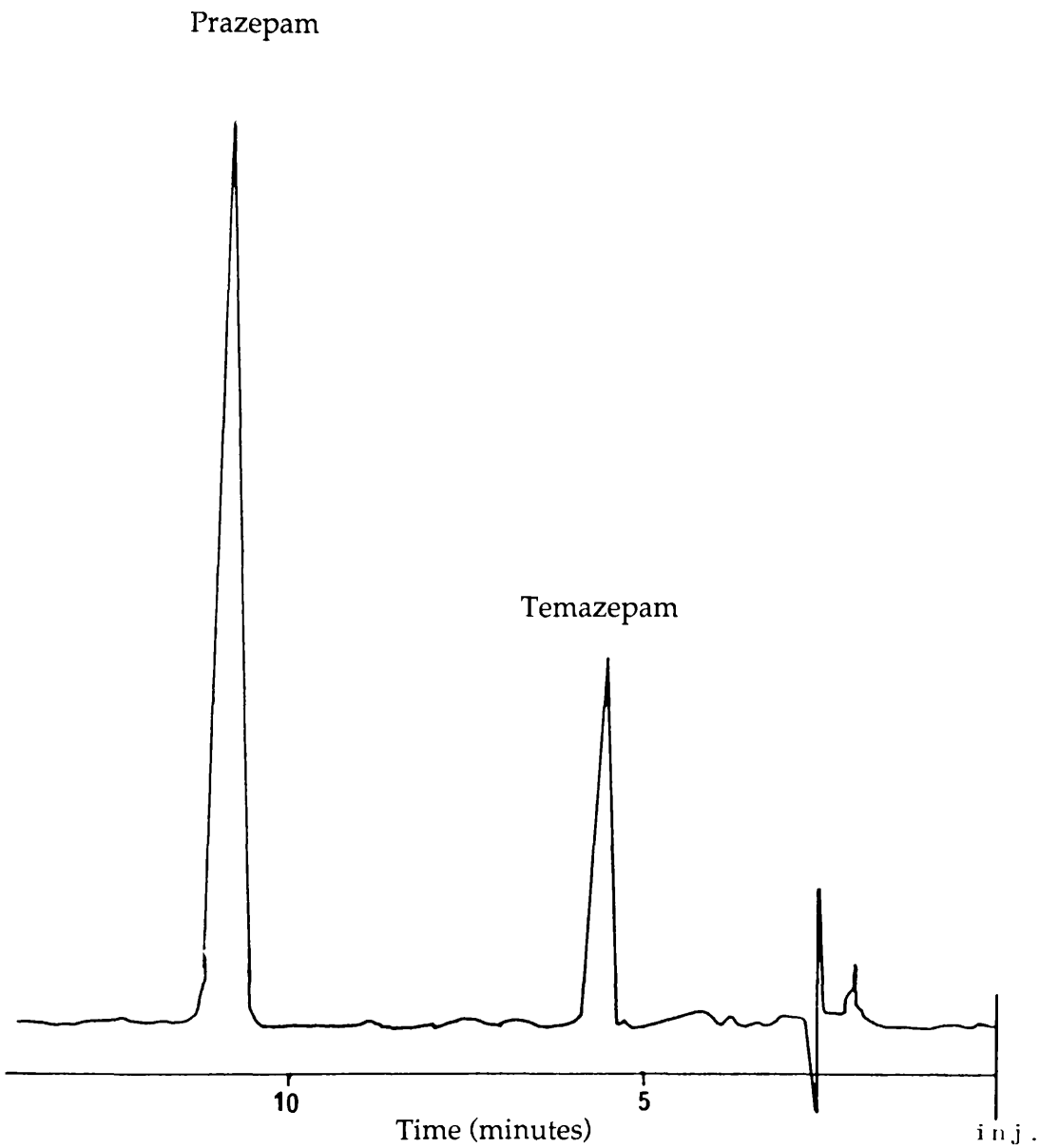


Figure 32. **The chromatogram of Temazepam and Prazepam (I.S) on benzodiazepine HPLC system.**

Results and Discussion

The derivatized morphine and buprenorphine molecular ions (MI) were recorded using selective ion recording (SIR) on the VG model 70-2505 mass spectrometer. The instrument was tuned on mass 454.9728 of perfluorokerosene (PFK) to a resolution of 1000.

The derivatized morphine has the molecular ion (m/z 457) as the most abundant ion on the mass spectrum. Derivatized buprenorphine has the most abundant ion at mass (464) formed by the loss of a neutral group (methanol) and the tertiary butyl group (57 a.m.v) from the molecular ion (m/z 553). The electron impact mass spectra of morphine and buprenorphine as EDMS derivatives are shown in Figure 33. The retention time of the morphine molecular ion (m/z 457) was 10:43 minutes and 10:42 for the internal standard D_3-morphine molecular ion (m/z 460). Buprenorphine molecular ion (m/z 464) had a retention time of 18:47 minutes and 18:46 for the internal standard D_2-buprenorphine molecular ion m/z (466). Figure 34 shows the chromatogram of morphine and buprenorphine. The day-to-day variation for morphine analysis were 4% and 5% for blood and aqueous solution respectively at 500 ng/ml, and at the same time, buprenorphine showed 6.2% and 5.8% for blood and aqueous solution respectively at a buprenorphine concentration of 250 ng/ml.

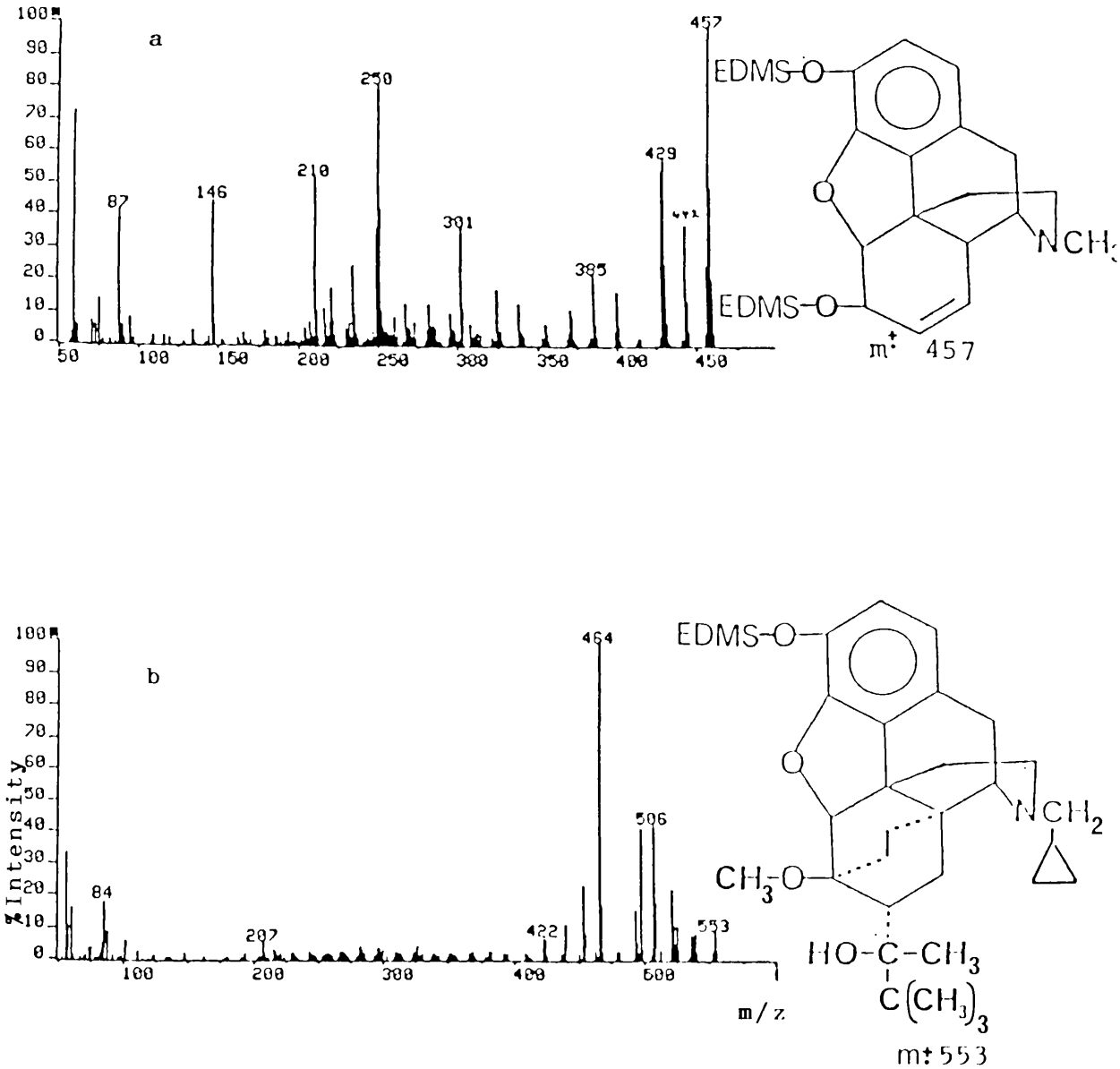


Figure 33. The electron impact spectra of (a) BIS-EDMS-Morphine and (b) EDMS-Buprenorphine.

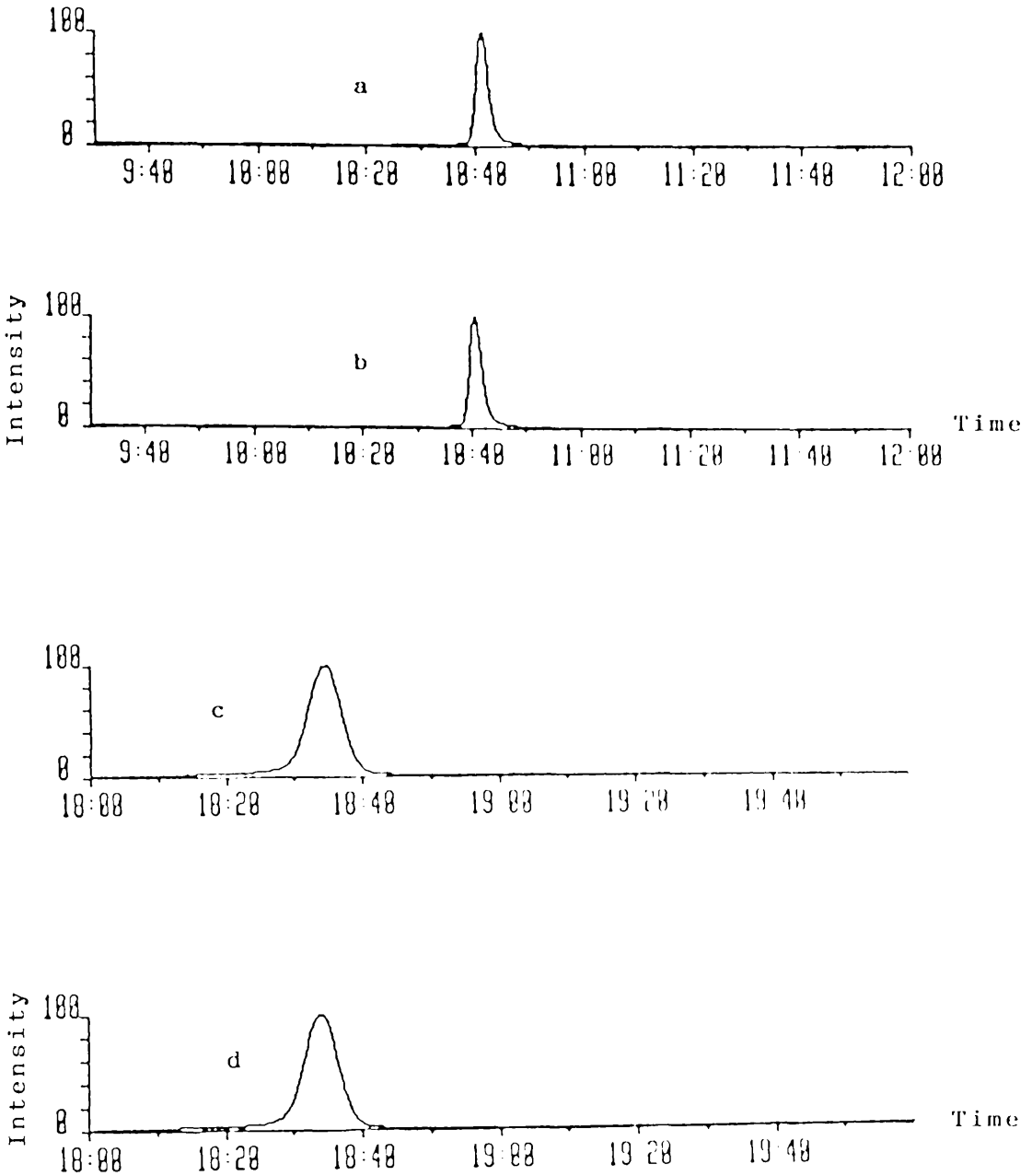


Figure 34. SIR trace of morphine (a) and the internal standard D3- morphine (b), Buprenorphine (c) and the internal standard D2-Buprenorphine (d) as EDMS derivatives obtained by electron impact, their SIR masses.

a. 457.2469
b. 460.2657
c. 464.260
d. 466.2747

Conclusion

The chosen methods for anticonvulsant, benzodiazepine and opiate analysis demonstrate a good reproducibility with a reasonable analysis time. The methods have been in use for a few years. The possible interference from putrefactive amines with drugs analysis using these methods will be investigated.

4.4 EXTRACTION OF INTERFERING SUBSTANCES

4.4.1 - Acid Interferences

Protein Precipitation Methods

Removal of protein by precipitation is an effective method of sample preparation. The main reason for removing proteins is that they can precipitate when in contact with the mobile phase and thereby block tubing, cause increases in back pressure or deterioration of HPLC column performance. The advantages of this technique are the speed at which the sample can be prepared and its simplicity. Different protein precipitation reagents have been tested for their suitability in sample preparation for solid phase extraction of Indole .

Result and Discussion

Methanol and Isopropanol are organic solvents that are miscible with water. They precipitate proteins by lowering their solubility. The recoveries of Indole from spiked whole blood using methanol as the protein precipitation reagent showed recoveries of about 80% in the supernatant using small volumes of blood and methanol in the ratio of 1:3 v/v. Using isopropyl alcohol as the protein precipitation reagent recoveries of about 90% were obtained using a sample/isopropanol ratio of 1:3. Zinc sulphate is also known to precipitate proteins in whole blood sample preparations. It acts by forming insoluble salts with the proteins. Zinc sulphate is mixed with methanol in the ratio of 7:3 v/v and two volumes of this are mixed with one volume of whole blood. After the sample was vortexed and centrifuged, the supernatant gave a recovery of Indole, of about 35%. Using two volumes of perchloric acid (0.4M) with one volume of whole blood spiked with Indole gives the poorest recoveries (<5%). Also, a very poor recovery was obtained when using the sulphosalicylic acid (10%) method. These acids precipitate proteins by forming insoluble salts. The low recoveries of Indole could be due to instability in the acidic environment or the formation of an insoluble salt. In the preliminary test using methanol and isopropanol in ratio of 5:1 v/v a recovery of $95 \pm 4.5\%$ was obtained.

Recovery of Indole with Methanol/Isopropanol

The recoveries of Indole and 5-chloroIndole (I.S) from blood using methanol/isopropanol as the protein precipitation reagent are shown in Table 12. The recoveries over the concentration range of 0.25, 1, 5 ug/ml Indole in whole blood was excellent for both Indole and 5-chloroIndole (I.S) with very good reproducibility.

Conclusion

A high recovery of Indole from spiked whole blood was obtained when using methanol and isopropanol in the ratio of 5:1 v/v as protein precipitation reagent. Poor recoveries (5 - 35%) were obtained when using one of acid protein precipitation reagents. Therefore methanol/Isopropanol protein precipitation was adopted in this study as the sample preparation for solid phase extraction of Indole.

TABLE 12.

The average recoveries of Indole and 5-chloroIndole (I.S) from blood treated with methanol/Isopropanol to precipitate the protein.

Indole and 5-chloroIndole Concentrations	5-chloroIndole % Recoveries	Indole % Recoveries	of Samples
0.25 ug/ml	93 $\pm$ 3.5%	97 $\pm$ 2.5%	n = 5
1 ug/ml	94 $\pm$ 4.7%	108 $\pm$ 4.1%	n = 5
5 ug/ml	112 $\pm$ 4.8%	111 $\pm$ 3.3%	n = 5

Solid Phase Extraction of Indole

The supernatant of whole blood spiked with Indole when treated with methanol/Isopropanol is not applicable for direct analysis on the HPLC system since the supernatant still contains other endogenous compounds and the sample volume is large. Therefore futher steps have to be considered to concentrate the sample and gave a cleaner extract. Solid phase extraction satisfies these criteria.

Bond Elute Certify

The Bond Elute Certify column was conditioned with 2 x 2 ml 0.1M phosphate buffer KH_2PO_4 (pH 6). The manufacturer, Analytichem International, claims that this column is capable of extracting acidic, basic and neutral components from biological specimens. Therefore this column was tested for its suitability in extracting Indole from blood as described in 3.6.1.

Result and Discussion

Table 13 shows the percentage losses of Indole when extracted through the Certify Column.

Bond Elute Certify exhibited a very poor extraction recovery for Indole since 58% of Indole was found in the column effluent when the samples was drawn through the column (unretained). 20% losses were observed during the rinsing step with 80:20 v/v of 0.1M phosphate buffer/methanol. Washing the column with hexane also caused a decrease in the Indole recovery. 18% of the Indole was washed from the column with the hexane. The total losses of Indole using the Bond Elut Certify column was about 96%. This makes the Certify column unsuitable for Indole extraction but would offer a clean extract in the case of drug extraction from putrefied specimens.

Non-Polar Sorbent C18

C18-column is known as a non-polar sorbent. Compounds with non-polar functional groups (e.g. alkyl chains, aromatic rings) can be extracted from polar solutions (water, buffer). The isolate should be neutral for the best non-polar retention.

TABLE 13.

The present loss of Indole during the extraction through Certify Column.

Indole Concentration	% Losses with Column Effluent Average	% Losses with Rinsing the Column with Phosphate/Methanol Average	% Losses through Washing with Hexane Average
1 ug/ml n = 5	58 $\pm$ 8.5%	20 $\pm$ 6.3%	18 $\pm$ 6.0%

Buffering System

The 6 ml capacity C18-column was activated by passing through 2 x 3 ml methanol followed by 2 x 3 ml deionized water and 2 x 3 ml phosphate buffer 0.05/0.5M disodium hydrogen phosphate (pH 9.0). 1 ml of Indole standard (5ug/ml in methanol) mixed with 2 ml of buffer were poured into the column which was then washed with 3 ml deionized water and was dried by leaving the vacuum on for five minutes. Then Indole was eluted from the column by 2 x 0.5 ml methanol and analysed by HPLC.

Result and Discussion

No data is available on the pka value of Indole but Indole is known as a tertiary amine and it has been successfully extracted from blood by organic solvent at high pH value with excellent efficiency [28]. Therefore, buffering the Indole samples with phosphate buffer with high pH value will enhance the non-polar properties of Indole and a non-polar interaction will be expected to occur between the non-polar carbon-hydrogen bond of the C18-column and the carbon-hydrogen bond and benzene ring of the Indole molecule. Indole was eluted by 2 x 0.5 ml methanol. The Indole recoveries are shown in Table 14. Un-extracted standard Indole of 5 ug/ml was used to calculate the recovery. When the Indole standard was prepared in methanol and mixed with the 0.5M Na_2HP0_4 it caused crystallization of Na_2HP0_4 in the column reservoir and blocked the column filter. This could be explained as methanol added to phosphate solution. It changes the polarity of the solution. Methanol is less polar than water, as a result methanol will reduce the

TABLE 14.

Recoveries of Indole using different ionic strength buffering systems.

Buffer used	Sample Concentration	Average Measured Concentration after Extraction	Average % Recovery	Number of Samples
0.5M Na_2HP0_4	5 ug/ml	4.7 ug/ml	96% $\pm$ 2.4	5
0.05M Na_2HP0_4	5 ug/ml	4.92 ug/ml	99% $\pm$ 3.3	5

solubility of Na_2HP0_4 and causes the precipitation of Na_2HP0_4. The 0.05M Na_2HP0_4 exhibited the same problem with less effect on passing the mixture through the column at high pressure. Therefore another buffer was chosen to overcome the precipitation problem. Sodium acetate at 0.1M was found to be a good buffering system which gave high recoveries.

Recoveries of Indole on C18 Bond Elut with Sodium Acetate Buffering

The disodium hydrogen phosphate buffer was replaced by low ionic strength 0.1M sodium acetate and the pH of the buffer adjusted to 12 by adding a few drops of 0.1M sodium hydroxide. Following the extraction procedure described earlier, Indole and internal standard were eluted with 2 x 0.5 ml methanol, and the eluant was analysed by HPLC. The efficiency of the extraction produced using sodium acetate to buffer the Indole sample was investigated.

Result and Discussion

The recoveries of Indole and 5-chloroIndole (I.S) at low and high concentrations are shown in Table 15. Un-extracted Indole and 5-chloroIndole was prepared at 0.5, 5 ug/ml to calculate the recoveries. C18-column conditioned with sodium acetate buffer showed good recoveries when standard Indole mixed with sodium acetate buffer in a ratio of 1:2 was passed through C18-column, Indole was eluted

from the column using methanol (2 x 0.5 ml). The recoveries of Indole was above 93% from both high and low Indole concentrations. The internal standard (5-chloroIndole) showed recoveries of 90 $\pm$ 5.9% at 0.5 ug/ml (n = 10) and 84 $\pm$ 6.5% at 5 ug/ml (n = 10).

TABLE 15.

Recoveries of Indole and 5-ChloroIndole (I.S) on C18-column using 0.1M sodium acetate buffer.

	Concentration ug/ml	Average % Recovery	Number of Samples
Indole	5 0.5	98 ± 3.7% 93 ± 5.7%	n = 10 n = 10
5-ChloroIndole	5 0.5	84 ± 6.5% 90 ± 5.9%	n = 10 n = 10

Day-to-day Recoveries of Indole on Bond Elut C18

The total number of samples used to estimate the day-to-day variation of the extraction procedure of Indole was 52 samples. Table 16 shows the average recoveries, coefficient of variation and number of samples used each time. Excellent recoveries were obtained for Indole samples at low and high Indole concentrations. The recovery was calculated by comparing the peak height of unextracted standard Indole with extracted samples.

TABLE 16.

Reproducibility of C18-column for Indole over three days at concentration of 0.5 and 5 ug/ml.

Days	Average %Recoveries	Indole Concentration ug/ml	Coefficient variation	Number of Samples Extracted
First	98.4% 92.6%	5 0.5	3.7% 3.7%	10 10
Second	96% 99.1%	5 0.5	8.7% 2.6%	6 6
Third	93.0% 91%	5 0.5	5.1% 7.2%	10 10

Re-using Bond Elut Columns

C18-Bond Elut^{R} columns have been tested for their ability to be re-used for Indole extraction (up to ten times). Different lot numbers of C18-column from the same supplier have been used in this investigation in duplicates. Their suitability for re-use was found to vary between 7 and 10 times. The columns were washed and conditioned before re-used with three bed volumes of methanol followed by three bed volumes of deionised water. The recoveries of Indole when the C18-column was used up to ten times was always very good and in the range of 93-98%. The recoveries did not decline, gradually but showed a very sharp drop to less than 5% in some columns after about 7 times. The reproducibility between the different column lot numbers was excellent with less than 5% variation, Table 17. The C18-column was re-used only four times in this work as a safety measure.

Indole Extraction Calibration Curve

Samples of spiked blood with Indole and 5-chloroIndole (I.S) were treated with methanol/isopropanol (5:1 v/v) to precipitate the proteins. The supernatant was mixed with two volumes of 0.1M sodium acetate and extracted through a C18-column as described earlier. The relation between Indole concentration and average peak height ratio (std./I.S) of samples analysed by HPLC are shown in Table 18.

TABLE 17.

The recovery of Indole extracted through C18-column of different lot numbers and the reproducibility of each column when used up to 7 - 10 times.

Column Lot Number	Recoveries	Number of samples Extracted through The Column
A	98.4 $\pm$ 3.7	n = 7
B	97.3 $\pm$ 2.9	n = 7
C	93.5 $\pm$ 4.5	n = 10

TABLE 18.

Relation between Indole concentration and average peak height ratio (Std./I.S) of samples extracted through C18-column and analysed by HPLC.

Indole Concentration ug/ml	Average Peak Height Ratio n = 6
0.1	0.045
0.25	0.095
0.5	0.21
2.5	1.07
5	1.9

Extraction Procedure

1. Sample, standard and blank mixed with 0.1 ml 5-chloroIndole (50 ug/ml) in 6 ml capacity screw-capped vials.

2. 2 ml of methanol/Isopropanol (5:1 v/v) added to the above vials. Vortex the mixture for one minute and then centrifuge on an angle-head centrifuge for three minutes.

3. Transfer the supernatant (2.5 ml) to a clean vial and mix with 5 ml 0.1M sodium acetate buffer.

4. The C18 column of 3 ml capacity was positioned in 10 ml column capacity Vac Elut^{R} system and conditioned by 2 x 3 ml methanol followed by 2 x 3 ml deionised water. The above mixture was transferred into a 10 ml syringe fitted above the conditioned column via adaptor.

5. The vacuum was applied and the sample was drawn slowly through the column.

6. Dry the column under full vacuum for two minutes.

7. Wash the column with 12% methanol/water.

8. Elute the Indole with 2 x 0.5 ml methanol into 3 ml capacity screw-capped vial. 500 ul injected into the developed HPLC system as described in 4.2.1.

Indole Losses with Storage

Indole standard solution of 0.6 ug/ml was stored at room temperature and analysed after five days. It showed losses of above 80%. This could be due to adsorption of Indole into the wall of the glass container.

Conclusion

The method developed for extracting the acid interference Indole from putrefied whole blood had a good recovery of above 91% using methanol/isopropanol as protein precipitation reagent and solid-phase extraction (C18-columns) with sodium acetate as the buffering agent pH = 12. Washing the C18-column with 12% methanol/water gave a cleaner extract without reducing the recovery. Extracted Indole should be analysed within the same day it is extracted and a freshly prepared Indole standard is desirable since there are significant losses on storage.

4.4.2 Acid Interferences in Blood Samples Spiked With Drugs

Indole formation in blood spiked with the anticonvulsant drugs has been monitored from the day of spiking up to 52 and 65 weeks for non-acid and acid drugs respectively using the HPLC system described in 3.4.1. The HPLC system was fitted with a 500 ul loop and the column effluent monitored at 273 nm. Indole was extracted from whole blood spiked with the drugs under study using the developed method described in 4.4.1. Spiked blood samples stored at the three different conditions and time intervals were analysed at preset times as described in the analysis protocol, 4.1.

Result and Discussion

Figures 37, 38 and 39 show the concentrations of Indole in whole blood spiked with anticonvulsant drugs, benzodiazepines and opiates respectively when stored at 5, 25, and -20^{o}C for different time intervals. The figures show low Indole concentrations $\leq$50 ng/ml regardless of the storage temperature. This value represents the detection limit of Indole analysis methods. A higher concentration of Indole was noticed in 22 - 25% of samples only at the end of the experiment and usually in the samples stored at 5, 25^{o}C.

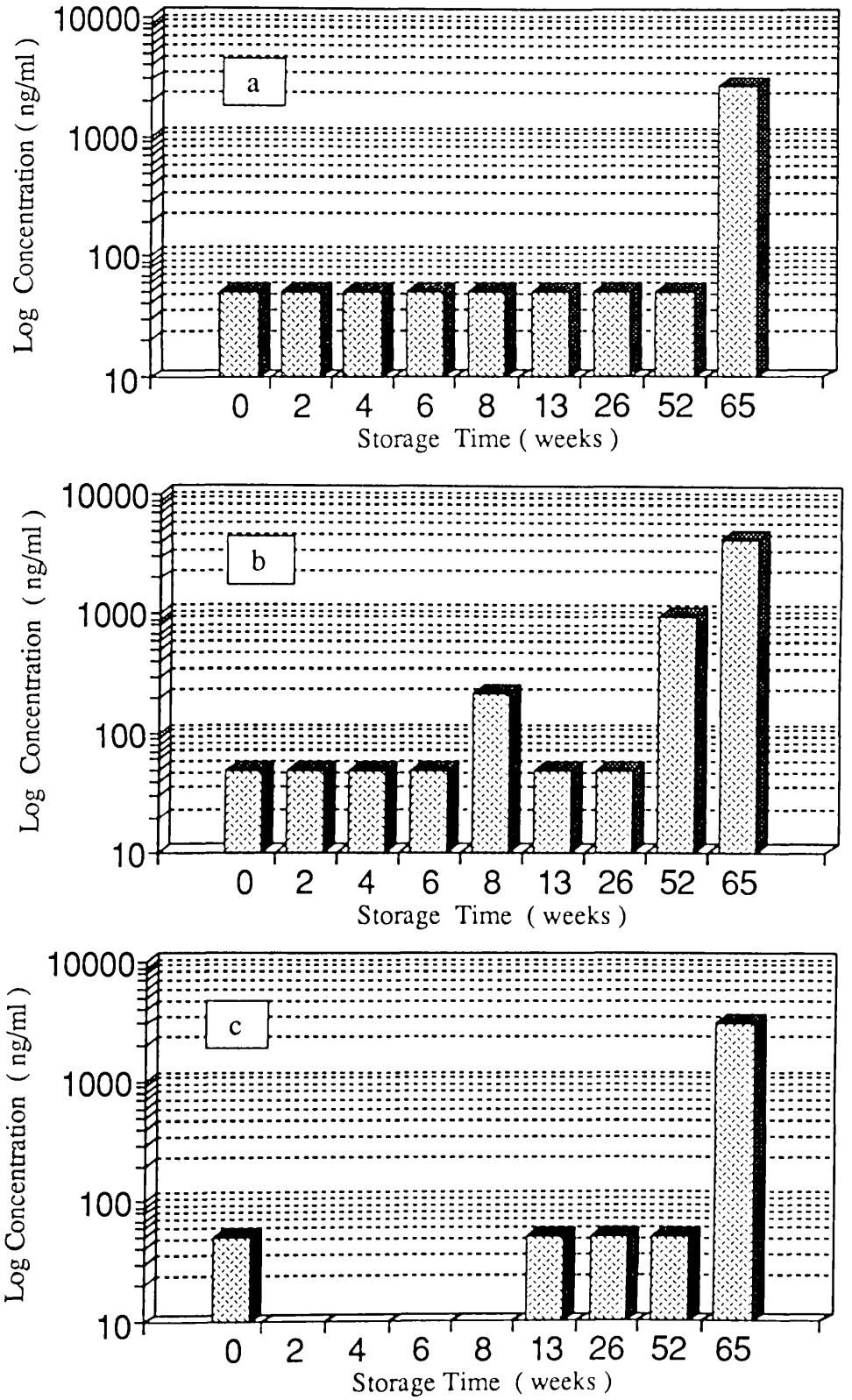


Figure 37. **Indole concentration in blood samples spiked with anti-convulsant drugs stored at: a) $5^{o}c$; b) $25^{o}c$; c) $-20^{o}c$ over different time periods.**

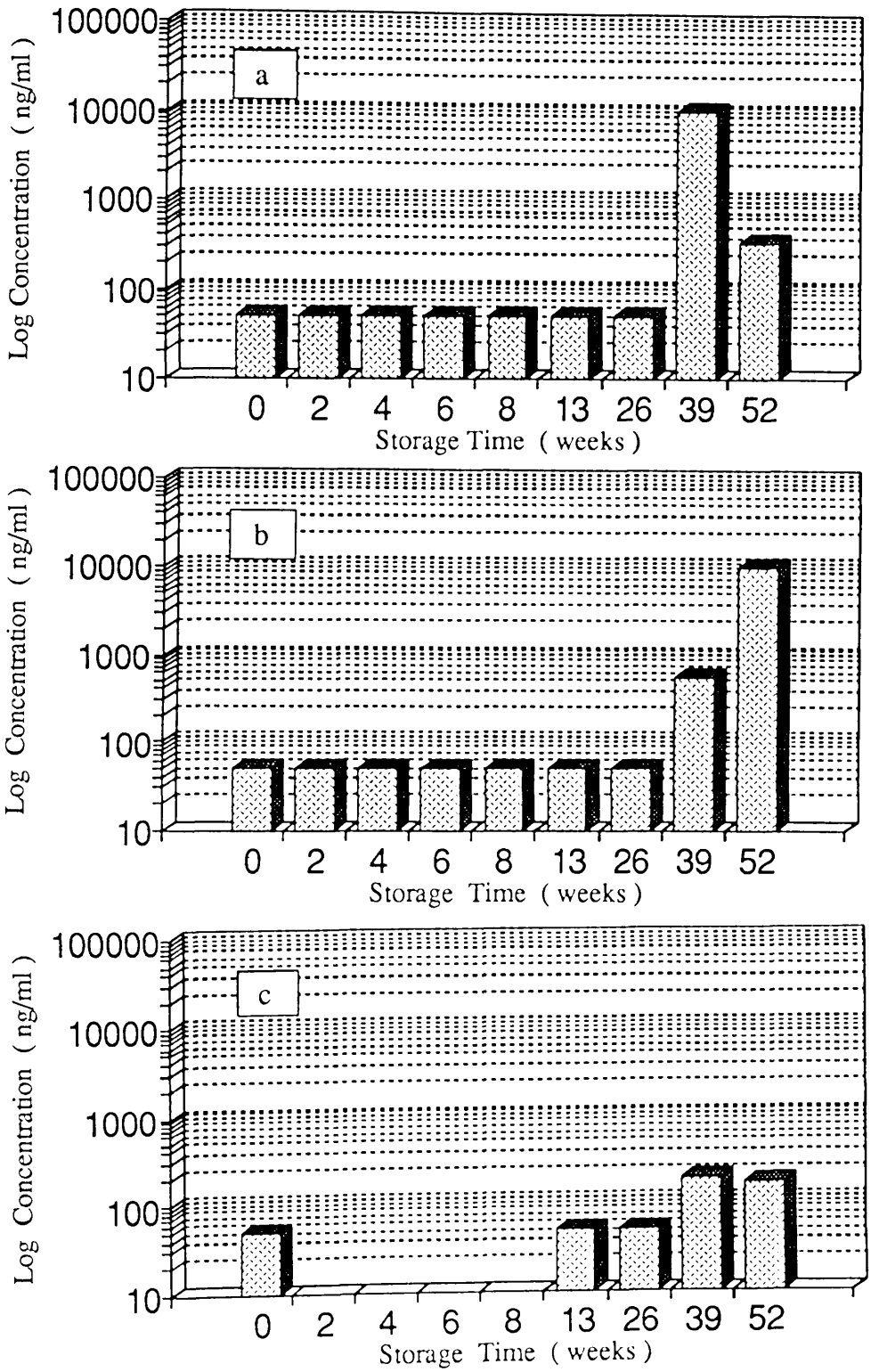


Figure 38. Indole concentration in blood samples spiked with Temazepam stored at: a) 5°c; b) 25°c; c) -20°c over different time periods.

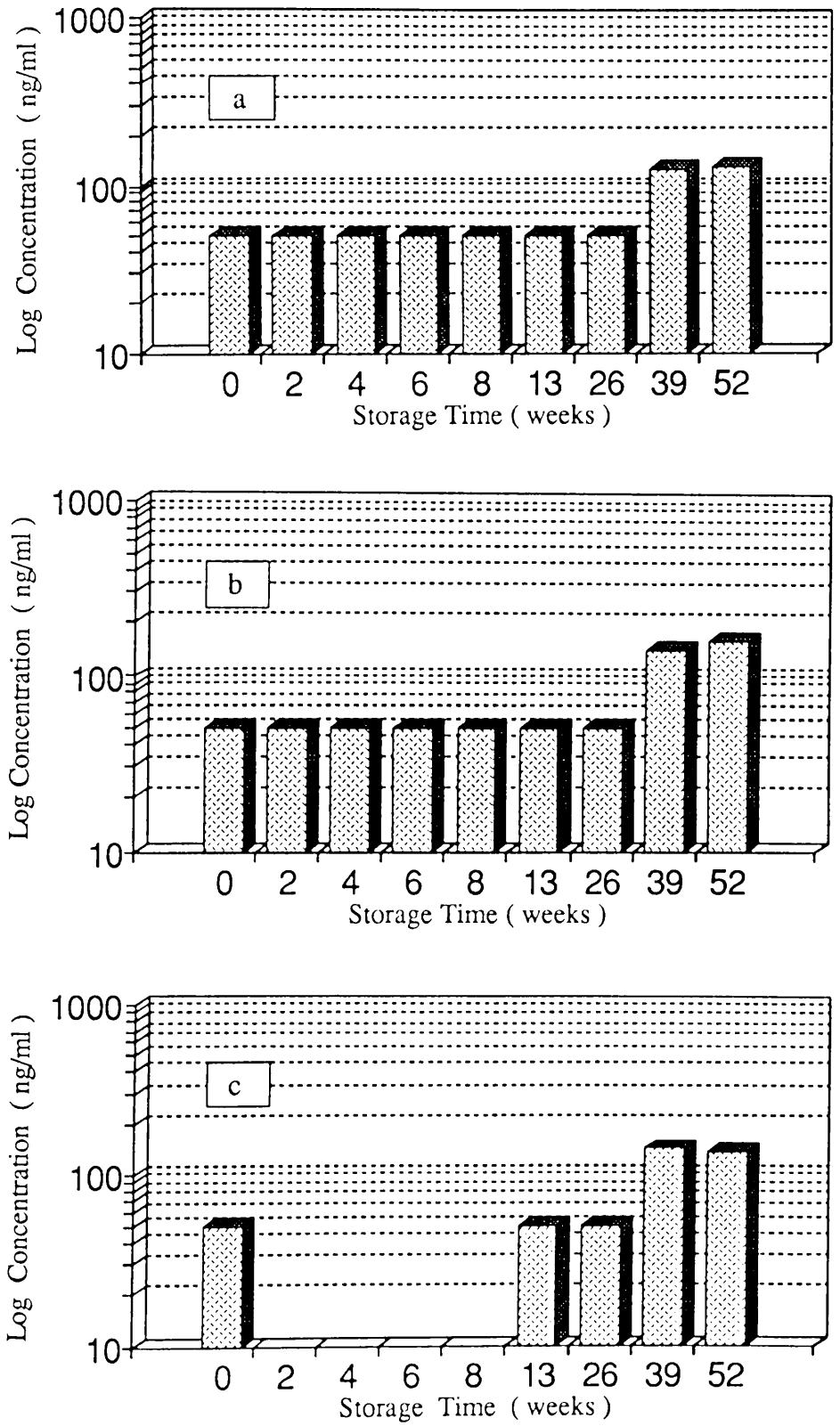


Figure 39. **Indole concentration in blood samples spiked with opiates and stored at: a) 5^{o}c; b) 25^{o}c; c) -20^{o}c over different time periods.**

Conclusion

Indole formation in whole blood samples spiked with anticonvulsant benzodiazepine and opiates was monitored. A considerable delay in Indole formation was noticed in all samples up to twenty-six weeks regardless of the storage temperature. Production of Indole was reduced by storing blood samples at -20^{o}C.

4.4.3 Non-acid Interferences

Protein Precipitation/Diatomaceous Earths (Extrelut^{R})

Two measures have been considered for sample preparation in the extraction of the three putrefactive amines through solid phase sorbent. Firstly using different protein precipitation reagents and secondly using diatomaceous earth (Extrelut^{R}).

Result and Discussion

Applying post-mortem blood to Bond Elut columns causes complete blockage of the column filter. Therefore different measures were considered to make the post-mortem blood usable with solid phase extraction. These were protein precipitation and diatomaceous earth extraction. Table 19 shows the recoveries of the three putrefactive amines with different protein precipitation reagent. In the case of whole blood spiked with 2-phenethylamine, the methanol protein precipitation failed to give a good recovery since 30 - 40% of the spiked putrefactive amine was lost Another known protein precipitation reagent, zinc sulphate, was tested on sheep serum spiked with 2-phenethylamine. It gave poor recoveries of (<10%) Acetonitrile/Isopropanol (5:1 v/v) recoveries (50%) of putrefactive amines from whole blood.

The diatomaceous earth Extrelut^{R}, is an alternative measure for whole blood sample preparation for solid phase extraction. Samples of 1 ml whole blood spiked with the three putrefactive amines in different concentrations were buffered with 2 ml 0.5M Na_2HP0_4 with a pH value of 9.0 and mixed well in a screw-capped vial then poured into 10 ml capacity syringes containing around 1.5 gm of diatomaceous earth. The column preparation methods were described earlier, [3.6.2]. The mechanism of extracting the three putrefactive amines by Extrelut^{R} involves adsorption of aqueous phase into diatomaceous earth, a porous material of which acts as a support for the aqueous phase. This provides a large surface area for partition into the elution solvent which flows through the immobilized specimen under gravity eluting the putrefactive amines. Tyramine has a pka values of 9.5 for the phenol and 10.8 for the amine group. The other two putrefactive amines have no pka values available in the literature, but these

TABLE 19.

The recoveries of the three putrefactive amines using different protein precipitation reagents.

Protein Precipitation Reagent	Putrefactive Amines Concentration	The Measured Concentration after Protein Precipitation	% Recovery	Number of Samples
Methanol	10 ug/ml of 2-phenethylamine in whole blood	6.1 ug/ml	61%	3
*Zinc Sulphate	10 ug/ml of 2-phenethylamine in sheep serum	0.9 ug/ml	<10%	3
Acetonitrile/ Isopropanol (5:1 v/v)	10 ug/ml of a) 2-phenethylamine b) Tyramine c) Tryptamine in sheep serum	a) 4.1 ug/ml b) 5.3 ug/ml c) 4.0 ug/ml	41% 53% 40%	5

compounds are known to exhibit basic characteristics due to the presence of amine group and are successfully extracted from blood at high pH [28].

A standard of the three putrefactive amines in blood buffered with 2 ml 0.5M Na_2HP0_4 were successfully eluted from Extrelut column with diethylether/Isopropanol (7:3 v/v) and collected in 10 ml capacity tubes to a total volume of 8 ml. The pH of the collected eluent was 8.5. The other buffering system was 5% w/v $NaHCO_3$ (pH 8.0) spiked blood samples extracted and eluted from the column as above. The pH of the collected eluent was 7.5. The recoveries of the three putrefactive amines for the two buffering systems was measured by injecting 100 ul of the eluant in the HPLC system. The un-extracted standard was injected into the HPLC to calculate the recoveries. Table 20 shows the recoveries of the three putrefactive amines for each buffering system.

TABLE 20.

The absolute recoveries for the three putrefactive amines from Extrelut using different buffering systems.

Putrefactive Amine	Average Recovery Using 0.5M Na_2HP0_4 n = 5	Average Recovery using 5% w/v $NaHC0_3$ n = 5
2-phenethylamine	86 ± 6.5%	95 ± 8.4%
Tyramine	72 ± 6.5%	81 ± 6.4%
Tryptamine	83 ± 5%	98 ± 4.3%

Conclusion

The recoveries using Extrelut for extracting the three putrefactive amines is good compared with their recoveries when using protein precipitation reagent for samples preparation. At the same time, the Extrelut^{R} eluent contains other endogenous compounds co-eluted with three putrefactive amines, and a large sample volume. Further purification and concentration steps are necessary to produce a small sample volume and a cleaner extract suitable for HPLC analysis.

Solid Phase Extraction

Different types of solid phase sorbent have been tested for their suitability to clean up the extracts from the Extrelut^{R} column prior to analysis.

Non-Polar-Sorbent C18

Three ml capacity C18-column was conditioned with 2 x 3 ml methanol to activate the column by opening up the hydrocarbon chain and as a result increasing the surface area available for interaction. Washing the column with methanol removes residue from the packing material that might interfere with the analysis. The column was washed with 2 x 3 ml deionized water to remove the excess methanol.

1 ml of standard 2-phenethylamine at a concentration of 10 ug/ml were prepared in methanol and deionised water and mixed with 2 ml sodium acetate buffer (pH12). These were poured into the column reservoir and the samples drawn slowly through the column by vacuum. The column was then washed with 1 ml deionized water. Elution of 2-phenethylamine was achieved by adding 2 x 0.5 ml. of methanol or a solvent mixture acetonitrile/methanol/ammonia (82:25:0.3 v/v/v). 20 ul of the recovered 2-phenethylamine was injected to the HPLC system described in 3.4.2.

Result and Discussion

Table 21 shows the recoveries of standard 2-phenethylamine prepared in methanol and in deionized water. 2-phenethylamine was eluted from the column using different elution solvents.

2-phenethylamine samples prepared in water and extracted through C18-colum showed a very good recovery due to the presence of 2-phenethylamine in a high polar environment. The recoveries obtained for 2-phenethylamine were above 90% when eluted with methanol and a solvent mixture of acetonitrile/methanol/ ammonia (82:25:0.3 v/v/v) respectively. On the other hand, samples of 2-phenethylamine prepared in methanol extracted and eluted as above showed a lower recovery of about 50% due to the presence of methanol which had enough non-polar character to disrupt the non-polar interaction between the 2-phenethylamine and the C18-column non-polar chain. Buffering the 2-phenethylamine samples at high pH value will increase the non-polar properties

TABLE 21.

Recoveries of 2-phenethylamine prepared in different solvents and eluted from C18-column using different elution solutions.

Standard 2-phenethylamine Prepared in	Average Absolute Recovery of 2-phenethylamine Eluted with Methanol	Average Absolute Recovery of 2-phenethylamine when Eluted with Acetonitrile/MeoH/Ammonia (82:25:0.03 v/v/v)
Methanol	60 $\pm$ 7% n = 4	52 $\pm$ 7% n = 4
Deionized Water	99 $\pm$ 5% n = 6	93 $\pm$ 9% n = 6

of 2-phenethylamine by suppressing the ionization of the amine group and as a result increase the lipophilicity of 2-phenethylamine and enhance the non-polar interaction with the non-polar side chain of C18-column.

Conclusion

Using the C18-column for extraction is limited since whole blood can not be applied directly to solid phase sorbent and non-polar sorbent C18 can not extract compounds present in organic solvents with a good recovery as noted in the case of 2-phenethylamine in methanol.

Bond Elut Certify Column

The recoveries of the three putrefactive amines using this relatively newly marketed Bond Elut Certify column was investigated by extracting a standard mixture of 2-phenethylamine, Tyramine, Tryptamine in a concentration of 10 ug/ml through the Certify column as described in 3.6.2.

Result and Discussion

The eluted amines were analysed by the HPLC system described in 3.4.2. Table 22 shows the recoveries of 2-phenethylamine, Tyramine and Tryptamine using the Certify column. The table shows a poor recovery of the three putrefactive amines when extracted through the Certify column.

Therefore, the Certify column can not be used as an extracting column for putrefactive amines. On the other hand this column would be a good extraction column for putrefied urine and blood since around 75-95% of the putrefactive amines would not be retained on the column and lost during the extraction procedure. Therefore a clean extract would be expected when this column was used for basic drug extraction from putrefied biological specimens.

TABLE 22.

Recoveries of the three putrefactive amines using Certify column.

Putrefactive Amines	Concentration in Sheep Serum	Average Recovered Concentration	Average % Recovery	Number of Samples
2-phenethylamine	10 ug/ml	2.5 ug/ml	25%	n = 4
Tyramine	10ug/ml	0.5 ug/ml	5%	n = 4
Tryptamine	10 ug/ml	1 ug/ml	10%	n = 4

Cationic Exchange Sorbent

In cationic exchange extraction the interaction occurs between an isolate molecule carrying a positive charge like "ionised amine" and the sorbent negative charge (sulfonic acid or carboxylic acid). Ion exchange sorbent in general is capable of extracting ionized molecules from a non-polar matrix. A Cationic exchange column was investigated for its suitability in retaining the three putrefactive amines.

SCX Sorbent

Benzene sulfonic acid (SCX) is known as a strong cation exchanger with very low pka, SCX exhibited ionic interaction, non-polar and ionic secondary interactions due to unbonded silanols on the silica substrates. A standard solution mixture of the three putrefactive amines, 2-phenethylamine, Tyramine and Tryptamine in a concentration of 1, 1, 0.5 ug/ml respectively, were prepared in the elution solvent of Extrelut^{R} to explore the possibility of a specific retention of the three putrefactive amines eluted from Extrelut^{R} with diethylether/isopropanol (7:3 v/v). One ml of the standard mixture was drawn through a preconditioned SCX-column as described in 3.6.2. The column effluent was collected and analysed by HPLC. The percent losses are shown in Table 23.

TABLE 23.

The losses of the three putrefactive amines during the extraction through SCX.

Putrefactive Amine	Standard Mixture Concentration	Measured Concentration in the Column Effluent	% Losses
2-phenethylamine	1 ug/ml	0.12 ug/ml	12%
Tyramine	1 ug/ml	0.16 ug/ml	16%
Tryptamine	0.5 ug/ml	0.08 ug/ml	8%

Elution of the Sample

Different elution solutions were used to disrupt the interaction between the sorbent and the putrefactive amines. Elution solvents with a pH of two units above the pka of the isolate are usually recommended to disrupt the cationic interaction and a non-polar solvent to disrupt the non-polar interaction. The first elution solvent tested was composed of 15% ammonia in diethylether/isopropanol (7:3 v/v) and the other was 20% ammonia in methanol; their pH values were 11.0 and 12.0 respectively. The recoveries of the three putrefactive amines from SCX-column using the three elution solutions are shown in Table 24. The first two elution solutions failed to give recoveries of more than 12% for the three putrefactive amines. Eluting the three putrefactive amines from SCX column by using a solvent with a low pH value of 2 was tried. Recoveries of less than 23% were obtained for the three putrefactive amines which means that the three putrefactive amines were strongly retained on the SCX-column. Therefore, using SCX-column for putrefactive amine extraction is of little value due to the strong interaction between the functional groups of the sorbent and putrefactive amines.

CBA-Column

Carboxylic acid (CBA) columns (weak cation exchangers) were tested as an alternative of SCX for retaining the three putrefactive amines from the elution solvent of Extrelut. Standard solution mixture of 2-phenethylamine, Tyramine and Tryptamine in a concentration of 1, 1, 0.5 ug/ml and 10, 10, 5 ug/ml respectively were prepared in diethylether (DEE)/isopropanol (IPA) (7:3 v/v). 1 ml of the standard mixture was passed through the precondition CBA-column.

TABLE 24.

Percentage recoveries of 2-phenethylamine, Tyramine and Tryptamine (1, 1, 0.5 ug/ml) respectively from SCX-column using different elution solutions.

Elution Solution	2-phenethylamine	Tyramine	Tryptamine	Number of Samples
15% ammonia in diethylether/ Isopropanol (7:3 v/v)	8%	12%	7%	3
20% ammonia in methanol	5%	10%	7%	3
Acid methanol (25 ul of 10% H_2S0_4 in 5 ml methanol	17%	23%	13%	3

Result and Discussion

Table 25 shows the recoveries of the three putrefactive amines extracted through CBA-column. The interaction between the carboxylic group of the CBA sorbent and the amine group of the putrefactive amines was easily disrupted using acidic methanol pH2 which was two units below the pka of CBA sorbent of 4.8. 2 x 0.5 ml acidic methanol successfully eluted the three amines. The recoveries were found to be excellent and above 95% for low and high concentrations of the three putrefactive amines.

It was noticed that direct injection of acidic methanol (CBA- column elution solvent) into the HPLC causes a temporary back pressure on the system and resulted in irreproducible retention time of the three putrefactive amines. This could be due to the presence of $SO_4^{=}$ ions on the CBA-eluent. This problem has been solved by passing the acid methanol through the NH_2-column. This is discussed later.

TABLE 25.

Percentage recoveries of the three putrefactive amines from CBA-Column.

Putrefactive Amine	Average % Recovery	Concentration
2-phenethylamine	97 ± 6.1% n = 6 98 ± 5.9%	1 ug/ml 10 ug/ml
Tyramine	112 ± 6.8% n = 6 95 ± 6.0%	1 ug/ml 10 ug/ml
Tryptamine	103 ± 7.4% n = 6 97 ± 5.9%	0.5 ug/ml 5 ug/ml

Re-using CBA-Columns

The possibility of using the CBA-column several times has been investigated. Six samples of standard 2-phenethylamine, Tyramine and Tryptamine in a concentration of 1 ug/ml were extracted through each CBA-column. Also a different lot number of CBA-column from the same manufacturer was used to investigate the reproducibility of different CBA-column batches. Unextracted standard solution (1 ug/ml) of the three putrefactive amines was prepared in acid methanol and analysed by HPLC to calculate the recoveries.

Result and Discussion

The recoveries of the three putrefactive amines were calculated by comparing the peak height of extracted to unextracted amines. Table 26 summarizes the recoveries and reproducibility of CBA of different lot numbers supplied by the same manufacturer. The recoveries after six extractions on different lot number CBA-colum was within the range of 96-105% and the variation between the three different lot number CBA-column was less than 8%.

TABLE 26.

The recoveries of the three putrefactive amines on CBA-column of different lot numbers and the reproducibility of the CBA-column when each column was used up to six times.

Lot No	2-phenethylamine Recovery ± COV	Tyramine Recovery ± COV	Tryptamine Recovery ± COV	Number of Samples extracted through each column
A	105 ± 6%	104 ± 6.8%	103 ± 7.9%	6
B	100 ± 6.7%	98 ± 7.2%	96 ± 6.6%	6
C	103 ± 6.5%	101 ± 7.4%	99 ± 8.0%	6

Anion Exchange Column

Amino propyl is known to have polar and anion exchange as a primary interaction and non-polar, cation exchange as a secondary interaction. Amino propyl NH_2 column was tested as a purification step of the CBA extract. Six samples of the three putrefactive amines mixture in a concentration of 1, 1, 0.5 ug/ml of 2-phenethylamine, Tyramine and Tryptamine respectively, were prepared in acid methanol.

Result and Discussion

The recoveries of the three putrefactive amines prepared in acidic methanol and passed through NH_2-column were excellent. These are shown in Table 27. This could be explained due to presence of the three putrefactive amines in organic solvent (methanol) which disrupts the polar and non-polar interaction between the sorbent and the amine. At pH2 the sorbent would not exhibit any cationic interaction but it will exhibit its primary interaction (anionic). In this environment anionic interaction between the $SO_4^{=}$ ions and ($\overset{+}{N}H_3$) of the sorbent is expected to occur resulting in a very low $SO_4^{=}$ ion in the column effluent. When the NH_2-column effluent was injected into the normal-phase HPLC system no back pressure was noticed due to the absence of $SO_4^{=}$ ions in the effluent.

TABLE 27.

The percentage recoveries "unretained" when the three putrefactive amines passed through NH_2 column.

Putrefactive Amine	Average Recoveries ± COV
2-phenethylamine	98 ± 3.5% n = 6
Tyramine	102 ± 4% n = 6
Tryptamine	96 ± 6% n = 6

Recoveries from Combined Extrelut-CBA-NH_2

The preliminary investigation of the extraction of the three putrefactive amines from whole blood using diatomaceous earth column and CBA-column individually showed good recoveries. Different standard concentrations (0.1, 1, 5, 10 ug/ml) of 2-phenethylamine, Tyramine and Tryptamine (six samples at each concentration in blood) were extracted using Extrelut-CBA-NH_2 to investigate the efficiency of the combined Extrut-CBA-NH_2 for extracting the three putrefactive amines, then the eluted amines were analysed using the described HPLC system, 3.4.2.

Result and Discussion

Table 28 shows good recoveries of the three putrefactive amines from spiked blood with the highest recoveries for 2-phenethylamine and the lowest recoveries for Tyramine over the concentration range of 0.1 - 10 ug/ml.

Calibration Curve

The relation between putrefactive amines concentrations and peak height ratio (std./I.S) is shown in Table 29. A series of standard putrefactive amines mixture

TABLE 28.

The recoveries of the three putrefactive amines from spiked blood extracted through the combines Extrelut-CBA-NH_2 over the concentrations range of 0.1 - 10 ug/ml.

Putrefactive Amine	Average recoveries n = 6
2-phenethylamine	94 ± 7.1% at 0.1 ug/ml 93 ± 4.5% at 1 ug/ml 95 ± 8.4% at 5 ug/ml 86 ± 7.0% at 10 ug/ml
Tyramine	71 ± 10% at 0.1 ug/ml 74 ± 8% at 1 ug/ml 81 ± 6.4% at 5 ug/ml 72 ± 6.5% at 10 ug/ml
Tryptamine	94 ± 8% at 0.1 ug/ml 81 ± 5.4% at 1 ug/ml 89 ± 8% at 5 ug/ml 83 ± 6.7% at 10 ug/ml

TABLE 29.

Relation between the three putrefactive amine concentrations and the peak height ratio (std./I.S) for samples extracted through Extrelut-CBA-column and analysed by normal phase HPLC system.

	Concentration ug/ml	Peak height ratio
2-phenethylamine	0.1 1 5 10	0.042 0.38 1.8 3.9
Tyramine	0.1 1 5 10	0.051 0.43 2.8 5.0
Tryptamine	0.1 1 5 10	0.048 0.57 2.3 4.7

were extracted through the combined Extrelut-CBA-NH_2-column. A standard calibration curve constructed between the peak height ratio (std./I.S) and the concentrations of each putrefactive amine a linear relations were obtained, Figure 40. The regression equation Y = A + BX. Where 'y' is the peak height ratio 'x' is the concentration of each putrefactive amine in ug/ml.

Table 30 shows the correlation coefficient (r) the slope (B) and the constant (A) for each amine.

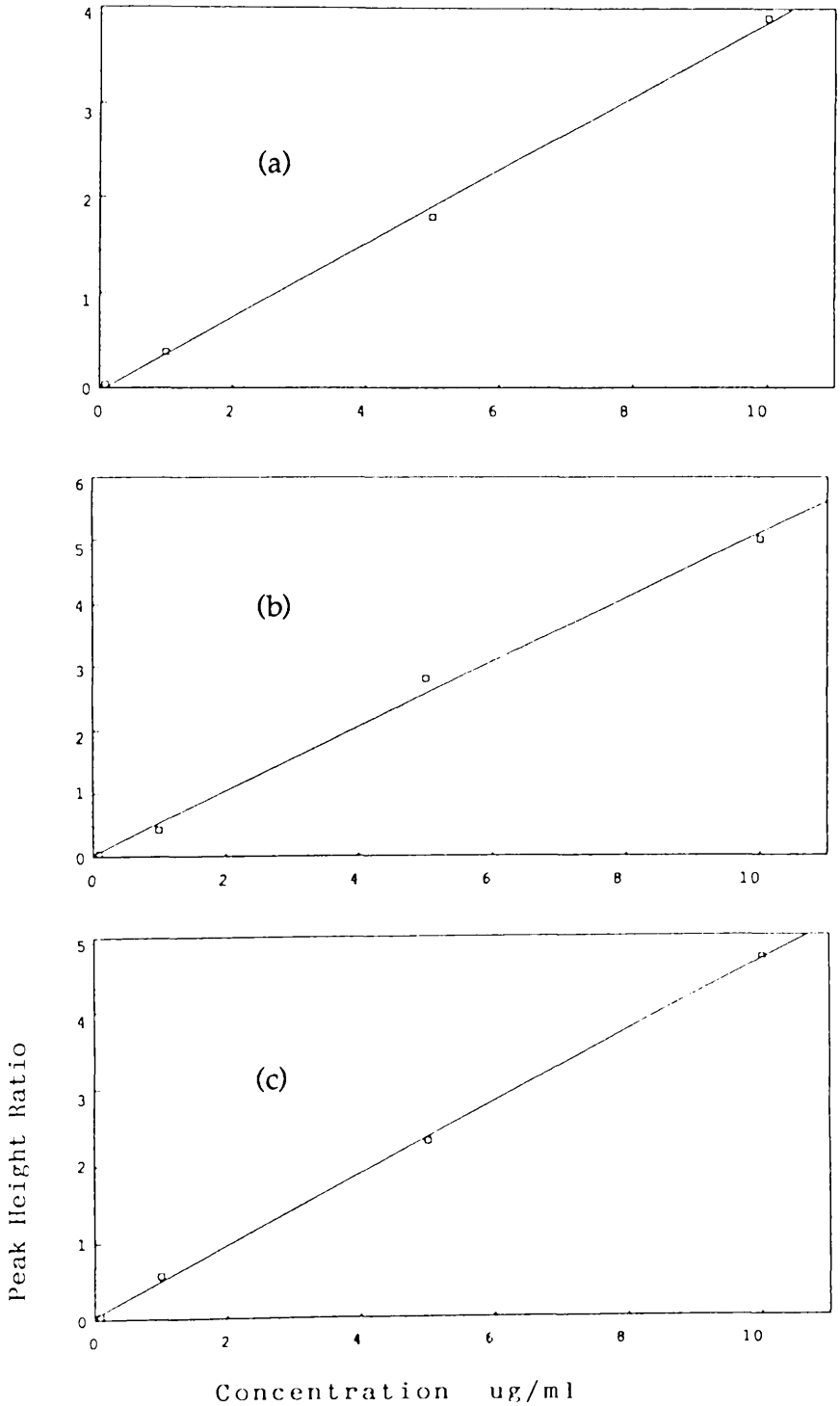


Figure 40. Standard calibration curves of the three putrefactive amines. The concentration of each amine is plotted against peak height ratio: a) 2-phenethylamine. b) Tyramine. c) Tryptamine.

TABLE 30.

The Linear Regression of the Three Putrefactive Amines extracted through combined Extelut-CBA and analysed by normal-phase HPLC.

	A	B	r
2-phenethylamine	0.04	0.44	0.998
Tyramine	0.07	0.78	0.999
Tryptamine	0.11	1.25	0.998

Experimental Details

The Resulting Method for Extraction of the Three Putrefactive Amines.

The search to develope a method for the simultaneous extraction of the three putrefactive amines from blood resulted in the following extraction procedure.

A. **Standard Preparation**

To 0.9 ml of blank blood, 0.1 ml of the standard mixture of three putrefactive amines (20 ug/ml in water) were added and mixed well in a 6 ml capacity screw-capped vial.

B. **Sample and Blank Preparation**

1 ml of blood sample and blank blood were transferred to 6 ml capacity screw-capped vial.

Extraction Procedure

1. 2 ml of 5% w/v $NaHC0_3$ and 0.1 ml of dihydrocodeine I.S (20 ug/ml) were added to the prepared standard, sample and blank, then mixed well.

2. Transfer the mixture on to the Extrelut-Column and allow to stand for 10 minutes.

3. Elute the three amines with 12 ml diethylether/Isopropanol (7:3 v/v) into a pre-conditioned CBA-Column.

4. Discard Extrelut Column and elute the three putrefactive amines from CBA-Column with 2 x 0.5 ml acidic methanol (25 ul 10% H_2S0_4 in 5 ml methanol) into

NH_2-column and collect the NH_2-column effluent and analyse using the developed HPLC system as described in 4.2.2.

Conclusion

A method for non-acid putrefactive amines extraction from putrefied whole blood has been developed based on Extrelut and solid phase sorbent as an alternative measure of liquid-liquid extraction. The combined Extrelut and carboxylic acid (CBA) sorbent has excellent efficiency for simultaneous extraction of the three putrefactive amines. The CBA-eluent should be passed through the NH_2-column to retain the $SO_4^{=}$ ions to overcome the back pressure on the normal phase HPLC. The CBA-column showed excellent recoveries and reproducibility even when they were used several times and came from different batches.

4.4.4 Non-Acid Interferences in Blood Samples Spiked with Drugs

Samples of whole blood spiked with anticonvulsants, benzodiazepine and opiates were analysed for the presence of the three putrefactive amines being stored at three different storage conditions and different periods of time according to the analysis protocol described in 4.1.

Result and Dicussion

A duplicate analysis had been conducted representing the standard, blank and blood samples extracted as described in 4.4.3. The samples were analysed using the developed HPLC system described in 4.2.2. The system was fitted with 100 ul loop at a range (AUFS) = 0.02, Filter = 2 sec.

Blood samples spiked with drugs under study showed low concentrations $\leq$50 ng/ml of the three putrefactive amines regardless of the storage temperatures and the period of time. Sealing the vials containing the blood samples and using sterilised blood from blood transfusion could minimise the possibility of bacterial contamination

Conclusion

Low concentrations of the three putrefactive amines were found in all blood samples spiked with drugs regardless of the storage temperature. Therefore, no interferences are expected from the three putrefactive amines on the stability of drugs in whole samples.

4.5 DRUGS EXTRACTION

Samples of whole blood and water spiked with drugs were extracted and analysed at the designated times. In each analysis a standard and blank were prepared to calculate the concentration of drugs under study. Data of each analysis were accumulated and tabulated at the end of the experiment. The standard for significant breakdown of drug to have occurred was a level measured which had decreased by greater than $\pm$ 3 SD for the analytical technique from the day zero concentration.

Blood and aqueous drug solutions were stored for the same period and temperature to investigate the stability of the drug in putrefying blood, the influence of putrefactive products on the analysis and the effect of storage temperature. For comparative purposes, fresh spiked samples were prepared on the day of analysis. Additonally, they were used to determine the within-day and day-to-day coefficient variation of the technique for the whole expected period.

Drugs Stability

There is a large number of possible reactions leading to drug degradation and most of those that do occur are classed as either hydrolysis or oxidation. In hydrolytic reactions temperature, pH and the presence of water are the major factors that influence drug decomposition, while oxidation reactions are strongly influenced by environmental factors such as light and metal ions. One major problem of

oxidation reactions is that some reactants such as oxygen need not be present in more than trace quantities to produce a significant stability problem, [222].

4.5.1 - Acid Drugs

4.5.1.1 Anticonvulsants

Phenobarbitone, carbamazepine and phenytoin are examples of anticonvulsant drugs whose stability in blood at different storage temperatures were investigated.

Phenobarbitone Stability

Samples of blood and water spiked with phenobarbitone were prepared to give a concentration of 10 ug/ml then stored for analysis according to the protocol described in [4.1]. The samples were extracted using the described method, 3.7.1.1 and analysed by the HPLC system described in 3.5.1.

Result and Discussion

The phenobarbitone concentrations following storage for the designated times and temperatures are presented in Table 31. The results for blood and water have been plotted in Figures 41 and 42. The per cent standard deviations for the entire

method for phenobarbitone analysis were 8.5% and 9.1% for blood and aqueous solution respectively.

Figures 41 and 42 display the decline in phenobarbitone concentration in blood and aqueous solution stored at the three different temperatures, 5, 25, -20^{o}C. Blood phenobarbitone samples analysed at twenty-six weeks (six months) showed no significant decrease in concentration with average recoveries of 79 $\pm$ 3.9% regardless of the storage temperature. The first significant decrease in blood phenobarbitone concentration was observed after one year of storage with recoveries ranging from 56 - 66%. At the end of the experiment (15 months) the average recovery for phenobarbitone was 46 $\pm$ 7.5% regardless of the storage temperature. The values for the rate of decrease of phenobarbitone concentration (Table 34) shows no significant difference in degradation rates at the three storage temperatures. An average loss rate of 102 ($\pm$ 9%) ng/ml/week was observed in the samples, (Table 34). On the other hand, the aqueous solutions of phenobarbitone stored for the same period of time showed a significant decrease in phenobarbitone levels. Samples stored at 5 and 25^{o}C had recoveries ranging from 57 - 79%, and at the end of the experiment the recovery of phenobarbitone ranged from 18 - 41%.

TABLE 31.

The concentration of phenobarbitone (ug/ml) in blood and aqueous solution stored at 5, 25, -20°C from day zero up to 65 weeks.

Storage	Concentration when stored at 5°C		Concentration when stored at 25°C		Time when stored at -20°C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueouse Solution
0	11.31	10.71	11.31	10.71	11.31	10.71
2	12.5	6.9	11.2	7.3		
4	10.7	9.0	10.1	9.0		
6	12.5	10.7	13.1	11.0		
8	11.4	9.2	13.0	8.07		
13	8.6	10.4	9.13	7.2	12.3	8.16
26	9.3	7.4	8.6	7.77	8.87	8.51
52	7.5	5.28	6.5	4.38	6.3	6.04
65	5.58	4.39	4.8	1.89	5.29	4.25

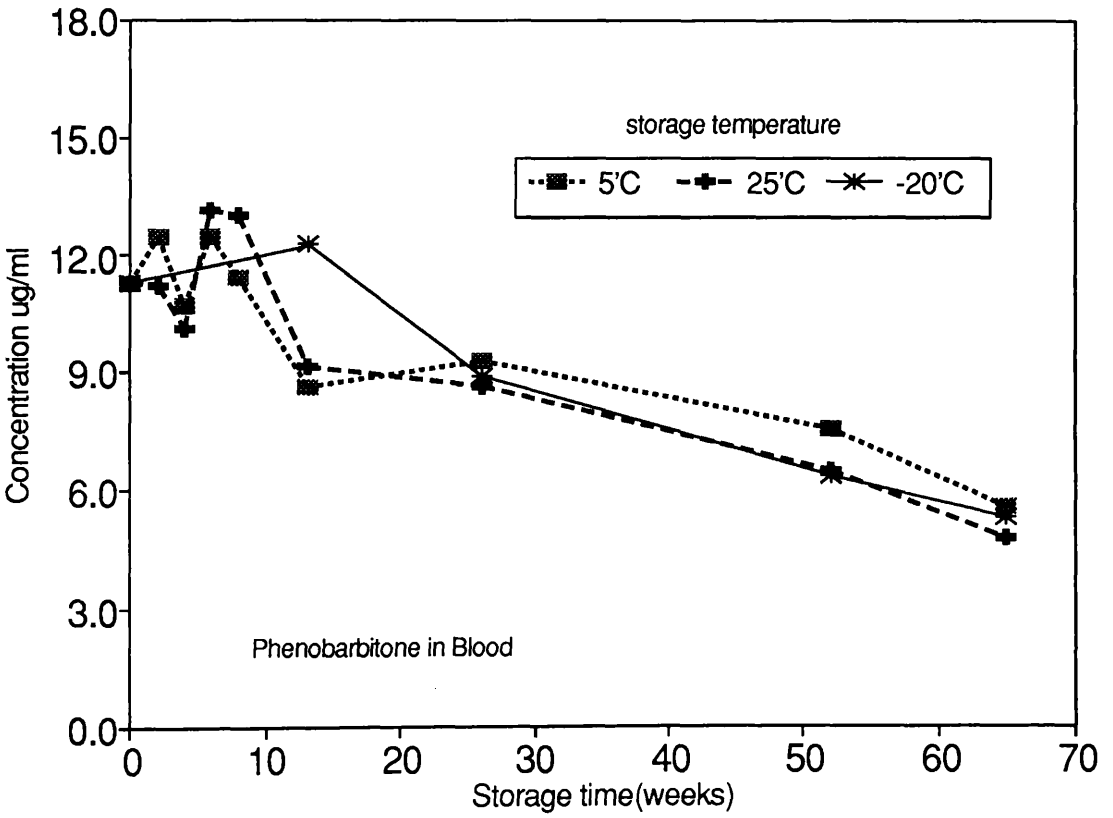


Figure 41. **Changes in phenobarbitone concentration with time in blood samples stored at 5, 25, -20^{o}C from day zero to 65 weeks.**

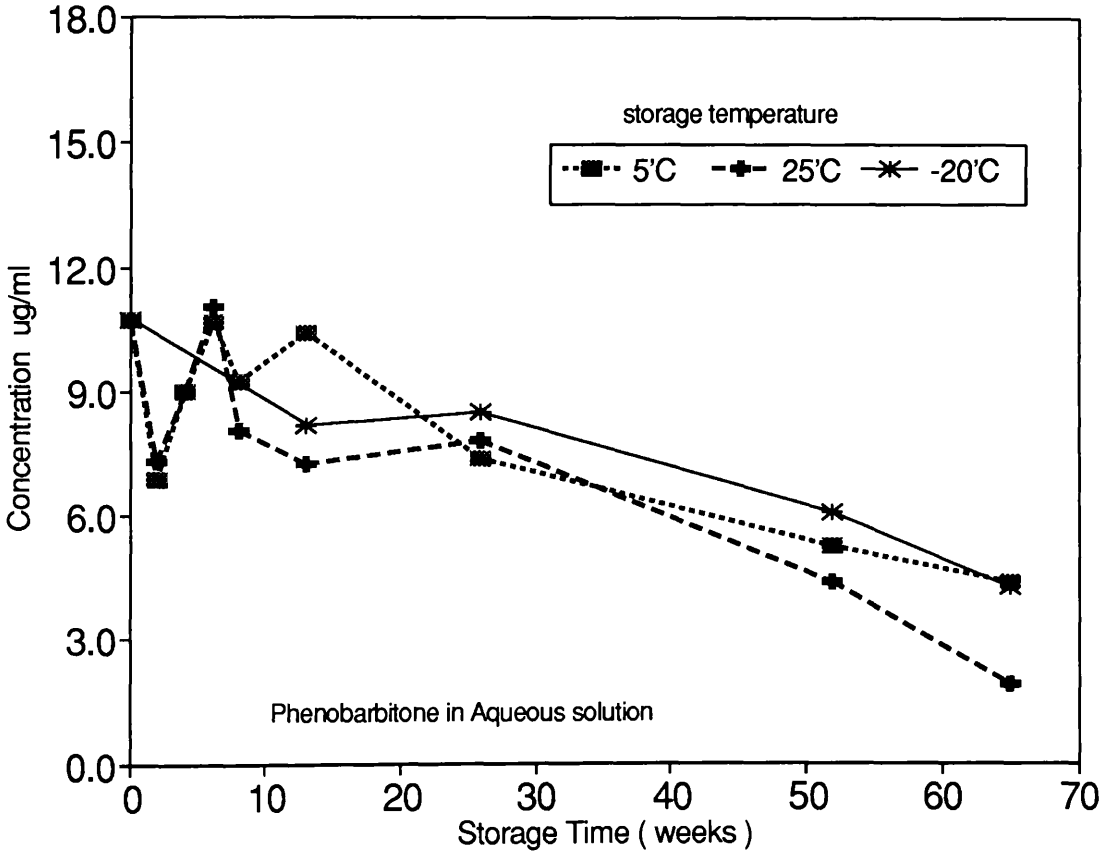


Figure 42. Changes in phenobarbitone concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 65 weeks.

From the above finding, phenobarbitone in blood showed a better stability compared with aqueous solutions stored at the same temperature for the same period of time. This could be explained by the hydrolysis of barbiturates in aqueous solutions. The hydrolysis mechanism of phenobarbitone in water [216, 217] has been shown to depend on whether the phenobarbitone was in ionized or unionized forms. Generally unionized barbiturates were hydrolysed at the 1,2 position Figure 45 while ionized barbiturates were hydrolysed at the 1,6 position Figure 46. The relatively better stability of phenobarbitone in blood was probably due to the reduced formation of the putrefactive amines in the blood and therefore the pH value for the blood would remain throughout the experiment were slightly acidic or neutral which favoured chemical stability of phenobarbitone since phenobarbitone hydrolysis is a base-catalyzed hydrolysis, [216].

Conclusions

Phenobarbitone in blood was shown to be more stable compared with phenobarbitone in aqueous solution when stored at the same temperature and time. The recovered phenobarbitone from blood samples at the end of the experiment were higher than those recovered from the aqueous solutions regardless of the storage temperature. Phenobarbitone in blood kept at the three storage temperatures for up to six months showed recoveries of 79 $\pm$ 3.9% regardless of the storage temperature, and even after 15 months of storage phenobarbitone was detected easily.

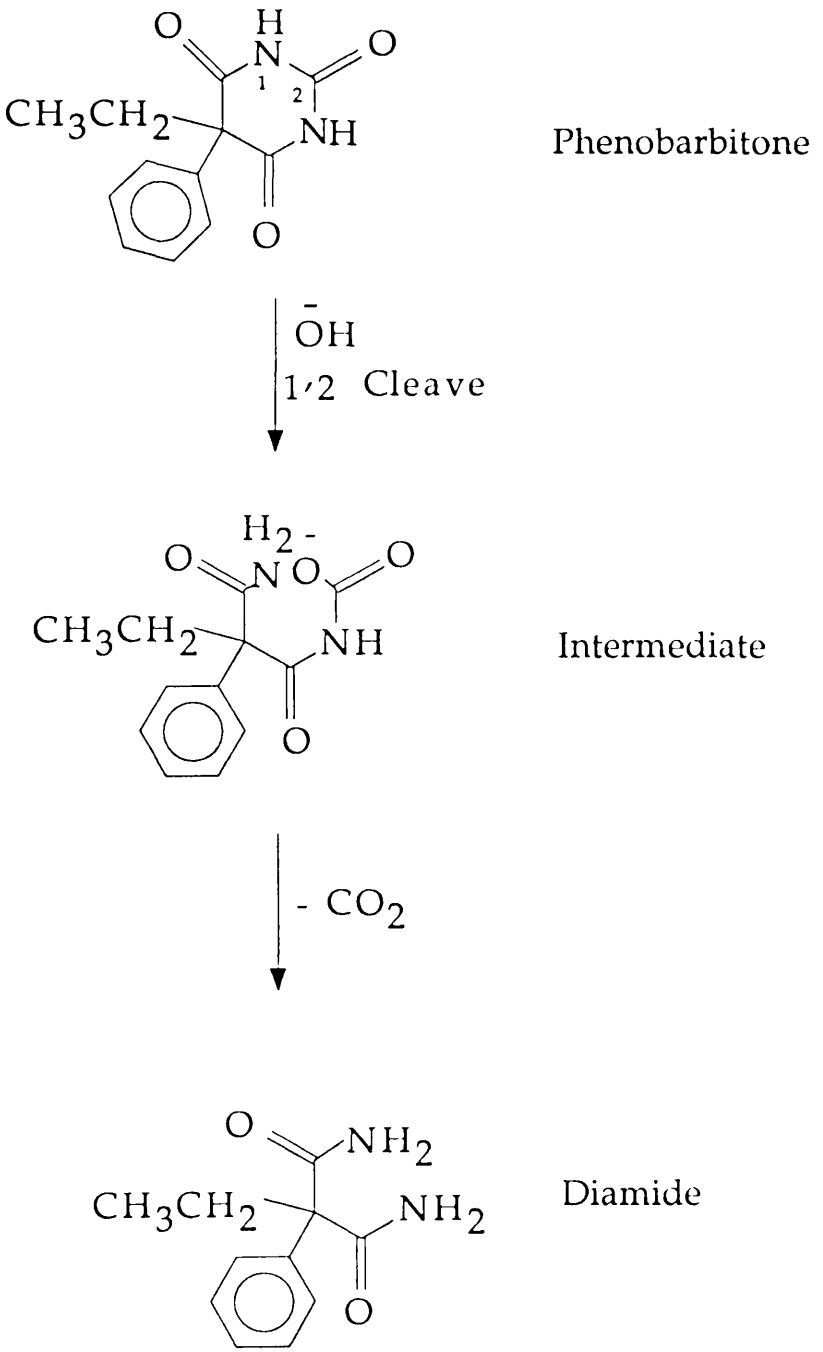


Figure 43. Degradation mechanism of unionised phenobarbitone in aqueous solution.

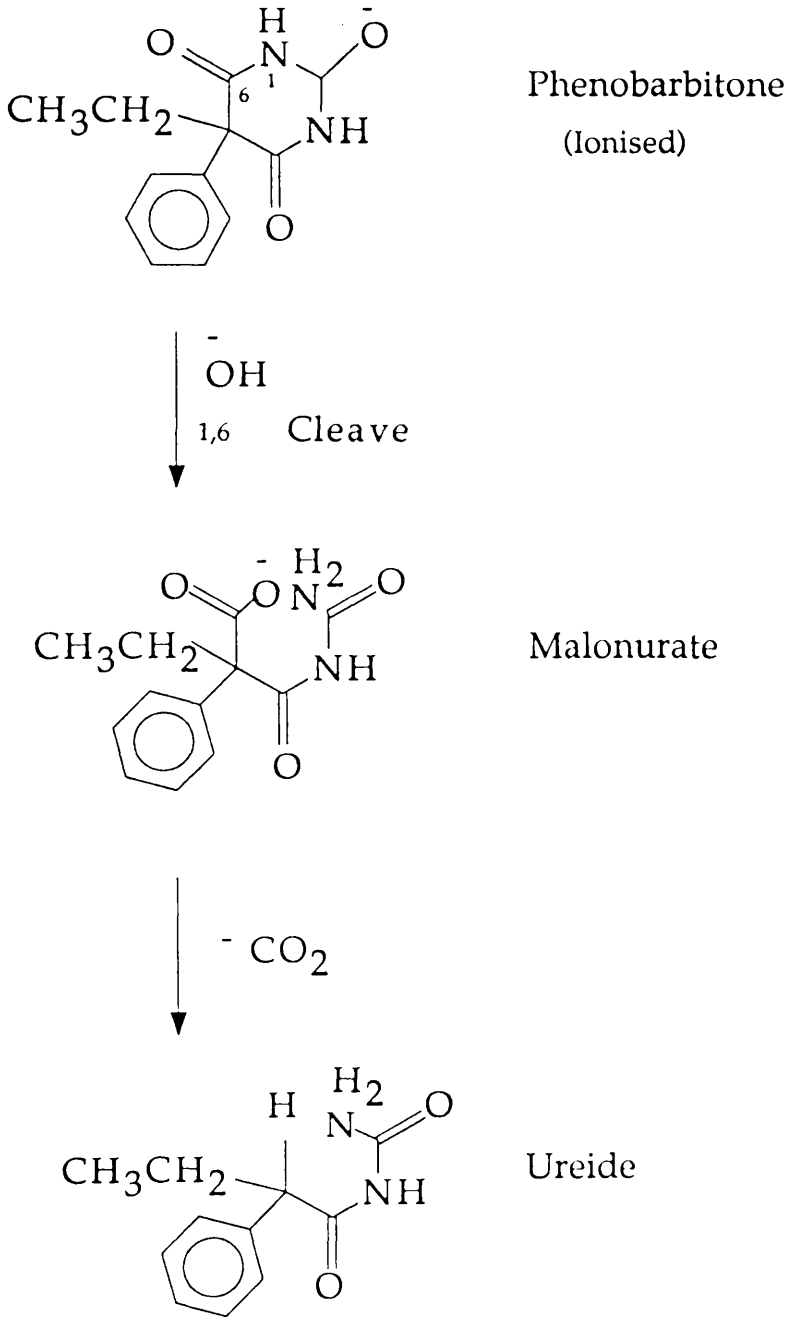


Figure 44. Degradation mechanism of ionised phenobarbitone in aqueous solution.

Carbamazepine Stability

Samples of blood and water spiked with carbamazepine were prepared in a concentration of 3 ug/ml and then stored for analysis according to the protocol described in 4.1. The samples were extracted using the described method (3.7.1.1) and analysed by the HPLC system described in 3.5.1.1.

Result and Discussion

Carbamazepine concentrations measured in blood and aqueous solutions at the three different storage temperatures and time periods are listed in Table 32. The per cent standard deviation for the entire method of extraction and analysis for both blood and aqueous solutions was found to be 9.5% for for carbamazepine.

The measured concentrations of carbamazepine in blood and aqueous solutions stored at the three storage temperatures is shown in Figures 45 and 46. The figures show a consistent decline in blood carbamazepine levels stored at 5, 25 and -20^{o}C from day zero to the end of the experiment (sixty-five weeks). A significant decrease in the blood carbamazepine concentration was found in samples stored at 25^{o}C only six weeks after of storage. Only 69% of the drug was recovered. After six months of storage, the recoveries at the three storage temperatures ranged from 60 - 84% and at the end of the experiment (15 months). The recovered

TABLE 32.

The concentration of carbamazepine (ug/ml) in blood and aqueous solution stored at 5, 25, -20^{o}C from day zero up to 65 weeks.

Storage Time	Concentration when stored at 5^{o}C		Concentration when stored at 25^{o}C		Concentration when stored at -20^{o}C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueous Solution
0	2.61	2.68	2.61	2.68	2.61	2.68
2	3.34	3.13	3.11	3.0		
4	2.44	3.14	2.18	3.11		
6	2.26	3.44	1.8	3.12		
8	2.54	2.92	2.2	3.04		
13	2.2	2.83	1.8	2.95	2.76	3.28
26	2.21	2.9	2.0	2.8	1.58	2.26
52	1.5	2.0	1.42	2.05	1.18	1.98
65	1.06	1.03	0.77	1.07	0.84	1.09

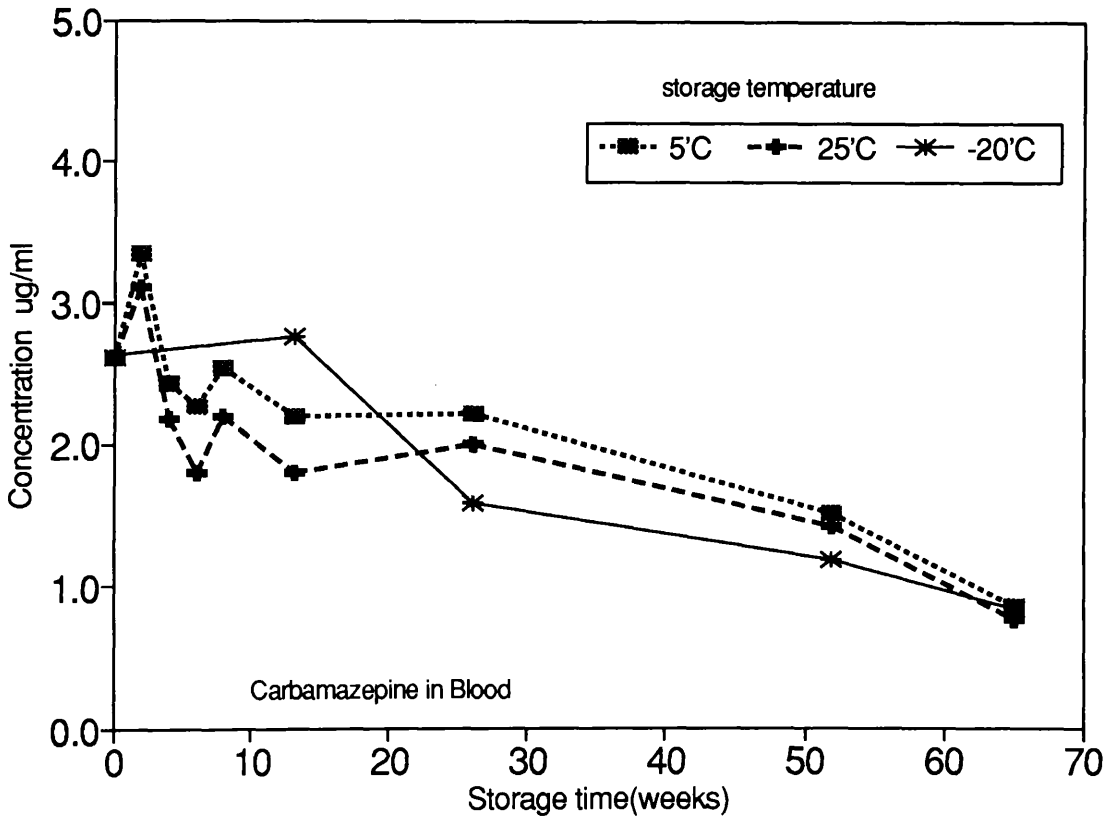


Figure 45. **Changes in carbamazepine concentration with time in blood stored at 5, 25, -20°C from day zero to 65 weeks.**

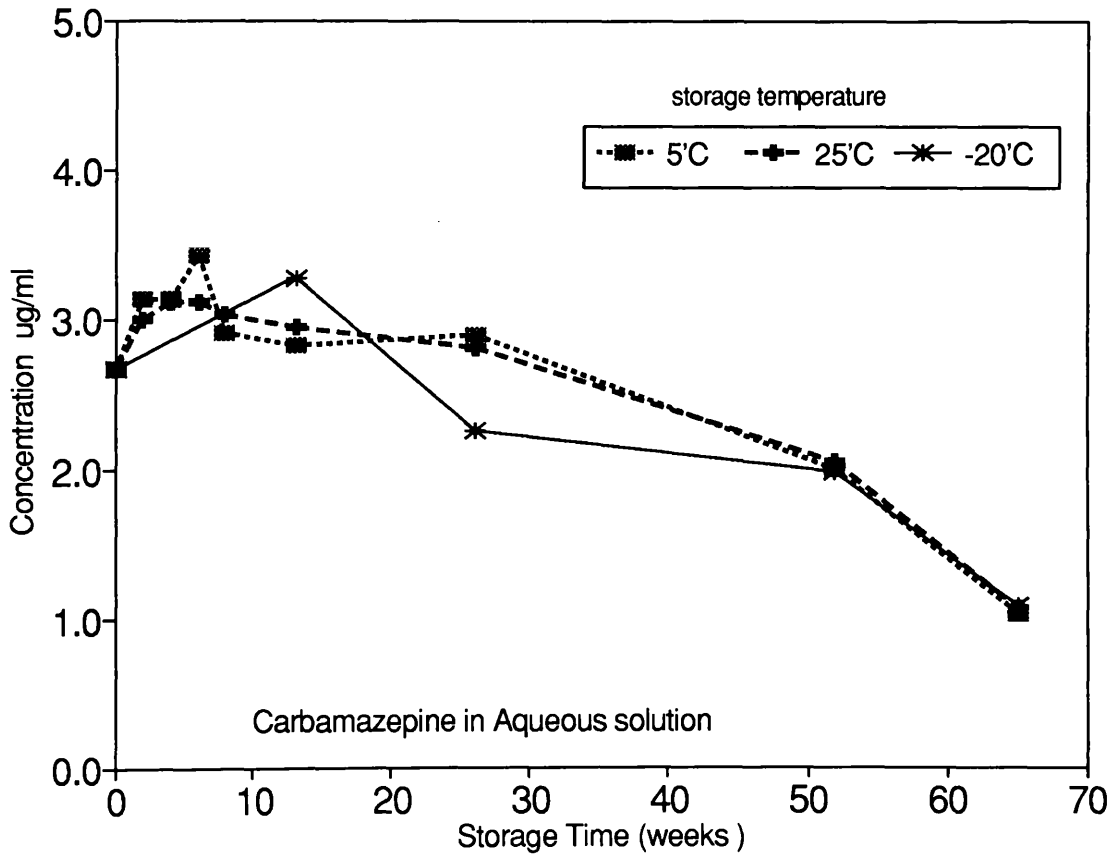


Figure 46. Changes in carbamazepine concentration with time in aqueous solution stored at 5, 25, -20^{o} from day zero to 65 weeks.

The recovered carbamazepine from blood samples was only 31% (± 5.7%).

Blood carbamazepine samples showed no significant difference in their degradation rates when stored at 5, 25, -20°C. The average loss was 27 (± 9.4%) ng/ml/week, (Table 34). Temperature, therefore, has no effect on the stability of carbamazepine in blood. In contrast, no significant decrease in carbamazepine levels in aqueous solutions was noticed when stored at the three storage temperatures up to six months. Recoveries measured ranged from 84 - 108%. At the end of the experiment (15 months) a significant decrease was observed in the aqueous solution of carbamazepine. An average recovery of 40 ± 2.8% for the three storage temperatures was obtained. For the aqueous solutions of carbamazepine no significant difference in the degradation rates regardless of the storage temperature was observed. An average loss of 27 (± 4%) ng/ml/week, (Table 34) was measured.

The above findings show that the loss of carbamazepine was similar regardless of the storage temeprature or the storage media. A reasonable amount of carmabazepine would be recovered even after 15 months of storage.

Conclusion

Carbamazepine was found to be reasonably stable in blood and aqueous solution up to six months regardless of the storage temperature, with recoveries ranging from 60 - 84% and 84 - 108% for blood and aqueous solutions respectively. After 15 months of storage at any temperature, a considerable amount of carbamazepine could be expected to be detected in blood samples from post-mortem cases involving a toxic dose of carbamazepine.

Phenytoin Stability

Samples of blood and water spiked with phenytoin were prepared to give a concentration of 10 ug/ml then stored for analysis according to the protocol described in 4.1. Samples were extracted using the described method, 3.7.1.1 and analysed by HPLC system as described in 3.5.1.1.

Result and Discussion

The levels of phenytoin measured in the spiked blood and aqueous solutions and stored at the three storage temperatures and analysed at the designated time are listed in Table 33. The per cent standard deviation of the phenytoin analysis was 8.6% and 9.3% for blood and aqueous solution respectively.

The measured drug levels have been plotted in figures 47 and 48 A significant fluctuation in phenytoin concentration in aqueous solution (Figure 50) was noticed. The same observation was reported [218, 219 and 220]. Phenytoin sodium, on exposure to air, absorbs carbon dioxide with the liberation of practically insoluble phenytoin [80].

Figure 47 shows less variation in blood phenytoin concentration compared to the aqueous solutions over the same period of time. Blood phenytoin samples showed a slower decline in the drug concentration over the period of twenty-six to fifty-two weeks of storage than the aqueous solutions for the same period. In blood the phenytoin showed no significant decrease in concentration with storage time or temperature over six months. An average recovery of 93 $\pm$ 5.4% was obtained. Over the next six month interval the recovery fell to 74 $\pm$ 10% (i.e. after one year of storage) At the end of the experiment the average recovery dropped to 36 $\pm$ 5%.

TABLE 33

The concentration of phenytoin (ug/ml) in blood and aqueous solution stored at 5, 25, -20°C from day zero up to 65 weeks.

Storage Time	Concentration when stored at 5°C		Concentration when stored at 25°C		Concentration when stored at -20°C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueous Solution
0	10.73	10.3	10.73	10.3	10.73	10.3
2	13.8	8.11	11.5	9.46		
4	10.0	11.9	9.55	11.4		
6	12.9	11.6	10.8	11.2		
8	10.4	8.97	11.2	7.69		
13	9.7	11.3	8.7	13.0	12.4	15.5
26	10.0	11.5	9.45	10.45	10.54	9.69
52	8.9	8.06	7.69	7.76	7.36	7.58
65	4.01	4.13	3.65	4.64	3.94	4.26

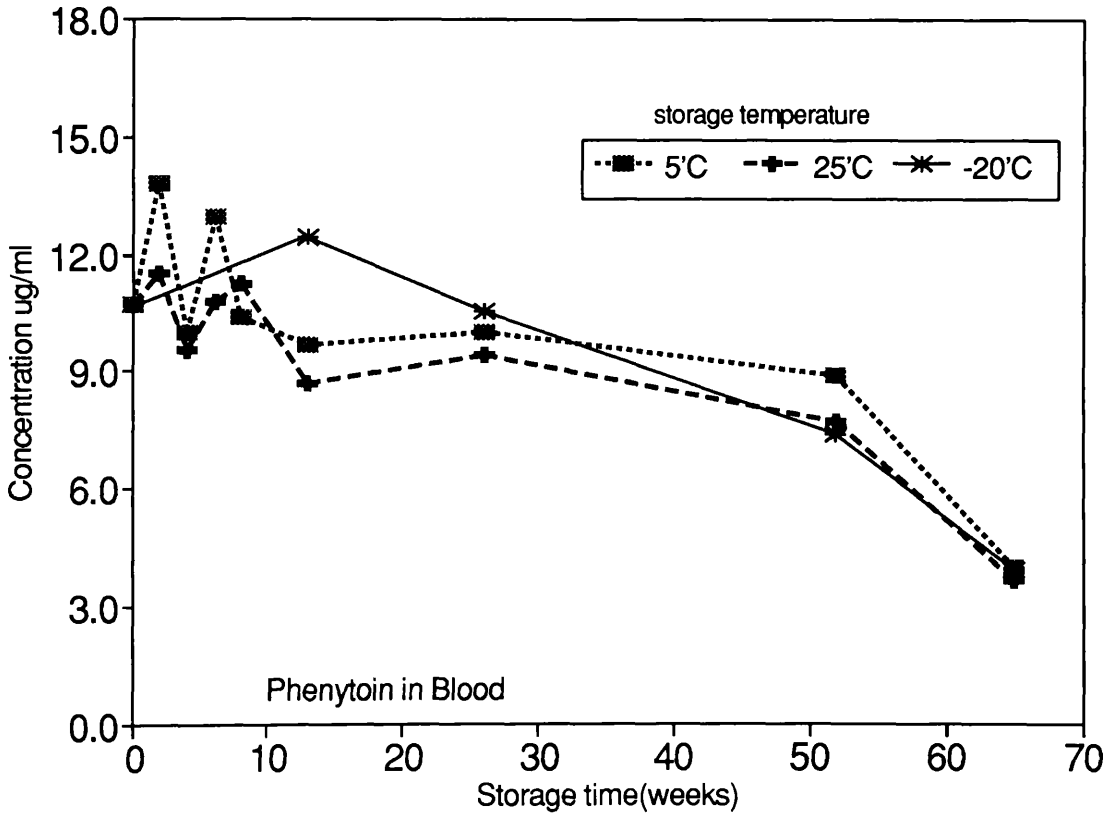


Figure 47. Changes in phenytoin concentration with time in blood stored at 5, 25, -20^{o}C from day zero to 65 weeks.

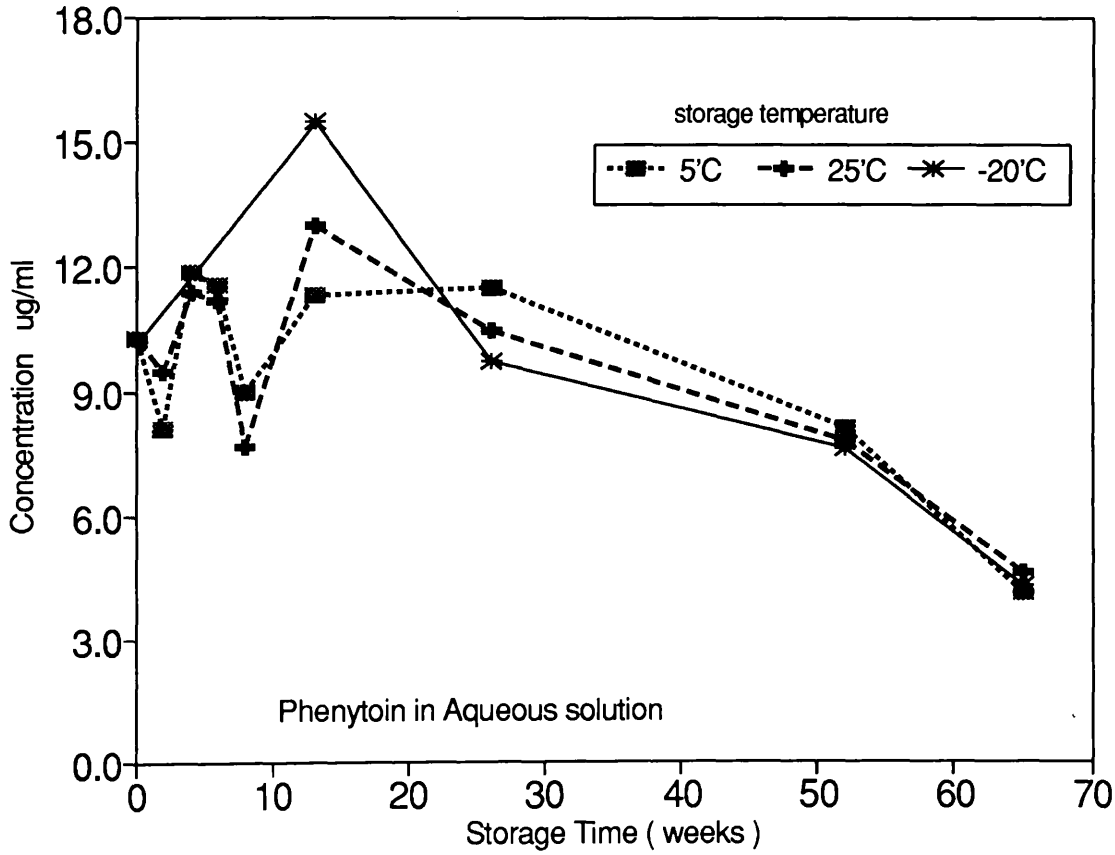


Figure 48. Changes in phenytoin concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 65 weeks.

A significantly high phenytoin concentration was measured in the aqueous solution samples stored at -20°C for three months. The sample was re-analysed several times and confirmed the high recovery. Further storage up to six months and one year at the three storage temperatures, resulted in average recoveries of 102 ± 8.6% and 76 ± 3% for the samples analysed after six months and one year respectively. At the end of the experiment the average recovered phenytoin was 40 ± 6% regardless of the storage temperature.

Conclusion

Phenytoin was found to be relatively more stable of the anticonvulsant drugs studied in this work. Blood and aqueous solutions at the three storage temperatures showed average recoveries of 93 ± 5.4% and 102 ± 8.6% respectively after twenty-six weeks of storage. After fifty-two weeks (one year) of storage 74 ± 10% and 76 ± 3% of phenytoin were recovered regardless of the storage temperature in the blood and aqueous samples.

TABLE 34.

The decrease rate in acid drugs concentration in blood and aqueous solution samples stored at 5, 25 and -20°C, from day zero up to 65 weeks.

Decrease Rate in Acid Drugs concentration ng/ml/week when stored from day zero up to 65 weeks at:			
Drugs	5°C	25°C	-20°C
Phenobarbitone in blood	91	107	107
Phenobarbitone in aqueous solution	83	109	88
Carbamazepine in blood	27	24	29
Carbamazepine in aqueous solution	28	26	26
Phenytoin in blood	96	93	112
Phenytoin in aqueous solution	76	76	121*

***High decrease rate in the phenytoin aqueous solution is due to the high phenytoin concentration in samples stored at -20°C and analysed after three months.**

4.5.2 - Non-acid Drugs

4.5.2.1 Benzodiazepines

Temazepam is a 1,4 benzodiazepine. It is subject to abuse by ingestion and injection. Its stability in post-mortem blood has not been reported.

Temazepam Stability

The stability of temazepam during the putrefaction process in blood was investigated. Samples of blood and water spiked with temazepam to a concentration of 1 ug/ml were prepared and then stored and analysed according to the protocol described in 4.1

Samples of blood and water spiked with temazepam were extracted as described in 3.7.2.1 and analysed by HPLC system as described in 3.5.2.

Result and Discussion

The levels of temazepam measured in the blood and water samples stored at the three storage temperatures and analysed at the designated time are presented in Table 35. The results for blood and water have been plotted in Figures 49 and 50.

The percent standard deviation for the entire temazepam analysis was found to be 4.8% for blood temazepam and 5.4% for aqueous solution temazepam. The standard for significant breakdown of temazepam to have occurred was a level measured which had decreased by greater than (±) 3 S.D from the day zero concentration.

TABLE 35.

The concentration of Temazepam (ug/ml) in blood and aqueous solution stored at 5, 25, -20°C from day zero up to 52 weeks.

Storage Time	Concentration when stored at 5°C		Concentration when stored at 25°C		Concentration when stored at -20°C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueous Solution
0	0.84	0.8	0.84	0.8	0.84	0.8
2	0.88	0.79	0.9	0.87		
4	0.9	0.86	0.88	0.85		
6	0.77	0.84	0.84	0.83		
8	0.71	0.83	0.81	0.85		
13	0.68	0.82	0.7	0.86	0.85	0.81
26	0.67	0.7	0.67	0.69	0.81	0.71
39	0.53	0.5	0.5	0.49	0.72	0.54
52	0.42	0.35	0.41	0.4	0.71	0.48

Figures 49 and 50 show the decline in temazepam concentration with time in blood and aqueous solution samples when stored at 5, 25, -20°C. Samples of blood temazepam stored at 5 and 25°C showed the first significant decrease in concentration after three months of storage and also showed the same rate of decline in their concentration with time, (Table 38). After six months of storage at 5, 25°C the average recovery of temazepam was 80%, while samples stored at -20°C showed no significant decrease in concentration with a recovery of 95%. This corresponded to a low decrease rate of 3 ng/ml/week, compared with the rate of 8.9 (± 9.5%) ng/ml/week for the samples stored at 5 and 25°C (Table 38). At the end of the experiment (one year) the amount of temazepam recovered from blood ranged from 48 - 84% regardless of the storage temperature. The temazepam stored in aqueous solution followed the same slow rate of decline in concentration with an average recovery of 88 ± 1.4% after six months. After one year of storage, the average recovery dropped to 41 ± 16%. The significant variation in the recoveries is due to the significant effect of temperature on the decrease rate which had an average of 8.4 (± 15%) ng/ml/week (Table 38). Samples of temazepam in blood and aqueous solution stored at -20°C were more stable. A low decrease rate compared to those stored at 5 and 25°C, (Table 38) was measured. This demonstrates the effect of temperature on temazepam stability.

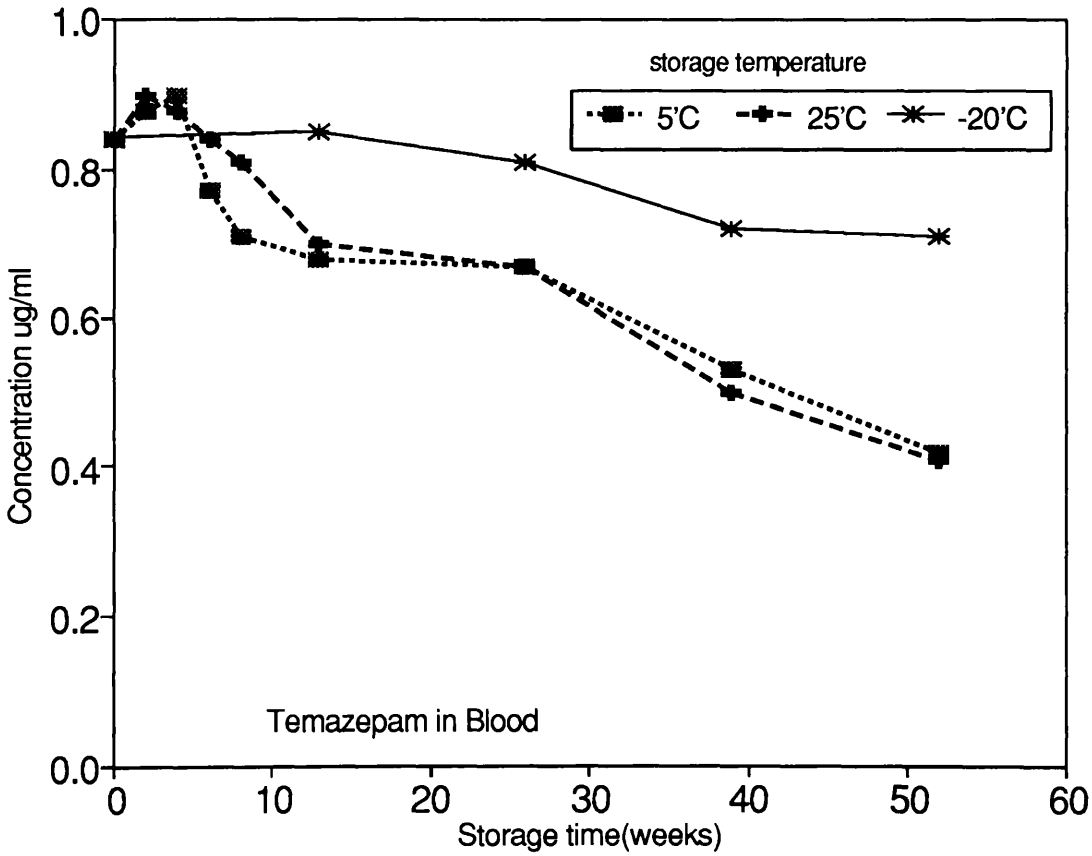


Figure 49. Changes in temazepam concentration with time in blood stored at 25°C from day zero to 52 weeks.

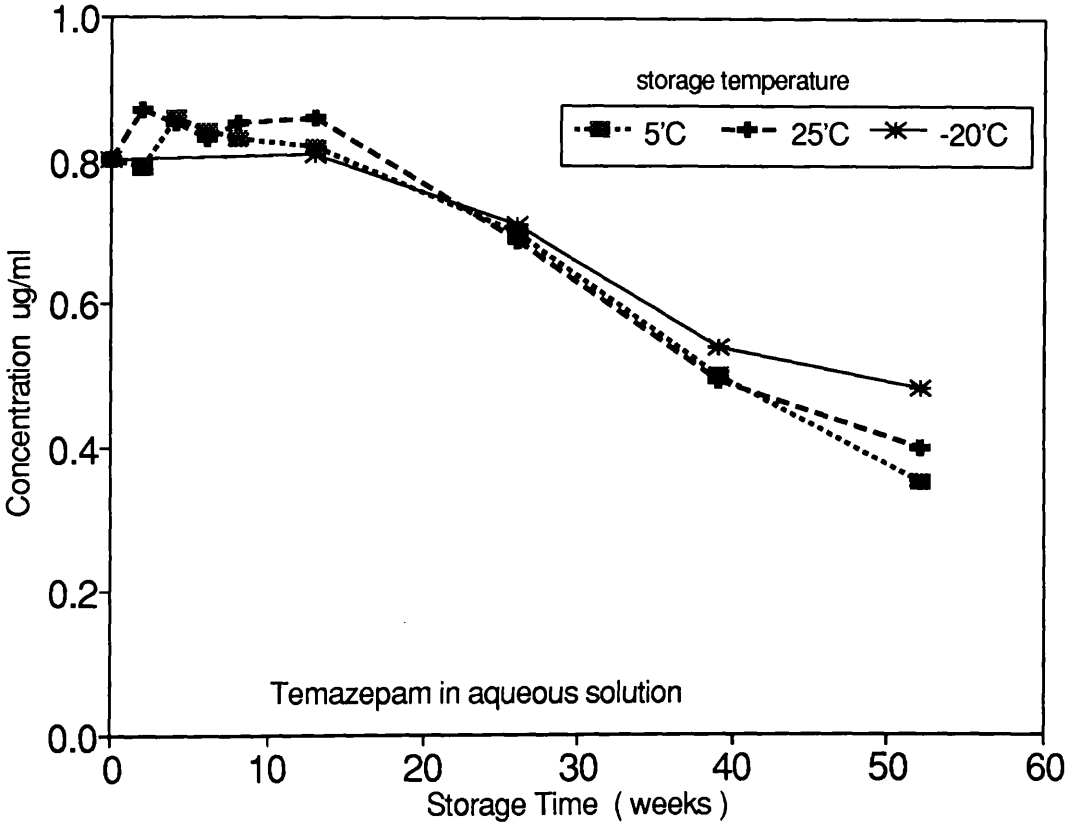


Figure 50. Changes in temazepam concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 52 weeks.

Temazepam (3-hydroxydiazepam) is one of the 1,4-benzodiazepine substitutes. It is a known metabolic product of diazepam. 1,4-benzodiazepine's substitutes are known to undergo hydrolysis in aqueous solution Figure 51 with the benzophenone as the major decomposition product [221, 222].

Conclusion

Although temperature has a marked effect on temazepam stability, after one year of storage the drug is still easily detected in blood regardless of the storage temperature, since samples stored at -20°C show the best recoveries and low decrease rate when compared with those stored at 5, 25°C regardless of the storage time.

Temazepam in water was shown to be stable for up to twenty-six weeks of storage regardless of the storage temperature with a recovery of 88%.

4.5.2.2 - Opiates

Morphine and buprenorphine are the chosen opiates in this study. Their stability when stored at the three different temperatures and time intervals were investigated.

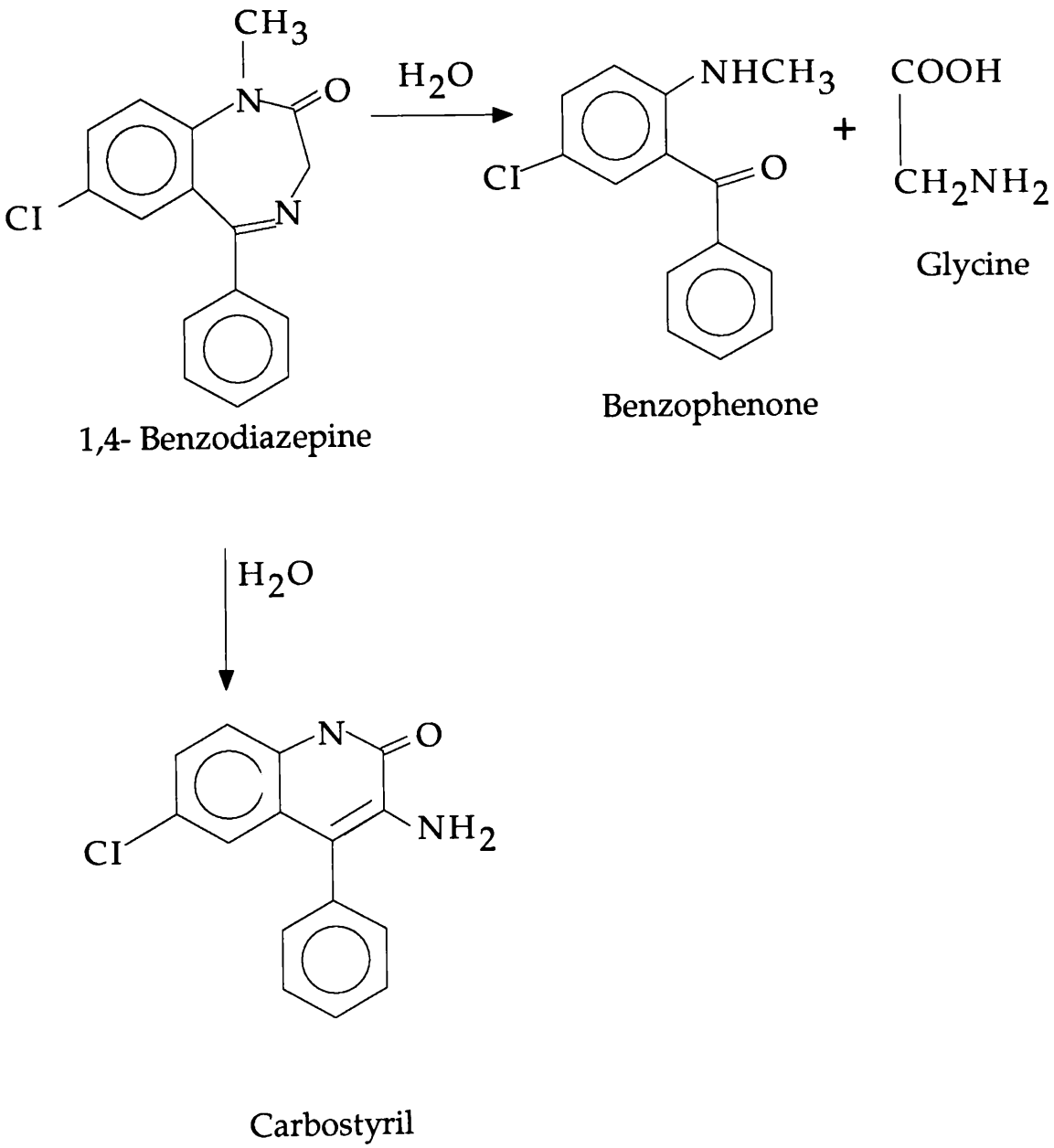


Figure 51. Degradation mechanism of 1,4 benzodiazepine in aqueous solution.

Morphine Stability

Samples of blood and water spiked with morphine were prepared in a concentration of 500 ng/ml and then stored and analysed according to the protocol described in [4.1]. For comparative purposes aqueous solutions of morphine were similarly prepared and studied. The samples were extracted as described in 3.7.2.2 and analysed by GC-MS using the described method in 3.5.2.2.

Result and Discussion

The morphine concentrations measured following storage for the designated times and at the set temperatures are presented in Table 36. The percentage standard deviations for the entire method for morphine analysis were $\pm$ 4% and $\pm$ 5% for blood and aqueous solution respectively.

TABLE 36.

The concentration of morphine (ng/ml) in blood and aqueous solution stored at 5, 25, -20°C from day zero up to 52 weeks.

Storage	Concentration when stored at 5°C		Concentration stored at 25°C		Concentration when when stored at -20°C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueous Solution
0	519	498	519	498	519	498
2	514	493	538	472		
4	525	512	532	411		
6	485	490	499	204		
8	456	487	498	230		
13	432	491	484	10	501	489
26	426	305	452	10	460	526
39	431	298	425	10	451	496
52	426	194	396	10	435	468

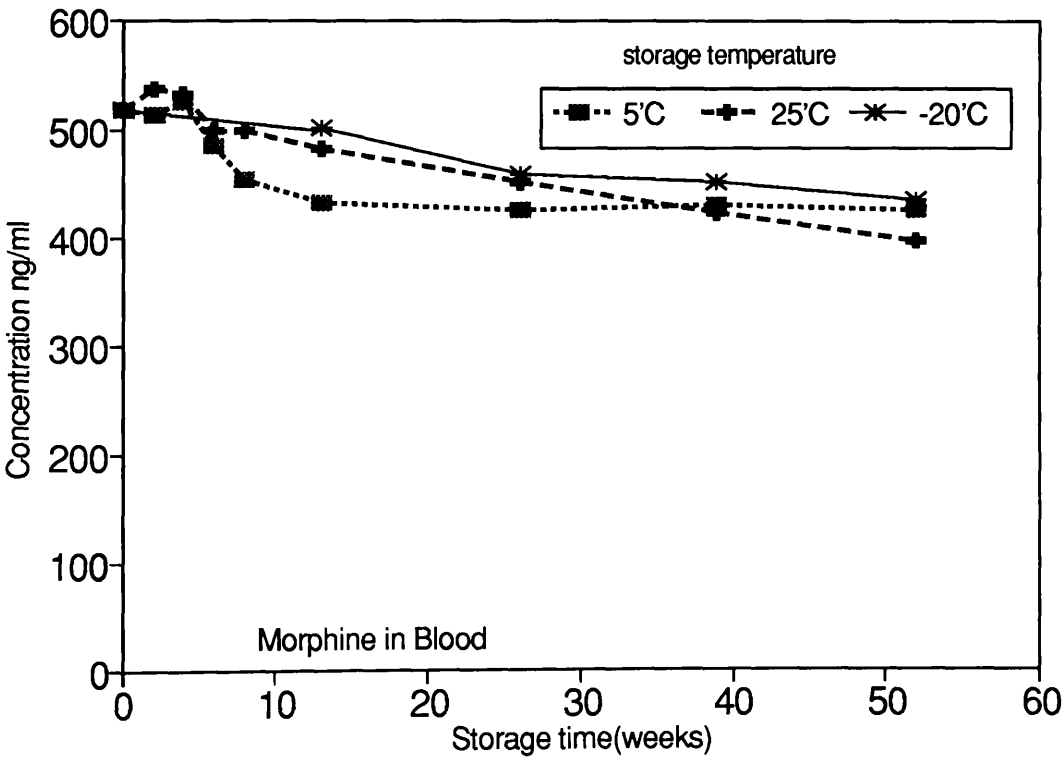


Figure 52. Changes in morphine concentration with time in blood stored at 5, 25, -20^{o}C from day zero to 52 weeks.

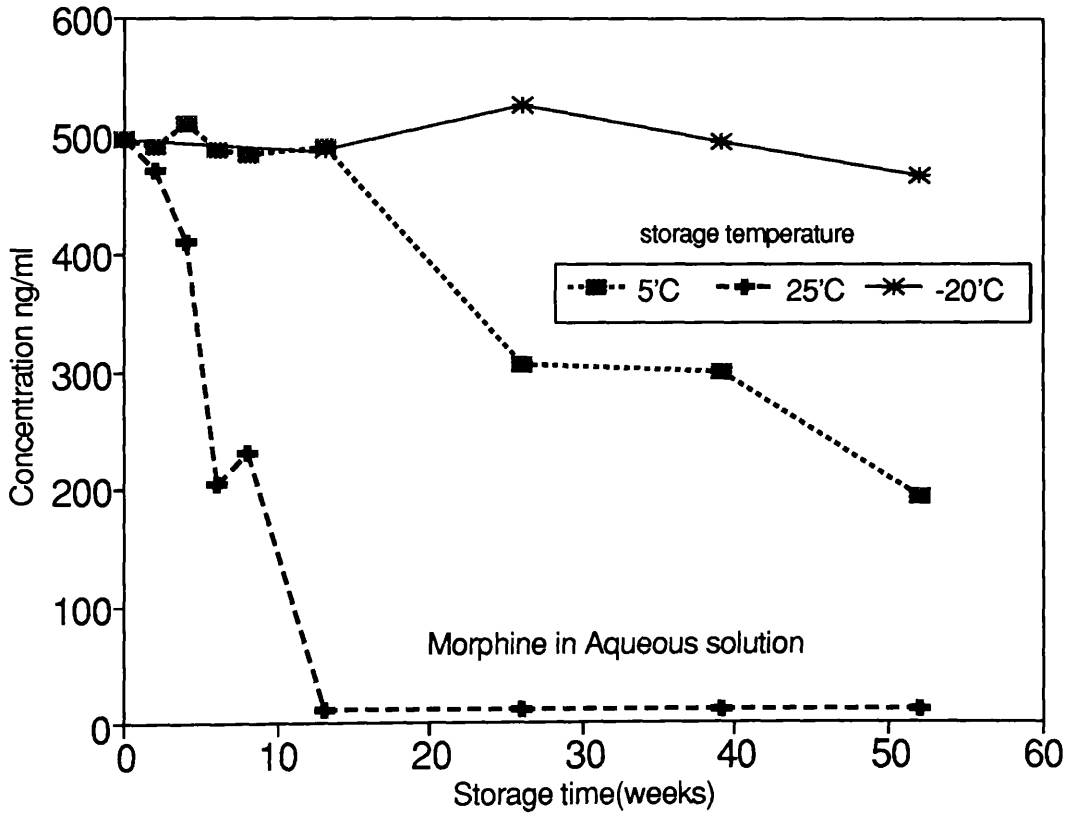


Figure 53. Changes in morphine concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 52 weeks.

The standard for significant breakdown of morphine to have occurred was a level measured which had decreased by greater than (+/-) 3.SD from day zero concentration.

A comparison between the measured blood and aqueous solution concentration stored at the three storage temperatures (5, 25, -20^{o}C) are displayed in Figures 52 and 53. The first significant decrease in blood morphine concentrations was found after 13 weeks of storage at 5^{o}C with a recovery of 83%. Figure 54 shows, however, that morphine in blood is relatively stable regardless of the storage temperature up to one year with recoveries of above 80 $\pm$ 4.8% measured. Morphine solutions are relatively stable at low pH values. It is known that post-mortem blood pH is slightly acidic which offers a good medium for morphine stability and as the pH goes up due to the formation of putrefactive amines, the rate of morphine degradation will increase. In this study, the formation of the putrefactive amines in the blood morphine samples were measured regularly at each designated time according to the drugs and putrefactive amine analysis protocol [4.1]. The amount of the four putrefactive amines present in morphine samples were shown to be minimal and around the detection limit of the developed methods. Only Indole showed a high concentration after 39 and 52 weeks of storage as discussed earlier (4.4.2.3). As a result, there were no increases in the pH of the blood because of the very limited formation of the putrefactive amines. The rate of morphine loss in blood was low (1.6 - 2.6 ng/ml/week) Table 38. In contrast, morphine stored in aqueous solution showed the effect of temperature on its stability with time. At a temperature of -20^{o}C morphine was shown to be very stable regardless of the storage time with a recovery of 99 $\pm$ 4.8% in all samples stored at -20^{o}C. As the temperature goes up the morphine stability in aqueous solution is affected dramatically. At 5^{o}C a steady decline of morphine concentration in water with time was observed.

The lowest recovery of 39% was reached after one year of storage. Morphine stored at a higher temperature 25^{o}C showed a sharp decline in morphine concentration to reach a recovery of 2% after thirteen weeks. The rate of morphine loss in aqueous solution was varied (0.4 - 40 ng/ml/week), Table 38. The instability of morphine at high temperatures has also been reported where morphine was estimated to have a half-life of 24 days at 25^{o}C and pH 6.0 [222].

Oxidation is the most important reaction contributing to the degradation of aqueous morphine solution. The phenolic moiety of morphine is known to be responsible for its instability in aqueous solution [222]. This group readily undergoes oxidation producing the major products pseudomorphine and morphine-N-oxide Figure 54. Morphine derivatives not possessing the free phenolic group as in the case of codeine and acetyl morphine, do not undergo this type of reaction [223]. As the temperature goes up the rate of degradation also increases. The decrease rate in morphine concentration shown in Table 38 indicated that morphine in aqueous solution has a higher rate compared with those of morphine in blood, and it also shows that the highest decrease rate was in morphine samples stored at a higher temperature, regardless of the storage media. Another factor affecting the stability of morphine solutions was the quality of the glass. Non-silanised glass allows the release of caustic materials increasing the pH and thus the rate of degradation. In this experiment, all the glass containers were silanised with dichlorosaline before use to minimise the effect of this phenomena.

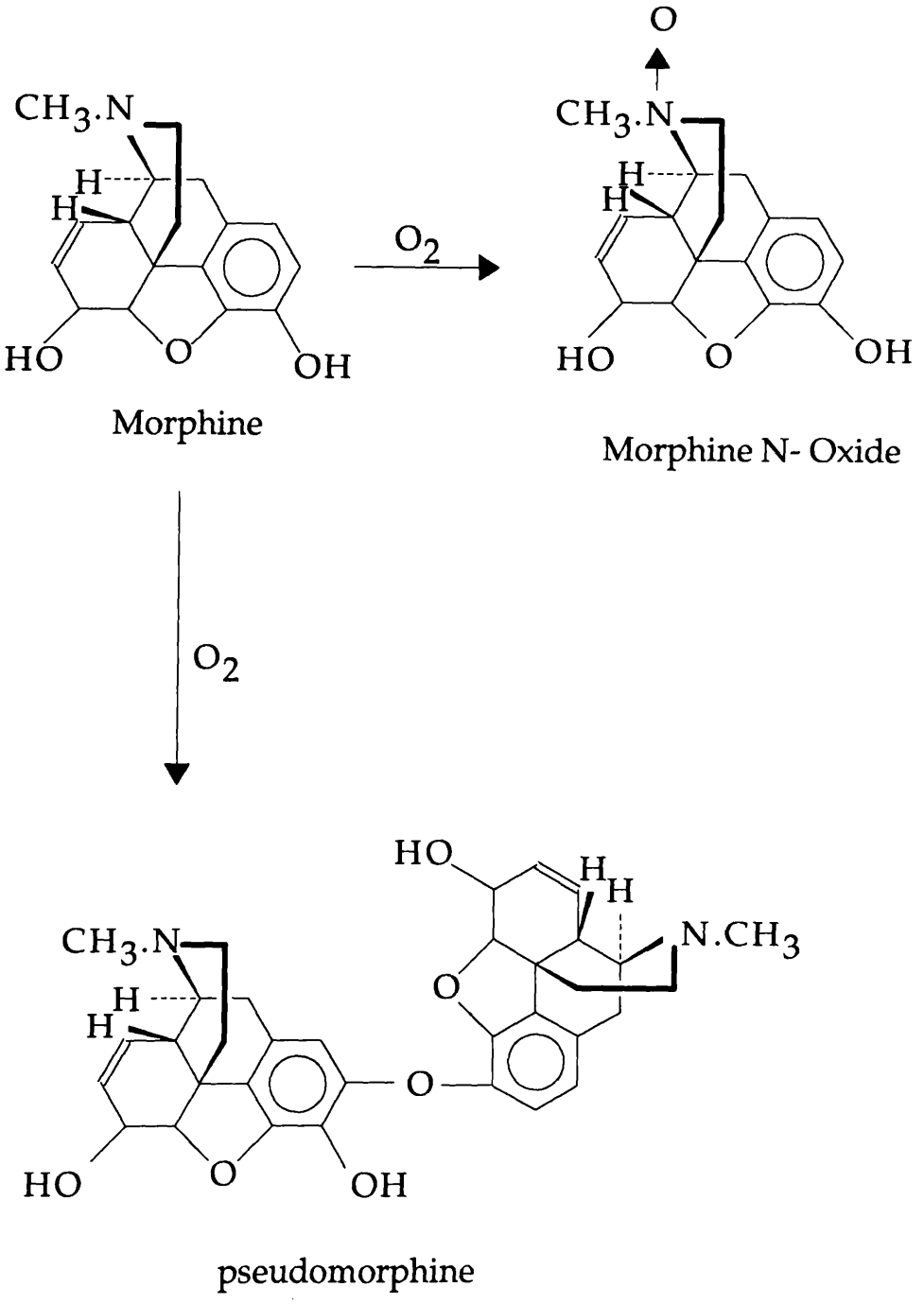


Figure 54. **Degradation mechanism of morphine in aqueous solution.**

Conclusion

Morphine in blood is shown to be reasonably stable regardless of the storage time and temperature with an expected recovery of 86 $\pm$ 4% and 80 $\pm$ 4.8% after six months and one year of storage respectively. Storage temperature showed a greater effect on morphine stability on aqueous solution samples. The recovery of morphine in aqueous solution varied with the storage time and temperature and ranged from 2-94%.

Buprenorphine Stability

Samples of blood and water spiked with Buprenorphine were prepared in a concentration of 250 ng/ml then stored and analysed according to the analysis protocol described, 4.1. The samples were extracted as described in 3.7.2.2 and analysed by GC-MS using the method described in 3.5.2.2.

Result and Discussion

Buprenorphine concentrations in blood and aqueous solution samples kept at the three storage temperatures for different periods of time were summarised in Table 37. The per cent standard deviation for buprenorphine analysis were 6.2% and 5.8% for blood and aqueous solution respectively.

The standard for significant breakdown of buprenorphine to have occurred was a level measured which had decreased by greater than ± 3. S.D from day zero concentration.

TABLE 37.

The concentration of buprenorphine (ng/ml) in blood and aqueous solution samples stored at 5, 25, -20°C from day zero up to 52 weeks.

Storage Time	Concentration when stored at 5°C		Concentration when stored at 25°C		Concentration when stored at -20°C	
Weeks	Blood	Aqueous Solution	Blood	Aqueous Solution	Blood	Aqueous Solution
0	270	249	270	249	270	249
2	284	252	280	232		
4	265	261	257	220		
6	270	260	262	206		
8	281	235	268	142		
13	281	240	253	159	286	251
26	257	120	207	150	271	261
39	239	132	192	148	247	255
52	221	108	185	145	268	247

A comparison between the recoveries of blood and aqueous buprenorphine kept at the three storage temperatures 5, 25 and -20°C from day zero to one year, is shown in Figures 55 and 56. A significant decrease in blood burprenorphine concentration was found only after six months of storage at 25°C. At the end of the experiment (one year) a reasonable amount of buprenorphine was recovered at the three storage temperatures and it was found to be in the range of 68 - 99%. Buprenorphine was expected to follow morphine degradation mechanism to some extent due to similarities in their structure. Buprenorphine and morphine have one phenolic moieties, which is known to be responsible for its instability. The decrease rates of buprenorphine in blood and aqueous solution samples are shown in Table 38.

The first significant decrease in buprenorphine concentration in aqueous solution was noticed only after eight weeks of storage at 25°C. On the other hand, samples stored at 5°C showed the first significant changes in buprenorphine concentration after six months, while samples stored at -20°C showed no significant changes in buprenorphine. At the end of the experiment, the buprenorphine recovery was in the range of 43 - 99%.

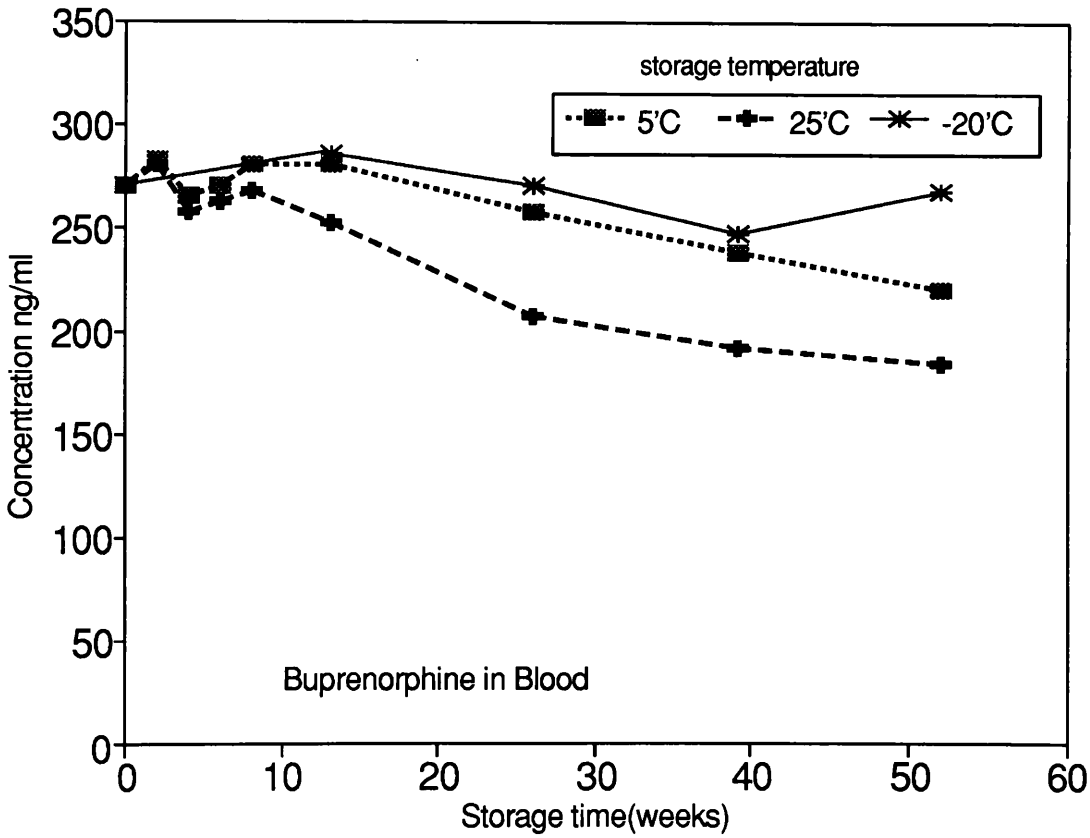


Figure 55. **Changes in buprenorphine concentration with time in blood stored at 5, 25, -20^{o}C from day zero to 52 weeks.**

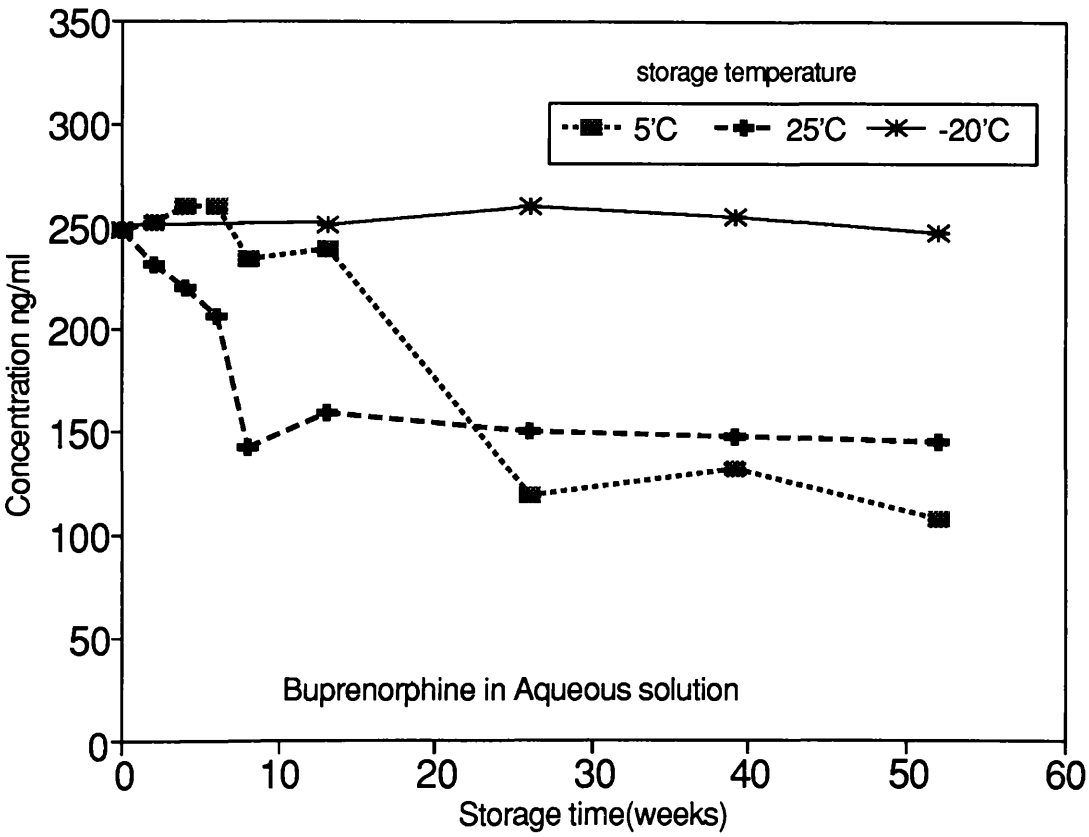


Figure 56. Changes in buprenorphine concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 52 weeks.

Conclusion

Storage temperatures have an effect on buprenorphine stability regardless of the storage time and the media as the temperature increased less buprenorphine recovered and the effect of media became important on the drug stability. Buprenorphine in aqueous solution was found to be less stable compared with those of blood for the same storage time and temperature.

TABLE 38.

The decrease rate in non-acid drugs concentration in blood and aqueous solution samples stored at 5, 25 and -20°C, from day zero up to 52 weeks.

Decrease Rate in Drugs concentration ng/ml/week when stored from day zero up to 52 weeks at:			
Drugs	5°C	25°C	-20°C
Temazepam in blood	8.3	9.5	3.0
Temazepam in aqueous solution	9.2	9.2	7.0
Morphine in blood	1.8	2.6	1.6
Morphine in aqueous solution	6.2	40*	0.41
Buprenorphine in blood	1.0	1.9	0.33
Buprenorphine in aqueous solution	3.3	1.6	0

***The high decrease rate is due to disappearance of morphine in aqueous solution only after three months of storage at 25°C.**

4.6 APPLICATIONS

4.6.1 - Interfering Substances in Post-mortem Samples

Eleven samples of post-mortem blood and two blank blood samples were analysed for the presence of acid and non-acid interfering substances according to the developed extraction and analysis methods described earlier in this thesis.

Result and Discussion

A summary of information related to the post-mortem samples analysed for the presence of Indole, 2-phenethylamine, Tyramine and Tryptamine, are shown in Table 39. The table shows the time of death, the date of receiving the samples for drugs screening and the time elapsed before the samples were analysed for the presence of putrefactive amines.

The concentration of four putrefactive amines in putrefied blank blood and the post-mortem blood samples was measured. Figure 57 shows that a high concentration of acid and non-acid interfering substances were observed in the putrefied blank blood samples stored at room temperature in open containers for two months. The two blank blood samples show different concentrations of interfering substance even when both were stored under the same conditions. These blank blood samples were from difference sources.

The formation of the four putrefactive amines in post-mortem cases stored for different time intervals between eight weeks to thirty-seven weeks was monitored. The post-mortem cases under investigation were usually stored at 5^{o}C for a few days until a full toxicological investigation was completed then stored at -20^{o}C. Table 39 shows the time elapsed before analysing the samples for the presence of Indole, 2-phenethylamine, Tyramine and Tryptamine.

TABLE 39.

A summary of the post-mortem case analysed for putrefactive amines.

	Time of Death	Post-mortem Date	Time Received for Toxicology	Toxicology Result for Drugs	Weeks elapsed when analysed for putrefactive amines
1	4.6.90	4.6.90	4.6.90	Positive	37
2	11.6.90	11.6.90	11.6.90	Positive	36
3	12.6.90	12.6.90	12.6.90	Positive	36
4*	18.7.90	18.7.90	27.7.90	Positive	31
5	1.5.91	2.5.91	3.5.91	Positive	11
6	15.5.91	16.5.91	17.5.91	Positive	9
7	14.8.90	14.8.90	17.8.90	Positive	26
8	3.9.90	5.9.90	6.9.90	Positive	24
9	20.5.91	20.5.91	20.5.91	Positive	8
10	29.11.90	30.11.90	30.11.90	Positive	12
11	22.5.91	22.5.91	22.5.91	Positive	8

*** Post-mortem conducted by External Pathologist.**

The results given in Figure 58 show a different concentration of the four putrefactive amines. This appears to be independent of the storage time. The figures also show that storing the samples at -20°C did not prevent the formation of the four putrefactive amines but reduced the rate of formation.

No significant correlation could be found between the production of the four putrefactive amines so it is probable that independent mechanisms of production exist. The study shows that the blank blood samples stored at room temperature in open containers for nine weeks resulted in a high concentration of the four putrefactive amines compared to post-mortem blood stored in closed containers at 5 and -20°C. The concentration of putrefactive amines in open containers were 10 - 27 times higher than those of post-mortem cases stored at 5 and -20°C in a closed container for different periods of time. The high concentration of the four putrefactive amines in blood samples stored in open containers could be due to samples being exposed to all types of bacteria which are known to be responsible for the progress of the putrefactive process.

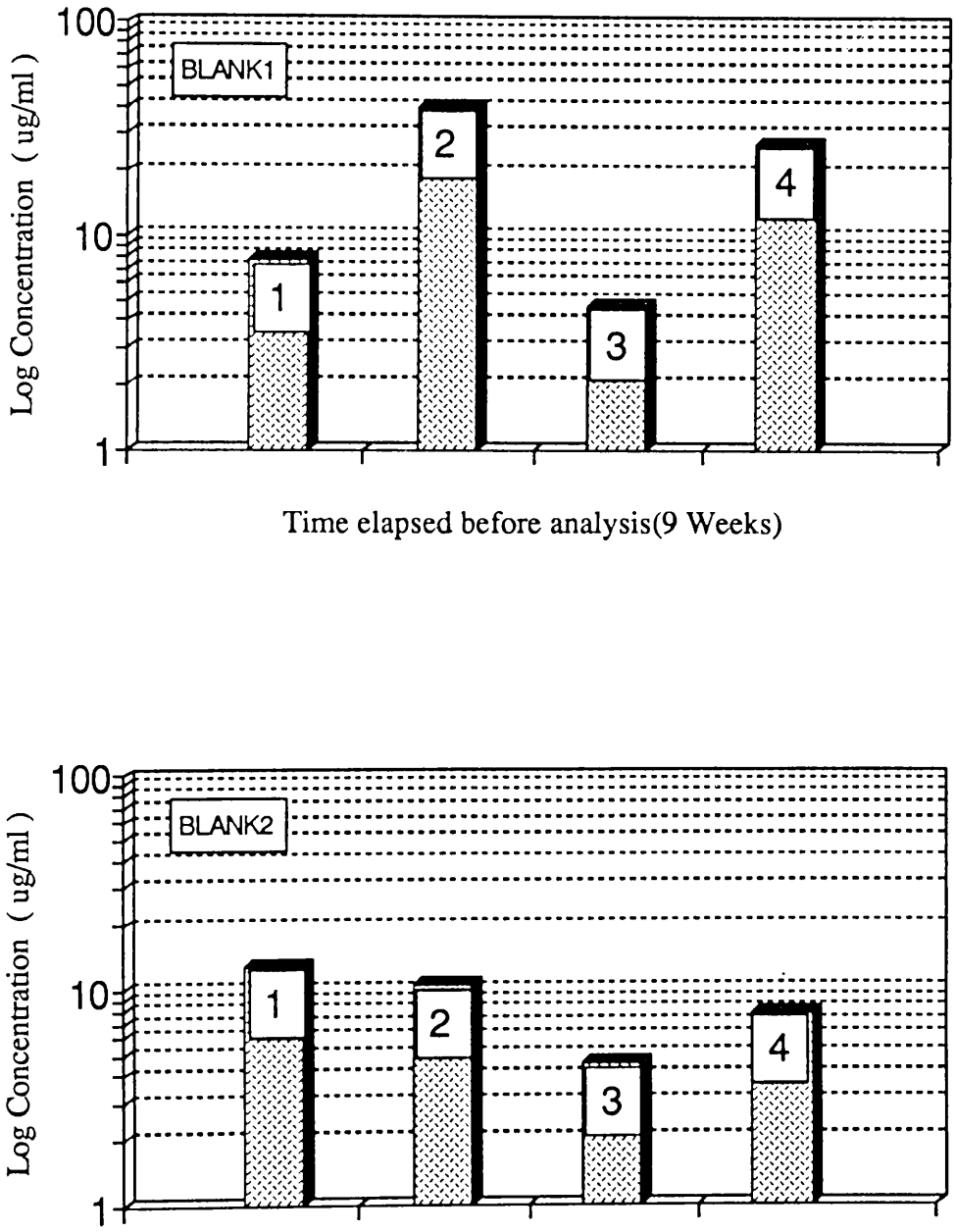
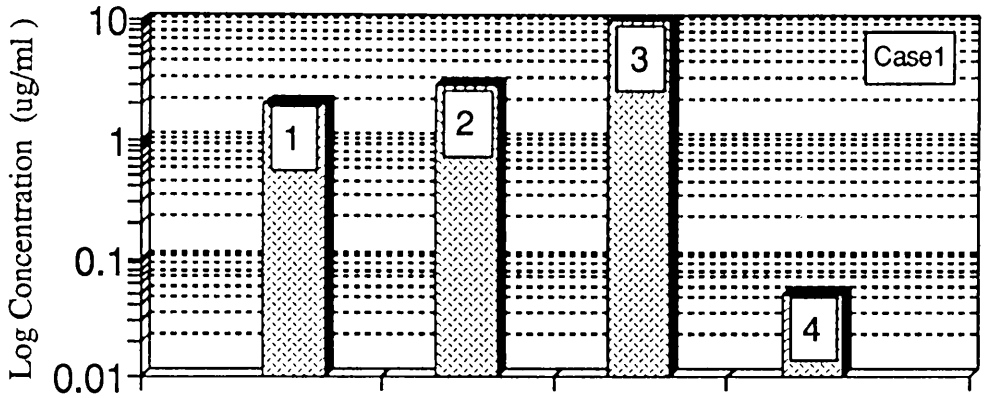
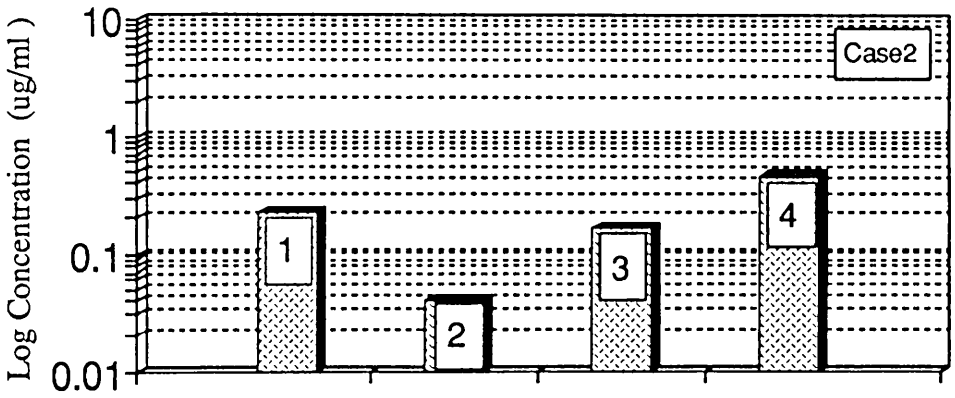


Figure 57. The concentration of the four putrefactive amines in blank blood samples stored at room temperature in open container for two months.

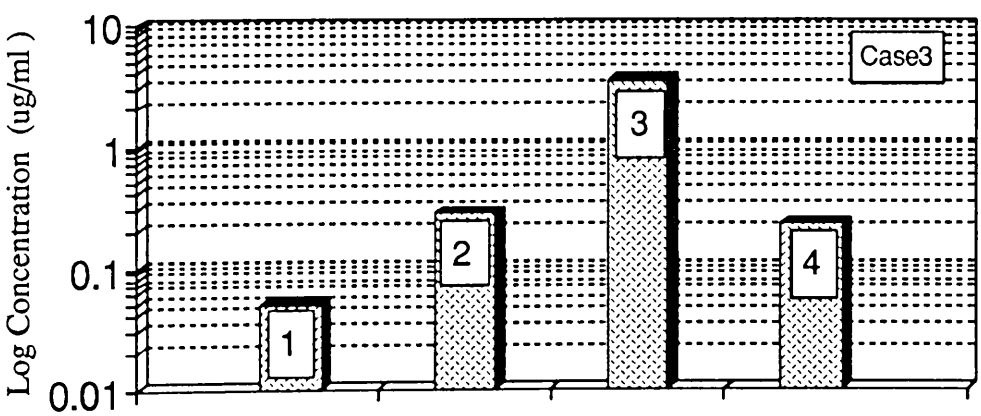
1. Indole. **2. 2-phenethylamine.**
3. Tyramine. **4. Tryptamine.**



Time elapsed before analysis(37 weeks)



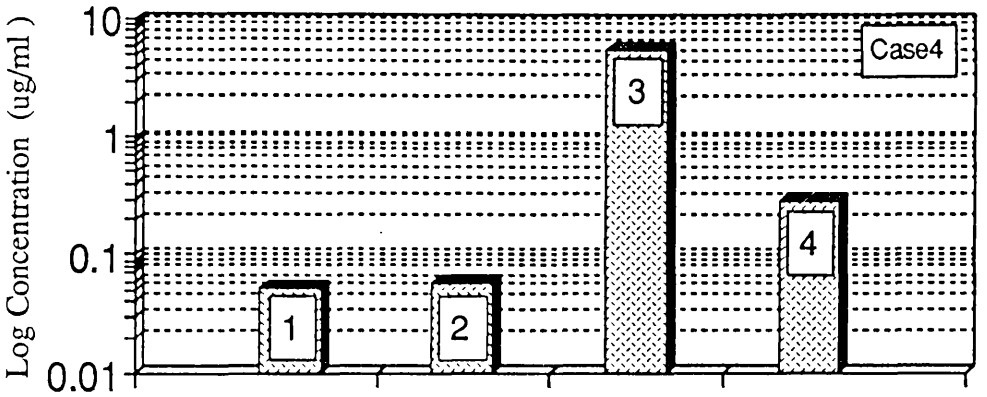
Time elapsed before analysis(36 Weeks)



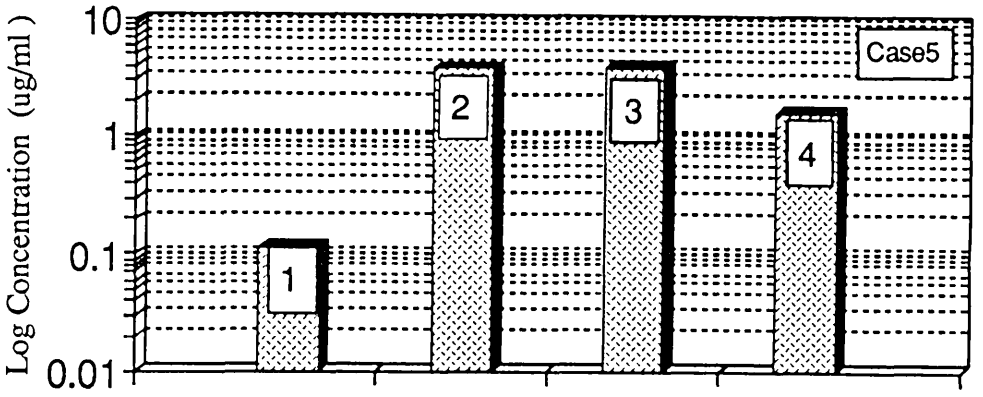
Time elapsed before analysis(36 Weeks)

Figure 58. The concentration of the four putrefactive amines in post-mortem blood samples.

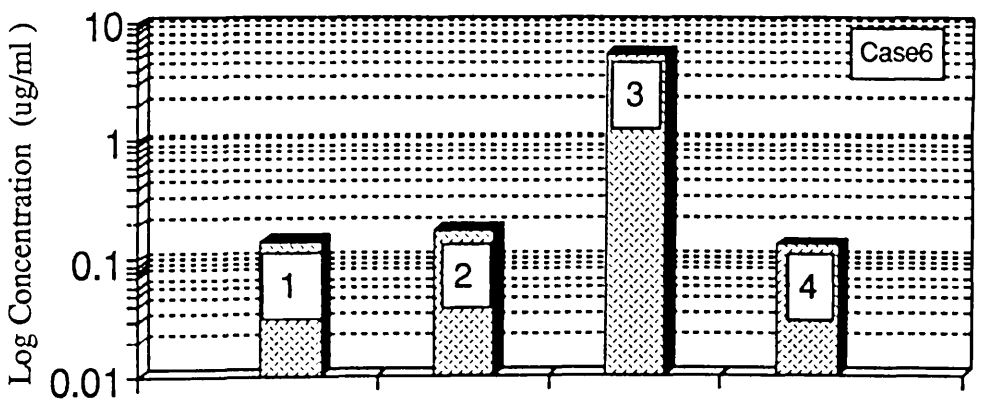
1. Indole. **2. 2-phenethylamine.**
3. Tyramine. **4. Tryptamine.**



Time elapsed before analysis(31 Weeks)

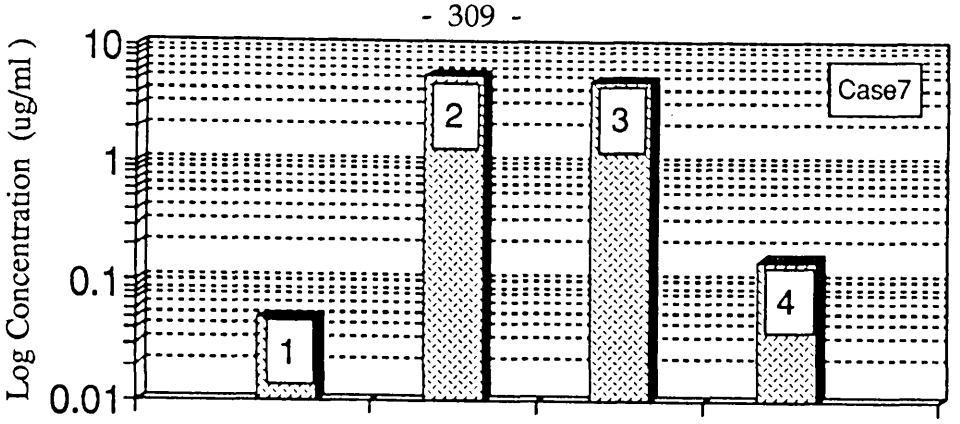


Time elapsed before analysis(29 Weeks)

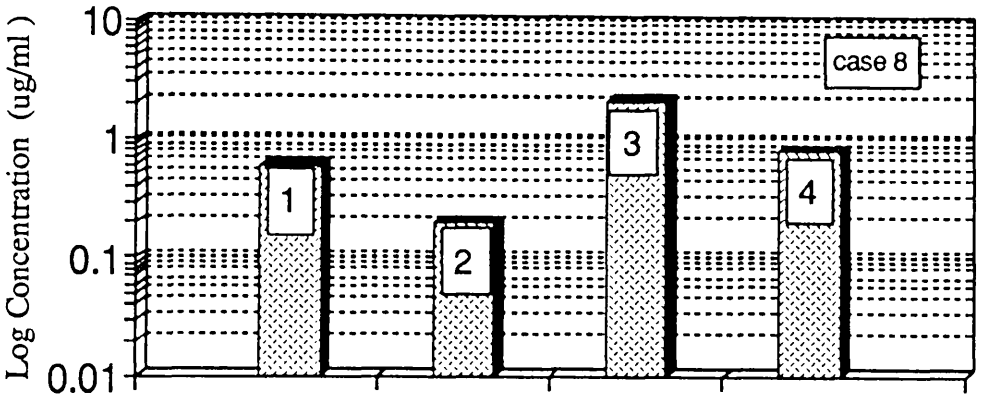


Time elapsed before analysis(9 Weeks)

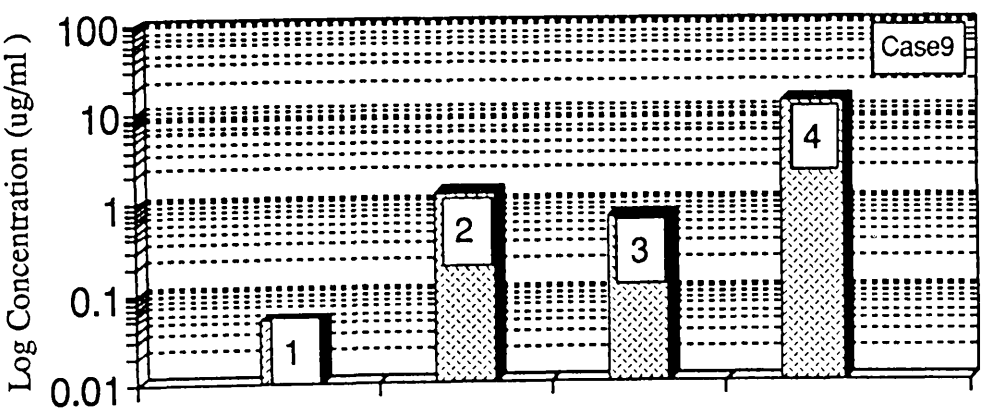
Figure .58 (continued) **The concentration of the four putrefactive amines in post-mortem blood samples.**



Time elapsed before analysis(26 Weeks)



Time elapsed before analysis(24 Weeks)



Time elapsed before analysis(8 Weeks)

Figure 58 (continued) **The concentration of the four putrefactive amines in post-mortem blood samples.**

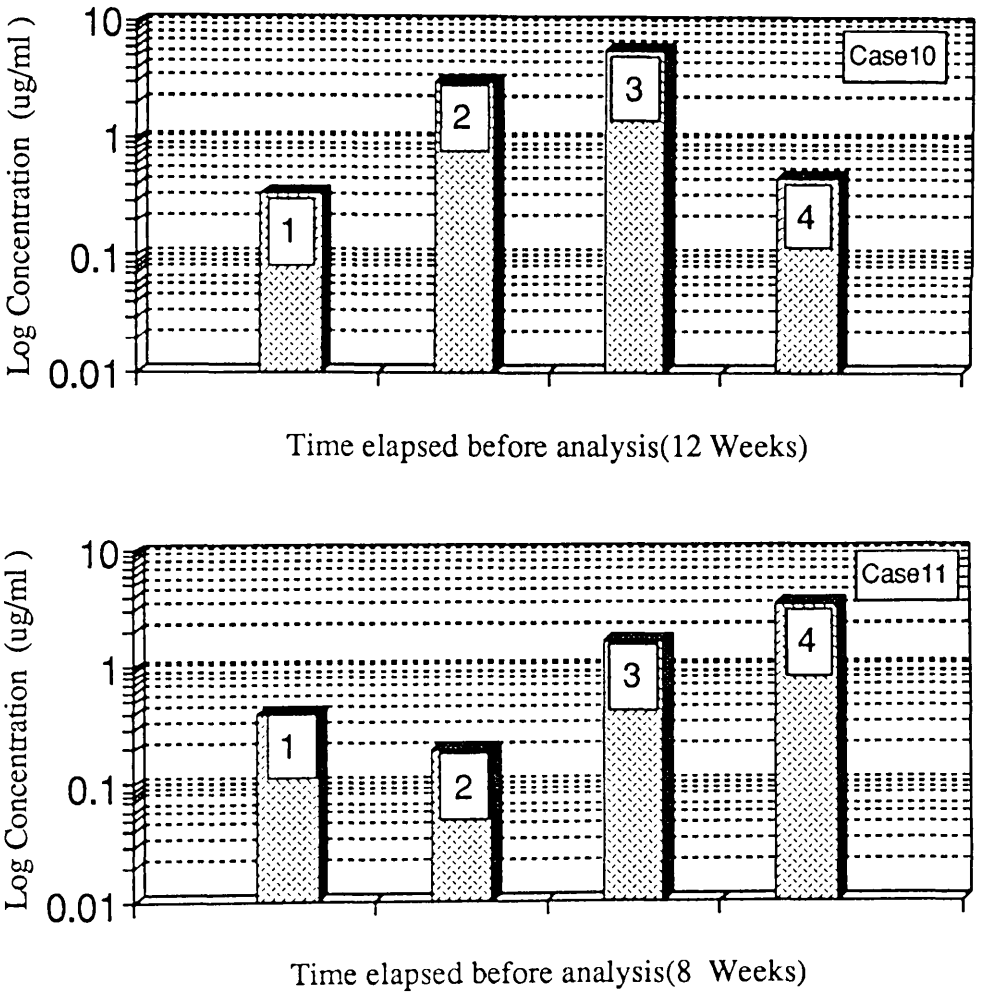


Figure 58 (continued) **The concentration of the four putrefactive amines in post-mortem blood samples.**

Conclusion

The investigation showed no relation between the production of different putrefactive amines and storage time in post-mortem samples stored in closed containers. On the other hand blank blood samples stored in open containers at room temperature showed higher concentrations of putrefactive amines compared to those of post-mortem samples stored at -20^{o}C for longer periods of time. This could be explained by the samples being exposed to different types of bacteria which appeared to be the main factor in promoting putrefaction. The speed of putrefactive decomposition was considerably reduced in post-mortem samples stored at -20^{o}C in a closed container.

4.6.2 - Analysis of Drug Positive Post-mortem Samples

Table 40 gives a summary of information on eleven post-mortem samples which were found to be positive for the drugs under investigation. The concentration of drugs in post-mortem samples was predicted using the decrease rates of acid and non-acid drugs concentration with time at a given storage temperature, Tables 34, 38. The predicted drug concentration after being stored for '**W**' weeks at a given storage temeprature calculated as follows:

$$P = D \times W \qquad \text{Equation 17}$$

When **P** is the predicted drug concentration, **W** is the storage time 'weeks' and **D** is the adjusted decrease rate (ng/ml/week) for a given drug at a given temperature. **D** can be obtained from the following equation:

$$D = \frac{R \times C}{S} \qquad \text{Equation 18}$$

Where **R** is the decrease rate (ng/ml/week) in spiked blood drug at a given temperature Tabes 34, 38, while **C** is the measured concentration of the drug in the post-mortem sample at the day of receiving the sample and **S** the concentration of the drug spiked blood at day zero which was obtained from Tables 31, 32, 33 in case of acid drugs and from Tables 35, 36, 37 in the case of non-acid drugs. The above two equations were used to calculate the predicted concentration in the eleven post-mortem samples, as a trial to examine the usefulness of the decrease rates obtained in this study.

TABLE 40.
A summary of the post-mortem cases re-analysed for drugs concentration.

Case No.	Time of Death	Date Received	Cause of Death	Drugs Present	Weeks elapsed before re-analysis
1	4.6.90	4.6.90	Liver Failure	Anticonvulsant	27
2	11.6.90	11.6.90	Drug Overdose	Benzodiazepine and Flupenthixol	26
3	12.6.90	12.6.90		Opiates	26
4*	18.7.90	27.7.90	Drug Overdose	Benzodiazepine Chlormethiazole Alcohol	19
5	1.5.91	3.5.91	Epileptic seizure	Anticonvulsant	11
6	10.8.91	13.8.91	Drug Overdose	Opiates	18
7	14.8.90	17.8.90	Drug Overdose	Opiates	17
8	3.9.90	6.9.90	Drug Overdose	Opiates, Benzodiazepine	13, 14
9	20.5.91	20.5.91	Epileptic seizure	Anticonvulsant	8
10	26.11.90	30.11.90	Acute Left Ventricular Failure	Benzodiazepine, Prothiaden Alcohol	2
11	22.5.91	22.5.91	Epileptic seizure	Anticonvulsant	8

*** Post-mortem conducted by External Pathologist.**

4.6.2.1 - Acid Drugs

Anticonvulsants

Four samples of post-mortem blood were found to be positive for phenobarbitone, carbamazepine and phenytoin. The samples were usually kept at 5°C until the full drug screening test was finished then the sample were stored at -20°C for a few months before being re-analysed for the concentration of drugs to investigate the stability of anticonvulsant drugs in post-mortem blood. The anticonvulsant drugs were extracted using the method described in 3.7.1.1 and were analysed by the HPLC system described in 3.5.1.1.

Results and Discussion

Table 41 shows the concentration of phenobarbitone, carbamazepine and phenytoin at the time of receiving the samples from the mortuary and the

TABLE 41.

Post-mortem acid drug samples re-analysed for phenobarbitone, carbamazepine, phenytoin concentration after being stored for different time intervals.

Case	Initial Concentration ug/ml	Storage Time and Temperature	Concentration after storage ug/ml	Adjusted Decrease Rate ng/ml/week	Predicted Concentration ug/ml	Predicted Recovery	Actual Recovery
1	**Phenobarbitone** 5 ± 0.42	27 weeks at $-20^{o}C$	3.93 ± 0.33	53	3.57	71%	78%
	Phenytoin 5 ± 0.43		2.45 ± 0.21	56	3.48	69%	49%
5	**Carbamazepine** 46 ± 4.3	11 weeks at $-20^{o}C$	27 ± 2.56	511	40.38	87%	59%
9	**Phenytoin** 18.3 ± 1.6	8 weeks at $-20^{o}C$	15.2 ± 1.3	2.5	16.66	91%	83%
11	**Carbamazepine** 3.1 ± 0.30	8 weeks at $-20^{o}C$	2.68 ± 0.25	34	2.82	91%	86%

concentration of the drugs after being left for a few days at 5°C then stored at -20°C for several weeks before being analysed again for the concentration of each drug.

Phenobarbitone sample (Case 1), Table 41, shows agreement between the concentration of phenobarbitone analysed after 27 weeks and the predicted concentration using the decrease rate in phenobarbitone concentration in blood samples stored at -20°C, Table 34. With phenytoin and carbamazepine (Case 1, 5, 9, 11) there was agreement between the predicted concentration using the decrease rate in carbamazepine and phenytoin concentration with time in blood samples stored at -20°C, Table 34, and the authentic concentration after storage only in 50% of the samples, Table 41.

4.6.2.2 Non-Acid Drugs

Benzodiazepine

Post-mortem blood samples found to be positive for temazepam were stored at -20°C for several weeks before being re-analysed for temazepam concentration. The storage time varied in the range of two weeks to twenty-six weeks. Post-mortem blood was extracted for the presence of benzodiazepine using the method described in 3.7.1.1 and was analysed by the HPLC system described in 3.5.2.1.

Results and Discussion

Table 42 shows the concentration of temazepam after being stored for different storage periods.

The cause of death in all of the cases was drug overdose. The drugs involved were benzodiazepine and flupenthixol in Case 2, benzodiazepine, chlormethiazole and alcohol in Case 4, opiates and benzodiazepine in Case 8. In Case 10, the cause of death was acute left ventricular failure with positive screening tests for benzodiazepine, prothiaden and alcohol.

In Table 42, Cases 4 and 10 show a significant difference in temazepam concentration between the predicted concentration using the decrease rate in blood temazepam concentration with time when stored at -20^{o}C at 5^{o}C while no significant difference within the method percent standard deviation was noticed between the predicted concentration and the measured concentration in Cases 2 and 8, Table 42.

The predicted concentration was calculated by multiplying the adjusted 'decrease rate' (D) in blood temazepam concentration stored at 5, 25 and -20^{o}C by the number of weeks the sample 'case' were stored, equation 17. The decrease rate in the study based on initial temazepam concentration of 0.84 ug/ml on day zero which is different from the initial concentration of authentic post-mortem cases, the adjusted decrease rate was calculated using equation 18. Therefore, each case has its adjusted decrease rate which was used to calculate the predicted temazepam concentration of each case.

TABLE 42.

Post-mortem benzodiazepine samples re-analysed for temazepam concentration after being stored for different time intervals.

Case	Initial Concentration ug/ml	Storage Time and Temperature	Concentration after storage ug/ml	Adjusted Decrease Rate ng/ml/week	Predicted Concentration ug/ml	Predicted Recovery	Actual Recovery
2	0.93 $\pm$ 0.045	26 weeks at -20^{o}C	0.8 $\pm$ 0.038	3.3	0.84	90%	86%
4	0.81 $\pm$ 0.039	19 weeks at -20^{o}C	0.99 $\pm$ 0.045	2.89	0.755	93%	122%
8	1.5 $\pm$ 0.072	14 weeks at -20^{o}C	1.49 $\pm$ 0.072	5.36	1.42	95%	99%
10	1.91 $\pm$ 0.092	2 weeks at 5^{o}C	2.36 $\pm$ 0.11	18.87	1.87	98%	123%

In Case 10 a high temazepam concentration was noticed when samples were stored at 5°C for two weeks. This could be explained as a result of temazepam being released from temazepam bound to protein.

Opiates

Four post-mortem cases were found to be positive for morphine. The samples were usually kept at 5°C until the full drug screen test was finished. The samples were then stored at -20°C for a few weeks before being re-analysed for morphine concentration. Post-mortem blood was extracted and was analysed for the presence of morphine using the method described in 3.8.2.2.

Result and Discussion

Table 43 shows no significant difference within the method per cent standard deviation between the concentration of morphine in samples analysed after 18 and 13 weeks. Cases 6, 8 and the predicted concentration, while in Cases 3 and 7 the morphine concentrations after 26 and 27 weeks of storage was found to be about 50% lower than the predicted concentration using the decrease rate.

TABLE 43.

Post-mortem opiates samples re-analysed for morphine concentration after being stored for different time intervals.

Case	Initial Concentration ug/ml	Storage Time and Temperature	Concentration after storage ug/ml	Adjusted Decrease Rate ng/ml/week	Predicted Concentration ug/ml	Predicted Recovery	Actual Recovery
3	5.2 ± 0.21	26 weeks at $-20^{o}C$	2.45 ± 0.1	16	4.78	92%	47%
6	0.34 ± 0.013	18 weeks at $-20^{o}C$	0.32 ± 0.013	1.05	0.321	94%	94%
7	$0.13 \pm 5.2 \times 10^{-3}$	17 weeks at $-20^{o}C$	$0.063 \pm 2.5 \times 10^{-3}$	0.4	0.123	94%	48%
8	$0.175 \pm 7.10 \times 10^{-3}$	13 weeks at $-20^{o}C$	$0.17 \pm 6.8 \times 10^{-3}$	0.54	0.167	96%	97%

Conclusion

Using the decrease rate in drug concentration to predict the authentic concentration of drug after being stored for a certain period of time at any of the three storage temperatures (5, 25, -20^{o}C) showed limited usefulness (50% agreement between the predicted and the authentic drug concentration). Increasing the number of post-mortem samples tested by this method could give more information on the agreement between the predicted concentration and the authentic concentration.

4.7 - THE EFFECT OF INTERFERING SUBSTANCES ON DRUG ANALYSIS

4.7.1 - Acid Drugs

Anticonvulsants

A standard solution of acid drugs containing the nine drugs were extracted as described in 3.7.1.1 20 ul was injected to the HPLC system which was usually used to analyse these drugs (described earlier 3.9.1). The four putrefactive amines were spiked in blank blood to give a concentration of 10 ug/ml and extracted using acidic drugs method, then analysed on the same HPLC system to establish their retention times.

Results and Discussion

Comparing the retention times of acidic drugs, Table 44, with the retention times of the four putrefactive amines, Table 45 showed a possibility of interferences from Indole with the qualitative and quantitative analysis of carbamazepine and amylobarbitone, since the difference in retention time between Indole and amylobarbitone is only 0.1 minutes while carbamazepine and Indole have the same retention times of 11.0 minutes. Ten ug/ml. of Indole could give a false positive of 21.12 ug/ml carbamazepine and 64.45 ug/ml amylobarbitone. The presence of Indole in putrefied blood analysed by HPLC for carbamazepine and amylobarbitone could give false positives or a four-fold increase of the authentic

TABLE 44.

The retention times of the eight acidic drugs and Internal Standard Butalbital on acid drugs HPLC.

Drug	Retention Times Minutes	Concentration ug/ml	*Peak Height Ratio
Primidone	4.27	12.42	1.97
Phenobarbitone	6.17	10.98	1.4
Butobarbitone	7.82	10.20	0.76
Butalbital (I.S)	8.29	5.76	-
Phenytoin	10.21	11.63	0.98
Carbamazepine	11.0	5.54	0.96
Amylobarbitone	11.1	13.39	0.76
Methaqualone	13.86	11.68	0.28
Quinalbarbitone	14.54	11.15	0.87

* Peak height ratio = drug peak height/butalbital (I.S) peak height

concentration of each drug. Therefore in the case of putrefaction post-mortem samples shown to be positive for carbamazepine or amylobarbitone by HPLC system another method such as GC or GC/MS should be used to confirm the HPLC results.

4.7.2 - Non-Acid Drugs

The possibility of interference between benzodiazepine and the four putrefactive amines has been investigated. The other group of non-acid drugs, 'opiates' is omitted because the method used to analyse the opiates in the study was GC/MS which provides unquivocal identification of morphine and buprenorphine with selective ion recording.

TABLE 45.

The retention times of the four putrefactive amines on acidic drug HPLC system.

Putrefactive Amines	Retention Time minutes	Concentration ug/ml	Peak Height Ratio	Comments
2-phenethylamine	11.29	10	0.05	Broad peak
Tyramine	3.93	10	0.13	Sharp peak
Tryptamine	10.49	10	0.03	Broad peak
Indole	11.0	10	3.66	Sharp peak

* The peak height ratio = putrefactive amine peak height/butalbital (I.S) peak height

Benzodiazepine

The standard benzodiazepines solution was injected into the HPLC system described in 3.9.2 at a flow rate of 1.5 ml/min.

Result and Discussion

The retention times of the five benzodiazepines and the internal standard are shown in Table 46. A separate analysis of each of the four putrefactive amines on the benzodiazepine HPLC system was conducted. The retention times of the four putrefactive amines are shown in Table 47.

Comparing the retention times of the five benzodiazepines and the four putrefactive amines, Tables 46 and 47, show that two of the four putrefactive amines eluted before the first benzodiazepine 'triazolam' while 2-phenethylamine did not elute from the column. Tryptamine has a retention time close to chlordiazepoxide but the possibility of a false positive chlordiazepoxide due to the presence of tryptamine is very slim, since chlordiazepoxide shows a good sharp peak with peak width of 0.2 cm while tryptamine shows a broad peak with peak width of 3.0 cm.

TABLE 46.

The Retention Times of Five Benzodiazepines and Internal Standard Prazepam on benzodiazepines HPLC System.

Benzodiazepines	Retention Times Minutes	Concentration ug/ml	Peak Height cm at AUFS = 0.16
Triazolam	5.9	2	7.6
Temazepam	7.1	2	7.0
Chlordiazepoxide	7.6	2	1.0
Desmethyldiazepam	8.3	2	9.3
Diazepam	9.2	2	8.1
Prazepam (I.S.)	15.7	2	4.2

TABLE 47.

The Retention Times of the Four Putrefactive Amines on Benzodiazepines HPLC System.

Putrefactive Amines	Retention Time minutes	Concentration ug/ml	Peak Height cm at AUFS=0.16	Comments
2-phenethylamine	-	50	-	Not Eluted
Tyramine	3.8	50	13.0	Sharp peak
Tryptamine	7.8	50	1.6	Broad peak
Indole	5	50	12.5	Sharp peak

Conclusion

Indole would be considered as a major interfering putrefactive amine with carbamazepine and amylobarbitone determination by HPLC. The possibility of interferences between benzodiazepines and the four putrefactive amines were found to be minimal.

CONCLUSIONS

a) Drugs Stability

The study was primarily concerned with the stability of drugs in putrefied biological material and was conducted for periods of time under three storage conditions. In this work the stability of some commonly encountered anticonvulsant, benzodiazepine and opiate drugs spiked in blood, was investigated for over one year of storage at three different temperatures (5, 25 and -20^{o}C). Phenytoin was found to be very stable over a period of six months with above 90% recovered regardless of the storage temperature. Phenobarbitone and carbamazepine showed a steady but slow decline in their concentration with time up to six months, when at least above 70% of the drugs were recovered.

The non-acid drugs, temazepam, morphine and buprenorphine were also found to be very stable under the storage conditions studied, with a very slow steady decline in their concentration with time up to six months. At least 85% of the drugs were recovered regardless of the storage temperatures. The storage temperatures showed an obvious effect on drugs stability in aqueous solution. 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 important information for forensic toxicologist to assist with the interpretation of results even in one year-old samples.

b) Putrefactive Amines Formation

It was found that the formation of putrefactive amines was dramatically retarded in blood spiked with the drugs under study when stored at 5, 25 and -20°C in sealed vials. Post-mortem blood samples showed that a reasonable amount of the four putrefactive amines were found. Storing the post-mortem blood at -20°C did retard the rate of formation of the putrefactive amines but no significant correlation could be found between the production of the four putrefactive amines with the storage time in the eleven post-mortem samples. On the other hand, the formation of the four putrefactive amines in blood samples stored at room temperature in open containers even for a short period of time showed 10 - 27 times higher concentration of the four putrefactive amines. This is probably due to the blood samples stored in open containers being exposed to different types of airbone bacteria which appeared to be the main factor in promoting putrefaction.

c) Methods of Analysis and Extraction of Acid and Non-Acid Substances

Acid interfering substance "Indole" is normally determined by measuring the ultraviolet absorbence of Indole in acidic or basic aqueous solution which contains other endogenous compounds. A method to analyse Indole by reversed-phase HPLC which separated Indole and the internal standard (5-chloroIndole) in less than 15 minutes was developed. The system was sufficiently sensitive for the determination of Indole in putrefied blood samples.

In the literature, no methods have been reported for the simultaneous analysis of 2-phenethylamine, Tyramine and Tryptamine using normal phase HPLC. Methods using reversed-phase separation with gradient elution or ion pairing agents have

been described. The analysis times, however, are unacceptably long (>15 minutes to separate the three putrefactive amines. In this study a method was developed to analyse the three putrefactive amines simultaneously using normal-phase HPLC. The separation was achieved in less than five minutes with sufficient sensitivity to determine the presence of the three putrefactive amines in putrefied blood samples.

Indole was usually extracted from blood using liquid-liquid extraction which is a tedious and time-consuming method. The extraction of Indole from putrefied blood was explored using methanol/isopropanol for protein precipitation and bonded sorbents. It showed that Indole could be efficiently extracted using non-polar sorbents. On the other hand, extracting the non-acid interfering substance from putrefied blood was usually achieved by liquid-liquid extraction. In the literature, there is some data regarding extraction of putrefactive amines from serum and urine using solid-phase sorbent. These methods have their limitations since post-mortem blood can not be applied directly on to solid-phase sorbent and the methods are not capable of extracting the three putrefactive amines simultaneously. Extracting the three putrefactive amines from putrefied blood was explored using Extrelut^{R} (diatomaceous earth) and different solid-phase sorbents. Ion-exchange sorbents showed the best efficiency to extract the three putrefactive amines. The ion exchange sorbent was shown to be suitable to extract primary amines from biological samples. The possibility of re-using the solid-phase sorbents was investigated and showed good efficiencies when used up to seven times in the case of non-polar sorbents for Indole extraction and up to six times in the case of ion-exchange sorbents for 2-phenethylamine, Tyramine and Tryptamine extraction.

d) **Applications**

The decrease rate in drug concentrations with time and storage temperature was tested in a trial to make a correlation between the authentic drug concentration and the predicted concentration using the decrease rates obtained in this study. This was found to be of limited usefulness (50% agreement between the authentic concentration and the predicted concentration).

The possible interferences from acid and non-acid interfering substance with extraction and analysis of acidic drugs and benzodiazepines by HPLC system showed Indole to be the most important interfering substance in carbamazepine and amylobarbitone analysis by HPLC system. Therefore, another method should be considered for qualitative and quantitative determination of the two drugs.

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PUBLICATION

STABILITY OF SOME DRUGS OF FORENSIC INTEREST IN WHOLE BLOOD STORED AT DIFFERENT CONDITIONS

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ABSTRACT

Whole blood spiked with phenobarbitone, carbamazepine and phenytoin was stored in sealed vials for different time intervals and at different temperatures prior to analysis. Phenytoin was found to be very stable over a period of six months. Also, phenobarbitone and carbamazepine were found to be reasonably stable since recoveries of more than 76% and 70% respectively were obtained for each drug after six months. At the end of fifteen months storage, however, for all of the drugs, less than 50% of the parent drugs were recovered. Drug losses appeared to be relatively independant of both media and temperature of storage.

INTRODUCTION

There is a shortage of data regarding the stability of drugs of forensic interest in human postmortem blood, especialy when the postmortem tissue sample has been subject to a variable degree of putrefaction or,in some cases, acquisition by the laboratory to perform a full drug screen is after a few months of storage due to a

requirement for new evidence. Therefore it is necessary to establish whether these drugs are stable over a period of time under different storage conditions. Attempts to study this problem date back to 1942 and from that time until the early seventies most of the work suffers from lack of sensitivity and specificity (2,3,4).

The stability of some drugs in human liver macerates spiked with drugs over different periods of time have been studied (1,9). The results show a large coefficient of variation within the homogenate(liver macerates) due to a variable distribution within the tissue. Intratissue differences in drug distribution are known for certain chemicals.

Studies in the early 1970s on the stability of some barbiturate drugs in blood from dogs(3) and humans(4,5) have been reported. The techniques used, however, still lacked sensitivity and specificity. They used ultraviolet detection to measure the barbiturates in the basic extract following a poor recovery liquid-liquid extraction procedure. Other findings at about the same time gave contradictory information on the stability of plasma and serum phenytoin and phenobarbitone stored at $4^{o}C$ for eight, twelve and twenty six weeks prior to analysis(10,11,12).

EXPERIMENTAL

Chemicals: Phenobarbitone was obtained from B.D.H., Poole, U.K., Carbamazepine from Geigy Pharmaceuticals, Macclesfield, U.K., Phenytoin from Parke Davis and Company, Hounslow, London, U.K. and butalbital (internal standard) from Sandoz, Basel, Switzerland.

HPLC grade methanol and acetonitrile and Analar grade sodium acetate and disodium hydrogen phosphate were supplied by B.D.H., Poole, U.K.

The blood used in this study was part packs supplied by the Scottish Blood Transfusion Service, Carluke, Strathclyde, Scotland.

Apparatus: The HPLC system used was isocratic based on a Pye Unicam PU 4015 pump and PU 4025 ultraviolet detector. The extracts were introduced using a Rheodyne 7125 valve with a 20*u*l sampleloop. The column (25cm * 4.6mm i.d.) and guard column (5cm * 4.6mm i.d.) stainless steel were packed with C18-Hypersil ODS, 5*u*m particle size, supplied by Jones Chromatography. The chromatograms were recorded on a strip chart recorder set at 10 mV full scale deflection with 1.0 cm chart speed

The separation and detection of each drug was achieved using the HPLC system with a mobile phase consisting of 0.02M sodium acetate/methanol/acetonitrile in a ratio of 400:180:180 v/v/v respectively, the pH of mobile phase was adjusted to 5.5 with glacial acetic acid. The flow rate used was 1.5 ml per minute. The detector was set to monitor 210 nm.

PROCEDURE

The blood used was screened for the presence of the drugs under study before use. A methanolic standard of each drug was evaporated to dryness at 60C under a nitrogen stream and the residue was reconstituted with a known amount of blood to produce a level above the therapeutic range of each drug in the sample. Duplicate portions of each spiked blood sample for each period of time under study were transferred to a clean vial, sealed, and stored under one of three different temperatures (5, 25, -20^{o}C). Duplicate control solutions of each drug at the same concentration as the blood samples, were made in water at pH7.4 with these control aqueous solutions were stored with the blood under the same storage conditions.

On the day of analysis duplicate specimens of fresh spiked blood and control aqueous standards were prepared and analysed with the stored specimen. They were extracted using a previously reported method (6) to measure the concentration of the drugs under investigation

Extraction procedure

Into a 5ml vial add the following;

1 0.4ml of specimen (spiked blood/control aqueous solution).
2 0.4ml of 0.5M disodium hydrogen phosphate buffer (pH 9.2).
3 0.1ml butalbital "internal standard."

4 Mix thoroughly using whirly mix (30 sec).

5. Transfer 0.65ml of the mixture to the extre-lut column (a pasteur pipette filled with diatomaceous earth granules washed with dichloromethane and dried off before use).

6 Allow to stand for 15 minutes.

7 Elute the drug with 3ml dichloromethane into a clean vial.

8 Evaporate to dryness at 60C under a nitrogen stream.

9 Reconstitute the residue with 200*u*l of mobile phase.

10 Analyse 20 *u*l aliquots by HPLC.

RESULTS AND DISCUSSION

Our standard for significant breakdown of drug to have occurred was a level measured which had decreased by greater than three standard deviations of variation for the method (Table 1). The stability of phenobarbitone in putrefied blood samples stored at three different conditions (5, 25, -20^{o}C) for different periods of time can be seen in figure(1).

5^{o}C is the temperature most frequently used for short term storage of biological specimens, while -20^{o}C is mostly used for their long term storage.

After eight weeks of storage, blood phenobarbitone levels at 5^{o}C were shown to be stable with no changes in their concentration outwith (+/-) 3 S.D for the method. The concentration of phenobarbitone aparently increased up to 15% from the initial concentration when stored at 25^{o}C for the same period of time.

Using this (+/-) 3 S.D parameter as an indicator of decomposition of the drug, blood containing phenobarbitone started to display losses after thirteen weeks of storage at 5^{o}C (24%). Samples stored at 25^{o}C exhibited losses of 19% while samples stored at -20^{o}C exhibited no losses over the same time period. Between 13 and 26 weeks no significant changes were observed.

A slow and steady decline in phenobarbitone concentration were observed for all samples stored at the three different storage conditions for 52 weeks and reached losses up to 34% for samples stored at 5^{o}C, 43% losses at 25^{o}C and 42% for samples stored at -20^{o}C.

After 65 weeks (15 MONTHS) of storage the concentration of phenobarbitone dropped to the half of it's initial concentration in all samples (losses of 51% at 5^{o}C, 58% at 25^{o}C and 53% for samples stored at -20^{o}C). The average loss in phenobarbitone concentration was 54% when stored at any of the three conditions for 65 weeks.

Figure 2 shows that the blood carbamazepine levels declines at different rates when stored at three different conditions. After 13 weeks blood carbamazepine samples stored at 5^{o}C showed no changes in their concentration outwith (+/-) 3 S.D for the method. 33% losses were found for storage at 25^{o}C for the same period of time, with no changes in concentration for samples stored at -20^{o}C. Losses in carbamazepine concentration of 41% were measured in samples stored at -20^{o}C after 26 weeks For samples stored at 5^{o}C over the period of 13 to 26 weeks the changes in carbamazepine concentration is still within (-/+) 3 S.D of the method variation, samples at 25^{o}C almost maintain the same percentage losses after 26 weeks (6 MONTHS).

At the three different storage conditions carbamazepine showed an average losses 49% after 52 weeks (ONE YEAR) and the average losses of carbamazepine reach up to 69% after 65 weeks (FIFTEEN MONTHS). Losses of carbamazepine in blood showed a steady decline over the period 26-65 weeks.

TABLE 1

The standard deviation for the entire extraction and analysis of anticonvulsant drugs.

Drug	Standard Deviation n = 8
Phenobarbitone in blood	± 8.5%
Phenobarbitone in water	± 9.1%
Carbamazepine in blood	± 9.5%
Carbamazepine in water	± 9.5%
Phenytoin in blood	± 8.6%
Phenytoin in water	± 9.3%

Phenytoin concentrations in blood samples stored at 5°C showed losses of less than 10% from the initial concentration after 26 weeks compared with 12% losses for samples stored at 25°C for the same period of time. At -20°C phenytoin showed no significant changes in concentration from the initial value. A very slow decline in phenytoin concentration was observed in samples stored at 5°C to reach 17% losses at 52 weeks, Figure 3.

At 25°C and-20°C phenytoin showed losses in concentration of up to 28% and 31% respectively from the initial concentration after 26 weeks. For the three storage conditions (5,25,-20°C), after 65 weeks (15 MONTHS), phenytoin had an average loss of 64%.

The decline in phenobarbitone aqueous solution stored at (5, 25, -20°C)for up to 65 weeks can seen in figure 4. It shows a linear decrease in concentration with time regardless the storage temperature and reaches a maximum loss in phenobarbitone concentration of up to 67% compared with the 54% average losses for phenobarbitone in blood samples for the same period of time. As the differences between blood and water losses are small and water occurs in both samples a hydrolysis of phenobarbitone in blood and water samples could explain the losses in phenobarbitone concentration with time. The mechanism of phenobarbitone degradation in aqueous solution has been studied (7,8) and it has been proposed that the degradation mechanism depends on whether the barbiturate (phenobarbitone) were in ionised or unionised forms. Unionised barbiturates were hydrolysed at the 1,2-position while ionised barbiturates were hydrolysed at 1,6-position. Different breakdown products could be formed.

Carbamazepine stored in aqueous solution under the three different conditions, see figure 5, showed a slow deterioration in carbamazepine to reach a maximum average loss of 24% compared with carbamazepine in blood which reached an average loss of up to 49% after 52(weeks). Carbamazepine therefore shows greater stability in aqueous solution than in blood. A factor of greater effect in blood than

in water is the presence of microorganisms which could exert a great effect on the deterioration of carbamazepine in blood with time. The decline in phenytoin concentration in aqueous solution stored at 5, 25, -20^{o}C for up to 65 weeks can be seen in figure 6 to show minimum average losses of up to 24% and 58% after being stroed for 52 and 65 weeks respectively.

Conclusions

Under the storage conditions studied, phenytoin was found to be very stable over a period of six months. Phenobarbitone and carbamazepine showed a steady slow decline in their concentration with time. Up to 6 months, at least 70% of the drugs were still recoverable. All three drugs could still be detected after 15 months irrespective of the storage condition demonstrating that an analysis could still provide useful information. The interpretation of the result would have to take into account both the storage conditions and the storage times. No interference with the analytical technique was experienced from the products of putrefaction.

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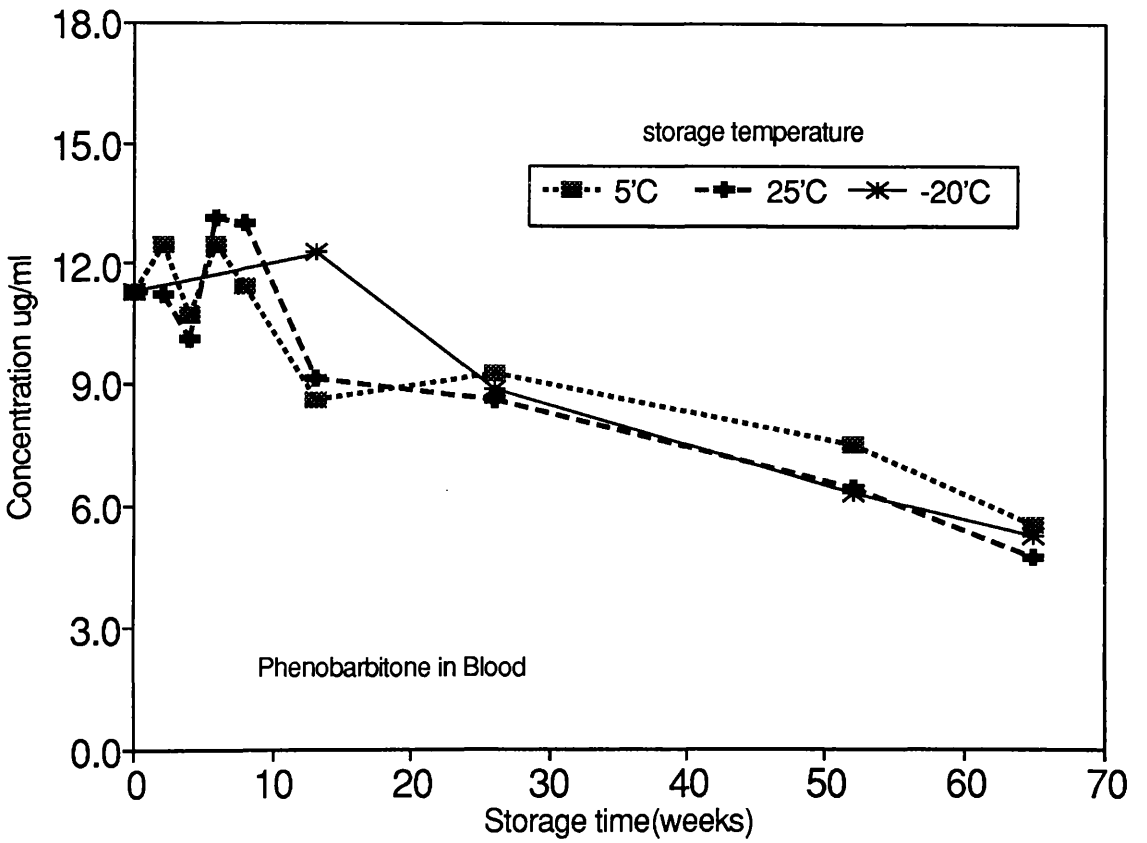

Figure 1. **Changes in phenobarbitone concentration with time in blood stored at 5, 25, -20^{O}C from day zero to 65 weeks.**

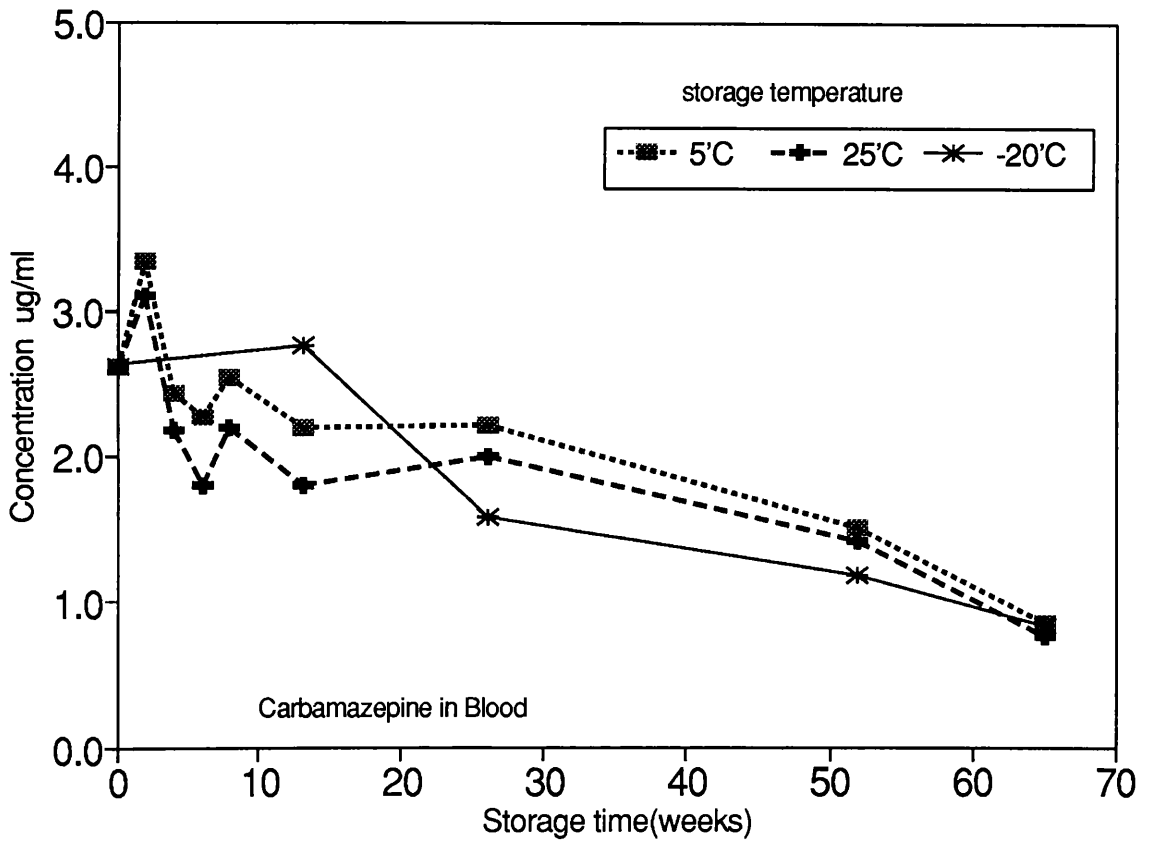

Figure 2. **Changes in carbamazepine concentration with time in blood stored at 5, 25, -20^{o}C from day zero to 65 weeks.**

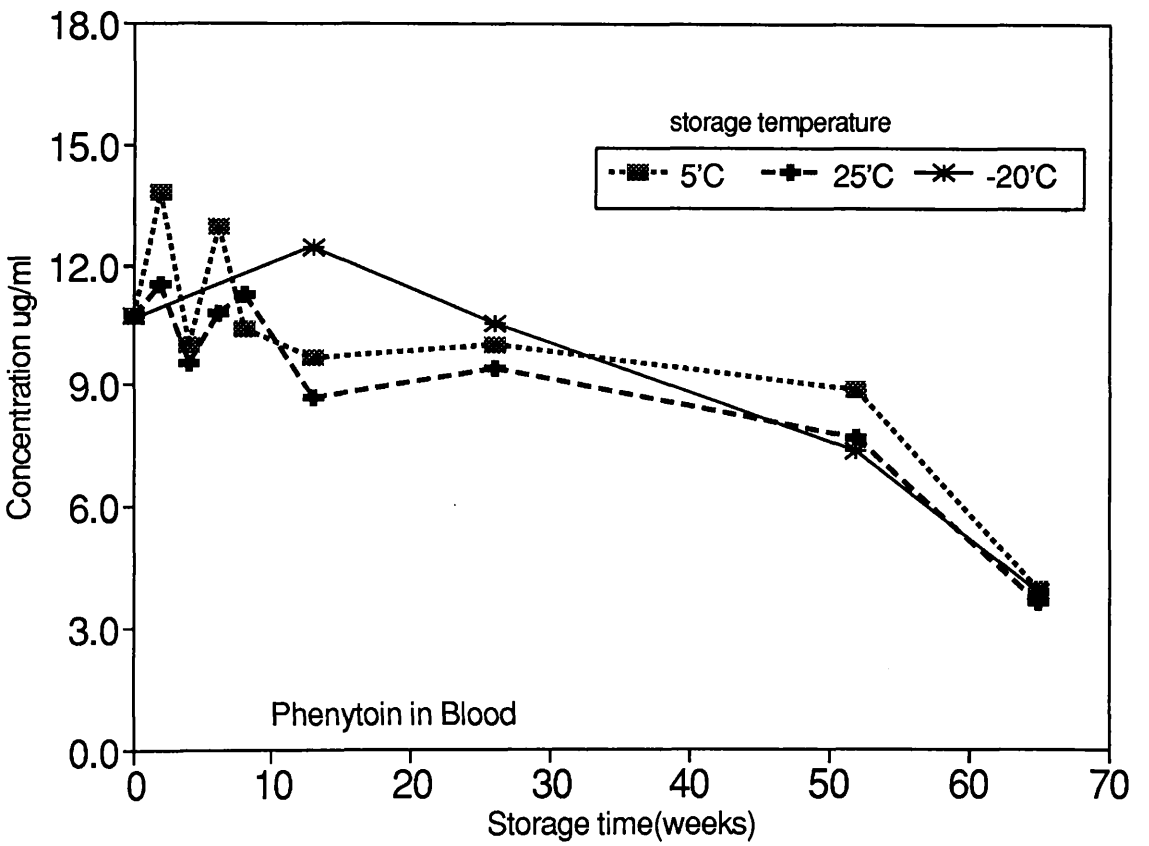

Figure 3. **Changes in phenytoin concentration with time in blood stored at 5, 25, -20^{0}C from day zero to 65 weeks.**

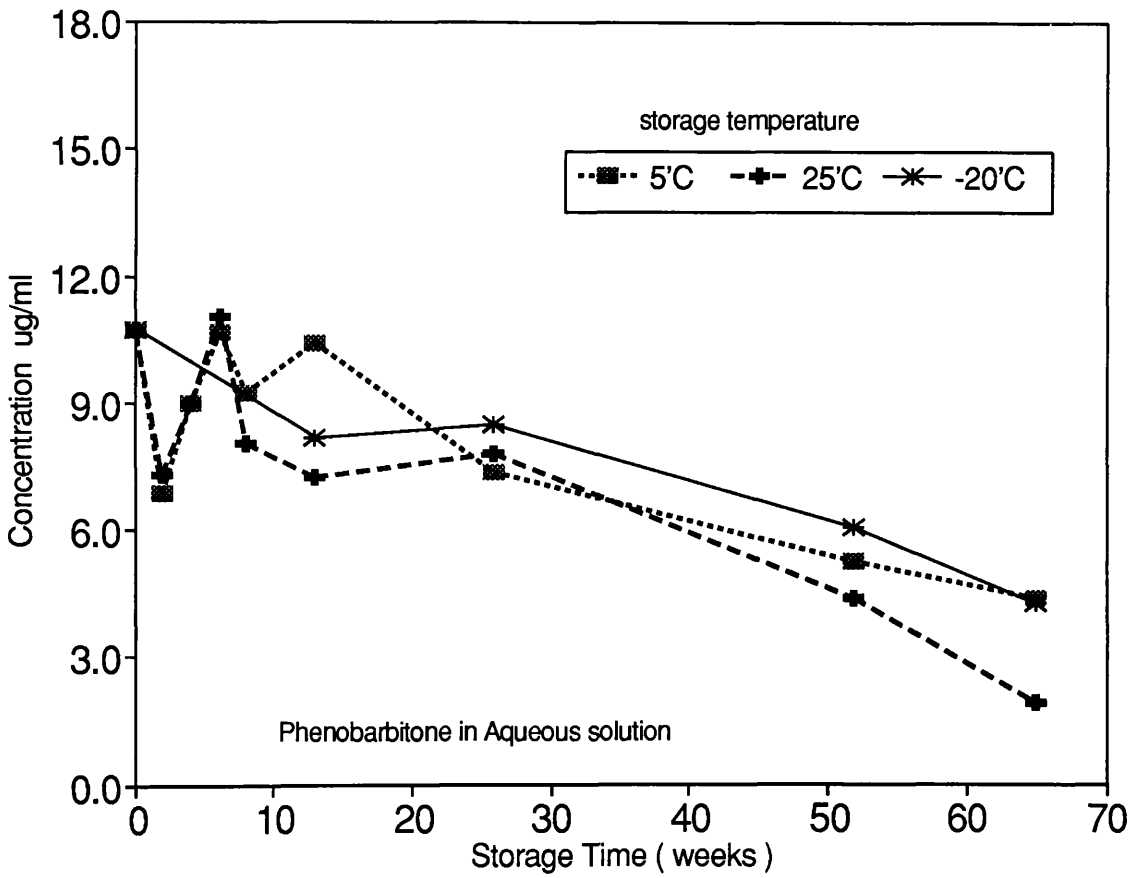

Figure 4. **Changes in phenobarbitone concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 65 weeks.**

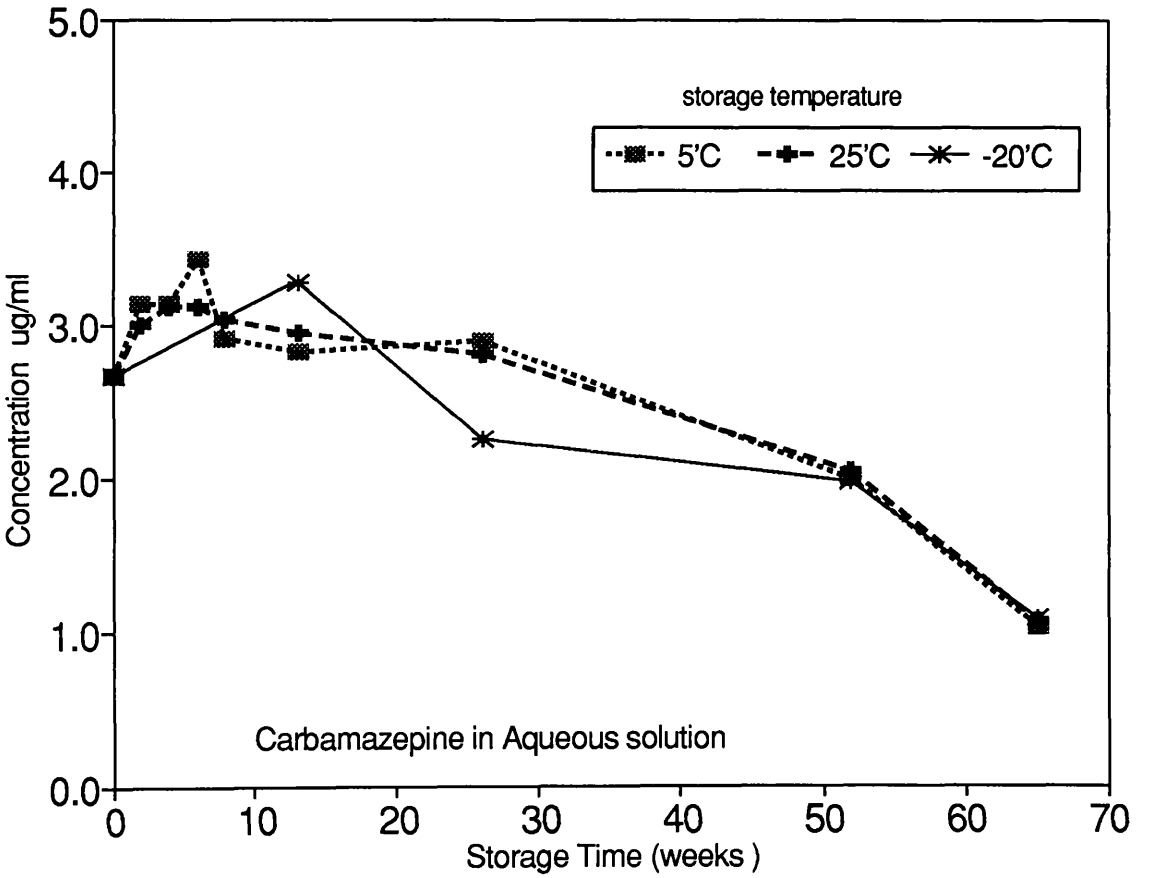

Figure 5. **Changes in carbamazepine concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 65 weeks.**

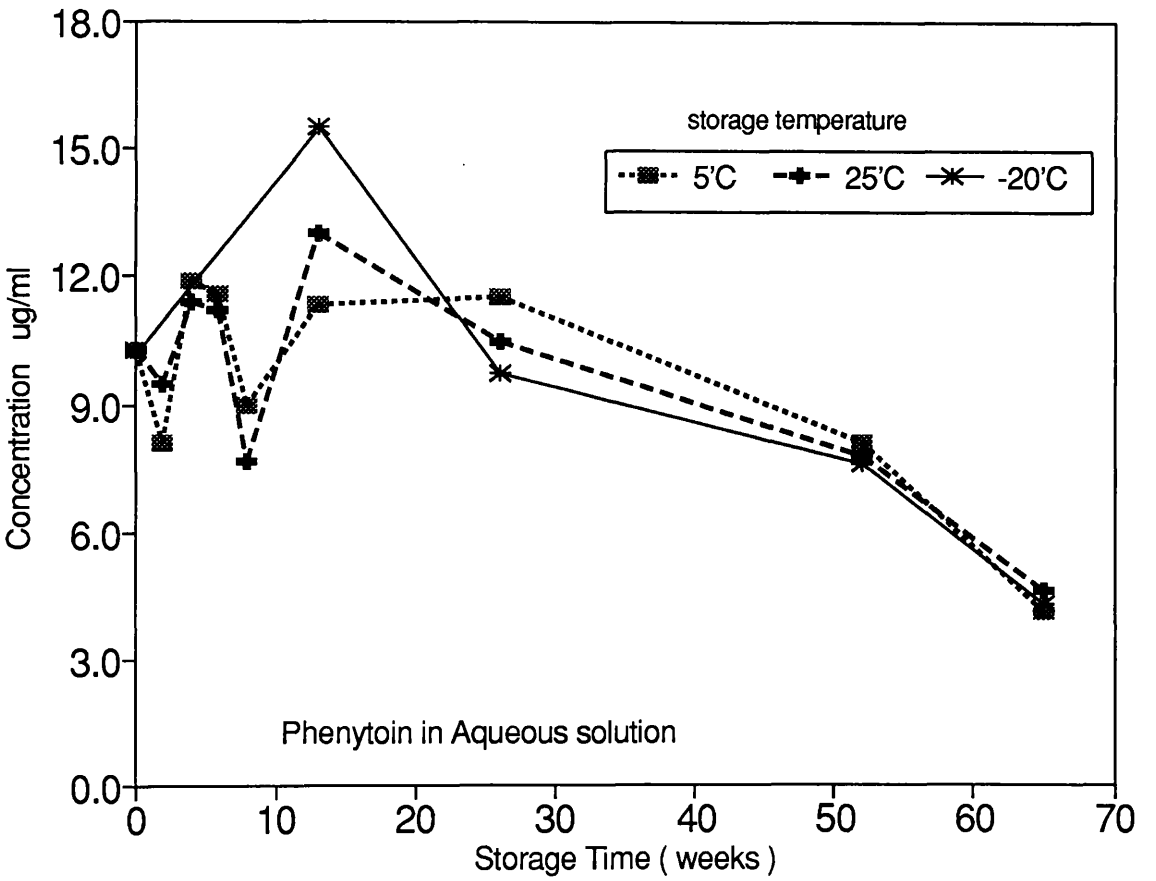

Figure 6. **Changes in phenytoin concentration with time in aqueous solution stored at 5, 25, -20^{o}C from day zero to 65 weeks.**