An Evaluation of Mixed-Mode Solid-Phase Extraction Columns for the Recovery of Basic Drugs from Whole Blood

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(***Forensic Toxicology***)

by
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Dedication

To Mum and Dad,
thanks for
Everything

and

To Ryan, Cheryl and Derek
and especially
to Baby Laing

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Abbreviations

ng	nanogram
µg	microgram
mg	milligram
µL	microlitre
mL	millilitre
µm	micrometre
mm	millilitre
m	metre
min.	minute
°C/min	degrees centigrade per minute
RI	Retention Index
DI	Deionised water
IS	Internal standard
PHR	Peak Height Ratio
GC-FID	Gas Chromatography with flame ionisation detection
GC-NPD	Gas Chromatography with nitrogen phosphorus detection
TLC	Thin layer chromatography
HPLC	High Performance Liquid Chromatography
GC-MS	Gas Chromatography with Mass Spectroscopy
EC-GC	Electron capture gas chromatography
HPLC-DAD	High Performance Liquid Chromatography with diode array detection
SPE	Solid-phase extraction
LLE	Liqid-Liquid extraction
STA	Systematic toxicological analysis
ASTED	Automated sequential trace enrichment of dialysate
PRS	Propylsulphonic acid, strong cation exchanger

Summary

A review of the methods currently used for the extraction and analysis of basic drugs from biological matrices has been carried out. The aims of this project were to develop a method for the extraction of basic drugs from whole blood using solid-phase extraction (SPE) and gas chromatography with flame ionisation detection (GC-FID).

A comparative evaluation was made of three experimental mixed-mode SPE columns (HCX-2, HCX-3 and HCX-4) to three mixed-mode SPE columns currently in use (HCX, ENV+ and Bond Elut Certify). HCX-2, HCX-3 and HCX-4 columns consistently gave better or comparable drug recoveries when compared to the other columns.

Two extraction methods were developed, which were able to produce recoveries of greater than 60% for each of the nine basic drugs using the experimental columns. Both methods involved a sample pretreatment step, including sonication (to break up the red blood cells and release the drugs) and dilution with a buffer (to reduce the viscosity of the blood and prevent blockage of the column). The methods differed in the pH of the 0.1M phosphate buffer used. Method A involved a buffer pH of 6.0, while Method B used a buffer pH of 7.4, both produced clean extracts, with no coeluting material.

For GC-FID analysis, an HP-1, fused-silica wide-bore capillary column (30m × 0.53mm i.d., film thickness 0.88μm) was chosen with a Helium carrier gas flow rate of 13mL/min. The temperature program used, was that stated by Chen *et al*[27]. The extraction methods separated the drugs into two fractions, Fraction A included all the

weakly basic drugs and Fraction B, the remaining basic drugs. Prazepam was used as the internal standard.

Linear calibration curves were produced for each of the nine basic drugs over a range of ng/mL and μg/mL levels and from a total of 1mL of blood, the limit of detection for the drugs ranged from 7.5 to 16 ng/mL. Due to time restrictions, only Method B in conjunction with an HCX-3, SPE column were used to demonstrate a linear response, for quantitative analysis. The procedure produced linear calibration curves for each of the drugs with correlation coefficients ranging from 0.99937 to 0.99985.

In conclusion, the optimum method established in this study, for the extraction and analysis of basic drugs from whole blood, involved the use of a phosphate buffer of pH 6.0 or 7.4, along with the extraction steps cited in Chen *et al*[27], followed by GC - FID as the end-step analysis.

Chapter One: Introduction

Drug abuse in the West of Scotland, as in society in general, has increased dramatically over the past decade (Figure1.1). As a result of this, the number of deaths caused directly from drug use, e.g. overdose[1] or indirectly, e.g. road traffic accidents[2] , has also increased. Drugs impair psychomotor skills and cause hazardous driving[3].

As a consequence of the increase in demand for drug identification, it is the role of the Forensic Toxicologist to develop a method which is both rapid and reproducible for the analysis of biological matrices.

Systematic Toxicological Analysis (STA) is defined as the logical chemical - analytical search for an unknown substance of toxicological relevance[3]. The first step is to establish whether the compound can be identified (screening) and secondly, to identify the compound (identification and confirmation).

Biological samples have to undergo a series of sample pre-treatment steps, to remove any endogenous materials and to increase the drug concentration, before being introduced onto a chromatographic column.

Technological advances in chromatographic analytical equipment have afforded the scientist with the means of separating a complex mixture of analytes and confirming their presence. However, sample preparation techniques have not improved as quickly and this remains the limiting factor for a totally automated analysis and is the source of greatest variability[4].

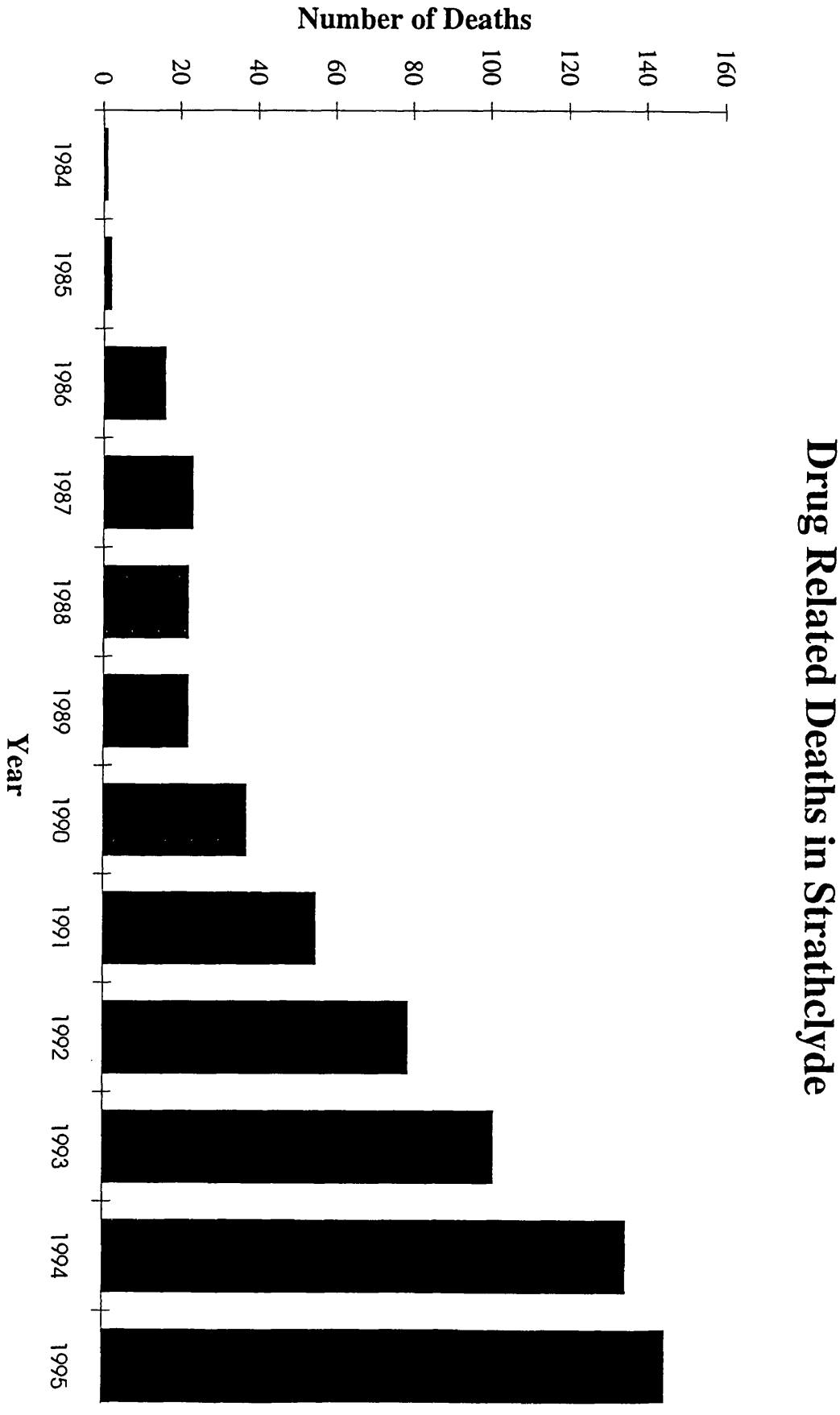

Drug Related Deaths in Strathclyde
Number of Deaths
0
20
40
60
80
100
120
140
160
Year
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995

Figure 1.1

Isolation of drugs from whole blood was until recently, time consuming and wasteful of sample due to inefficient preparation and extraction techniques. The use of Solid-Phase Extraction (SPE) as an alternative to Liquid-Liquid Extraction (LLE) has demonstrated the advantages of this technology in producing better extraction results. This is of great importance as whole blood is the most widely available sample for forensic toxicological analysis.

LLE continues to be the method of choice in most Forensic Toxicology laboratories, despite the obvious advantages of SPE. Scheurer and Moore[4], gave one explanation for this being that, Forensic Labs are in the unique position where their results are subject to severe scrutiny in courts. In the past SPE had problems with reproducibility, due to lack of quality control on the packing materials used.

However, with the advent of mixed-mode SPE columns, the problems of not being able to extract a wide range of drugs with varying physical and chemical properties became a thing of the past as did the problems with reproducibility.

The majority of SPE methods reported, involve the selective extraction of individual drugs or groups of similar drugs. Of those methods which screened a large group of drugs[5,6,7], most analysed samples other than blood and none of them included all of the drugs included in this research project.

The aims of this study were to:

(1) Compare and evaluate the extraction efficiencies of three experimental mixed-mode SPE columns using whole blood,

(2) Compare the three experimental mixed-mode SPE columns to mixed-mode

columns currently in use, and

(3) Develop a validated method for screening basic drugs in whole blood samples using SPE and GC-FID.

1.1 Drug Abuse: An Overview

The abuse of drugs both legal and illegal has increased alarmingly throughout the United Kingdom. Tobacco smoking is thought to contribute to 100,000 premature deaths in the UK every year and alcohol abuse is prevalent in a society where 90% of the adult population drink.[8]

Although relevant data relating to the prevalence of drug usage in the United Kingdom is not readily available, it can be assumed to be on the increase, due to the increase in drug related deaths[1] and the number of notified drug addicts[1]. From 1992 to 1993 their was a 13% increase in the number of drug addicts who were notified to the Home Office, 40% of which were newly registered. This increasing trend has been constant over the past few years. The number of deaths attributed to drug misuse of notified drug addicts was reported to be 83 in 1981 and this figure had increased to 206 in 1991. Of those, opiates and methadone overdose, accounted for the vast majority of deaths[1].

Drug misuse databases reported that 17,800 people from across England started treatment between October 1st 1992 and March 31st 1993.[9] Heroin accounted for approximately 50% of the main reported drug problem, methadone(15%), amphetamines(11%), cannabis(7%), benzodiazepines(4%) and cocaine(3%).

Looking specifically at the Scottish Drug Scene[10], only 2 deaths were recorded in 1984 from the misuse of heroin. However, for the Strathclyde region alone, this had increased to 101 drug related deaths in 1993 and 145 in 1995. Deaths occurring in the late 1980's were attributed to Heroin abuse, but at the start of the 1990's the abuse of benzodiazepines featured much more[11].

The nine drugs chosen for this project, are representative of the different classes of drugs which are more commonly abused in the UK. Table 1 summarises these drugs along with their drug type and effects[12].

Table 1: Drugs and their effects.

Drug Name	Drug Type	Effects
Amitriptyline	Tricyclic Antidepressant	Sedative effects, elevates mood, increases physical activity and appetite.
Cocaine Methamphetamine	Local Anaesthetic Central Stimulant	Central Nervous System stimulants: increases alertness and diminishes fatigue.
Codeine Dihydrocodeine Methadone Propoxyphene	Narcotic Analgesic Narcotic Analgesic Narcotic Analgesic Narcotic Analgesic	Reduces sensitivity and emotional reaction to pain, discomfort and anxiety.
Diazepam Desmethyldiazepam	Tranquilliser Tranquilliser	Central Nervous System Depressants: promote relaxation, impair the efficiency of mental and physical functioning.

1.2 Forensic Toxicology

Toxicology is the study of poisons and the term 'poison' is used to describe any substance which has a harmful effect on a living system. For the purposes of this

project, we are looking specifically at the toxicology of drugs, primarily, illegal drugs.

It is of great importance to know whether someone died due to the effects of a drug, in the case of suicide, homicide (very rare), or accidental poisoning.

The toxicity of a drug does not only depend on its toxic properties but also on the dose administered. However, there is no direct relationship between the dose and the effects that it elicits. Many factors contribute to the effects of a drug from one individual to another, e.g. tolerance and what would constitute a fatal dose to one person may have little or no effect on another.

A summary of the therapeutic, suggested toxic and fatal levels of the nine drugs used in this study (μg/mL) are presented in Table 2.

Table 2: Therapeutic, Toxic and Fatal Levels of Drugs(μg/mL)[13].

Drug	Therapeutic	Toxic	Fatal	Half-Life(Hrs)
Amitriptyline	0.05 - 0.2	0.05 - 0.17	0.55 - 3.3	8 - 51
Cocaine	0.05 - 0.3	0.9	1.0 - 20	0.7 - 1.5
Codeine	0.01 - 0.250	0.3 - 0.5	> 1.6	2 - 4
Diazepam	0.125 - 0.75	1.5 - 5.0	-	20 - 100
Dihydrocodeine	0.03 - 0.25	1.0	2.0	4
Methadone	0.1 - 0.75	1.0 - 2.0	> 4.0	10 - 55
Methamphetamine	0.01 - 0.075	0.2 - 1.0	10 - 40	9
Propoxyphene	0.1 - 0.75	1.0	> 2	8 - 24
Desmethyldiazepam	0.17 - 1.84	-	-	25 - 200

Amphetamines are the most popular illicit stimulant, second only to cannabis in the overall league table of illicit use[9]. Methadone is often used in the treatment of opiate addiction. Propoxyphene is a synthetic analgesic agent and misuse induces toxic overdose reactions involved in accidental and suicidal poisonings[14]. Amitriptyline along with other tricyclic antidepressants are among the most commonly prescribed drugs for the treatment of endogenous depression[15].

Benzodiazepines are the most commonly prescribed drug in the world, used as antianxietics, hypnotics and antiepileptics. They are also the most frequently encountered drug in Forensic and Clinical Toxicology[16]. Codeine and dihydrocodeine are two of the markers found after opiate abuse. Cocaine and heroin are reported to be the most commonly found drug combination with the exception of alcohol[17].

1.3 Systematic Toxicological Analysis

The main purpose of Systematic Toxicological Analysis (STA), is to detect and identify unknown substances in a given matrix[18].

Drug screening in STA is an extremely important preliminary step in the analysis of biological specimens and involves two main steps;

1. Sample Preparation (clean-up and extraction) and
2. actual analysis for determination and confirmation.

Sample pretreatment produces a cleaner sample and leads to greater selectivity and precision, by eliminating any matrix effects which may interfere with the analysis.

The extraction is optimised through adjustment of the pH, ionic strength and sample concentration.

Traditionally, liquid-liquid extraction (LLE) techniques were used to separate analytes from biological matrices. However, they were found to be tedious, time consuming and were not always sufficiently reproducible.

Solid phase extraction (SPE) emerged in the mid 1970's[19] as a simpler alternative to LLE and has since proved to be an extremely effective means of extracting drugs from biological matrices with several distinct advantages over LLE[18]. These include reduced solvent usage, cleaner extracts, no emulsion formation and considerable reduction in analysis times.

Several analytical techniques have been used for the screening of drugs, these include, Thin Layer Chromatography (TLC), Immunoassay, High Performance Liquid Chromatography (HPLC) and Gas Liquid Chromatography (GLC).
The ideal analytical method, should be specific, statistically defined (accuracy, precision and sensitivity), and routinely reliable[20].

In forensic Toxicology, capillary GC and capillary GC/MS are often the preferred methods for analyte confirmation and quantification[21]. A number of authors have reported the benefits of using GC, for drug screening[22], and when used in conjunction with Retention Indices[23]. Chen et al[24] reported GC to have good separation efficiency, high sensitivity, inertness and especially valuable for drug screening.

For the purposes of this project, Gas Liquid Chromatography connected to a Flame Ionisation Detector was used in conjunction with retention indices (RI) for the identification of basic drugs.

1.4 Solid-Phase Extraction

1.4.1 Introduction

Solid-Phase Extraction (SPE) was introduced as a simpler alternative to Liquid-Liquid Extraction (LLE) in the mid 1970's, to try and combat the problems encountered with this method[18], i.e.,

1. Phase emulsion
2. Large solvent volumes
3. Impure and wet extracts
4. Non-quantitative and irreproducible extractions
5. Time consuming
6. High costs
7. Several sample handling steps.

The first bonded phases used were of hydrophobic character, e.g. C18 and XAD resins[25]. These single phase columns showed great promise for the analysis of biological samples, but problems occured with co-extraction of endogeneous materials.

Ion-exchange columns were introduced in the 1980's and successfully extracted a wide range of compounds of differing chemistries, of both basic and acidic character. Improvements with sample clean-up and recovery were also reported, however extraction of neutral compounds still remained a problem.

Mixed-mode bonded silicas, consisting of both hydrophobic and ion-exchange properties in one single column e.g., Clean Screen®, first introduced in 1986[25], enabled the extraction of a range of drugs, including neutral compounds[7].

SPE is a powerful pretreatment technique for the clinical and toxicological drug analysis of biological samples[26] and over the years has become more and more acceptable in Forensic Toxicological laboratories[18,27], having very distinct advantages over LLE. These advantages include:

1. High selectivity
2. Cleaner extracts
3. No emulsion formation
4. Better reproducibility
5. Reduced solvent usage
6. Higher throughput due to automation[7,21,24]
7. Less contamination of GC system.

1.4.2 Principles of Solid Phase Extraction

The extraction procedure in SPE, involves the interaction between a solid and a liquid phase, and is a physical extraction process, similar to Liquid

Chromatography[28]. However, the aims of SPE are to isolate and then concentrate the compounds of interest, while Liquid Chromatography aims to separate the compounds, with good peak shape and height, and short retention times[28].

There are many different sorbent materials available, e.g diatomaceous earth, silica and bonded silica gels. Bonded silicas are the most frequently used sorbent as they can be modified with different functional groups to produce highly stable non-swelling stationary phases which allow for a wider range of extraction capabilities. Secondary interactions also take place where the isolate molecules interact with the silica substrate as opposed to the functional group[28].

The extractions can be separated into three types, depending on the functional groups bonded to the silica; non-polar, polar and ion-exchange.

Non-Polar

Non-polar interactions result between the carbon-hydrogen bonds of the sorbent and of the isolate and are caused by "Van der Waals" or "dispersion forces". The majority of organic molecules have some non-polar character and so are retained on the surface of sorbents containing non-polar functional groups.

Octadecyl silane (C18) is the most frequently used non-polar sorbent and is non-selective, resulting in the retention of a variety of structurally unrelated isolates. Retention of isolates onto non-polar sorbents is facilitated by the use of polar solvents, while elution requires a solvent of sufficient non-polar character to disrupt the non-polar interactions between the isolate and the sorbent.

Polar

Groups with polar character include, hydroxyls, amines, aromatic rings and hetero-atoms, e.g. oxygen and nitrogen. These are retained on sorbents containing polar functional groups due to hydrogen bonding, dipole/dipole, induced dipole/dipole, pi-pi and other polar interactions resulting from the uneven distribution of electrons between individual atoms in the functional group.

Secondary interactions due to the polar character of the silica substrate are present in all bonded silicas. Retention of isolates onto polar sorbents is facilitated by the use of non-polar solvents, while elution requires a polar solvent of sufficiently high ionic strength to disrupt the polar interactions between the isolate and the sorbent.

Ionic

Ion-exchange occurs between groups present on the isolate and the sorbent with opposite charges. There are two classes of these groups: cationic (positively charged), e.g. primary, secondary, tertiary, and quarternary amines, and anionic (negatively charged), e.g. carboxylic and sulphonic acids. Depending on the pH of the solvent environment, these molecules can exhibit cationic or anionic character. Two criteria have to be met before the retention of an isolate by an ion-exchange mechanism can take place;

1. the pH of the system must be such that both isolate and sorbent are charged, and
2. the system must not contain high concentrations of strongly competing ionic species of the same charge as the isolate.

The pKa is defined as the pH at which half of the molecules in solution are charged and the other half are not. Increasing the pH to a value above the pKa of anionic molecules, increases the number of charged anionic groups and decreasing the pH, decreases the number of charged molecules. The opposite is found with cationic groups.

In order to achieve retention of ionic groups, both the sorbent and the isolate have to be charged, i.e. at a pH below the pKa of the cation and at a pH above the pKa of the anion. A pH of at least two pH units below the pKa of the cation and two pH units above the pKa of the anion is required for approximately 99% of the groups to be charged.

Other factors which are important in ion-exchange are ionic strength and selectivity. Ionic strength is a measure of the total ionic concentration of ionic species present within the solvent/matrix environment. Ion-exchange is a competitive process and so low ionic strength encourages retention of the isolates while high ionic strength disrupts it. Selectivity describes the preference shown by ion-exchange sorbents for certain types of ionic groups over others. Ionic secondary interactions also occur due to unbonded silanols on the silica substrate.

Retention of an isolate onto a sorbent matrix occurs through the interaction of a combination of the three extraction mechanisms, i.e. non-polar, polar and ion-exchange, these interactions are dependent on the sample matrix and solvent environments.

Table 3, contains information on the non-polar and polar phases of the three experimental mixed-mode columns and the standard HCX column.

Table 3: Information on Column Packing Materials

Product	Part#	Compostion	Comment
HCX	902	LC C8 (UC) + PRS	Standard mixed sorbent product, made with low carbon loading C8 (non-end capped) + propyl-sulphonic acid strong cation exchanger.
HCX-2	912	C12 (UC) + PRS	Non-end capped C12 + propyl-sulphonic acid strong cation exchanger.
HCX-3	913	C18 (UC) + PRS	Non-end capped C18 + propyl-sulphonic acid strong cation exchanger.
HCX-4	914	C8 (UC) + PRS	Standard C8 (non-end capped) + propylsulphonic acid strong cation exchanger.

HCX contains low carbon loading C8 non-end capped sorbent which would result in the least non-polar retention characteristics of the four columns, followed by HCX-4, HCX-2 and then HCX-3 having increasingly more non-polar character.

Each of the columns contains a 4:1 mixure of the non-polar sorbent to PRS.

1.4.3 Procedural Steps in Solid Phase Extraction

In its simplest form, a solid phase extraction procedure consists of five steps: column preconditioning, sample application, column wash, column drying and drug elution.

1. Column Preconditioning

Before applying a pretreated sample to an extraction column, the active sites on the column have to be activated with a suitable solvent. Bonded silica sorbents are dried and packed and so are not available to interact with the analytes. Application of the sample before solvation would result in low and irreproducible recoveries.

2. Sample Application

Once the column is solvated, the sample is applied onto the column under a light vacuum. The flow rate of the sample through the column is very important, as a low flow rate allows the necessary time for the maximum amount of drugs to interact with the active sites on the column. Normally this would be 1.5mL/min.

3. Column Wash

The wash step allows the removal of any endogenous components that were retained by the active sites. Water or an appropriate solvent is used to selectively remove any material which could interfere with the analysis. Selection of a solvent which will not remove any of the relevant drugs can be difficult and so a compromise must be reached between acceptable recoveries and removal of interfering compounds.

4. Column Drying

This is an extremely important step, especially when Gas Chromatography is used for the final analysis, as any residual water may cause damage to the GC column. The water may be removed by applying a vacuum to the column or by the addition of a solvent, e.g. Methanol[27].

5. Elution of Relevant Drugs

In order to elute the drugs for analysis, a suitable solvent is chosen which will extract only the drugs of interest and leave any remaining matrix interferents on the column. The solvent should also be strong enough to displace all of the drugs into a small eluent volume. Large eluent volumes have the disadvantage of diluting the extract, extracting more impurities and increasing the extraction time. An evaporation step is usually included at this stage to concentrate the drugs, however care must be taken to prevent the loss of more volatile drugs, e.g. amphetamines.

Chapter Two - Literature Review

2.1 Introduction

Drug screening in Systematic Toxicological Analysis (STA) is an important preliminary step in the analysis of biological materials. In order to analyse a sample, two steps are required: **1.** clean-up and extraction, and **2.** detection.

Developments in the analysis of biological samples have resulted in, more efficient drug recoveries due to better clean-up steps, new extraction techniques and advances in chromatographic separation. These improvements have permitted methods which have greater reproducibility, and are quick and easy to use.

A review of the recent literature has revealed the increasing use of Solid-Phase extraction (SPE) as an alternative to Liquid-Liquid extraction (LLE) and there has been extensive research using both techniques for the extraction of drugs from urine[29]and plasma[30] as well as unconventional tissues, e.g. meconium[31], hair[17] and vitreous humor[32].

SPE was primarily used for the analysis of urine and plasma samples, as whole blood tended to block the columns and cause reduced extraction efficiencies. This was resolved by sonication of the blood to fragment the red blood cells[33], then diluting the sample with an appropriate buffer[34], to ease the flow through the column. This is of great importance, as whole blood is the most widely available sample for forensic toxicological analysis and postmortem blood samples are very rarely able to be separated due to degredation (putrefaction).

Previous work carried out using SPE of whole blood, has concentrated on the extraction of individual or closely related groups of drugs[35,36,37,38]. As a consequence of this, the methods which are available are very specific and are inappropriate for the simultaneous screening of various classes of drugs.

2.2 Liquid-Liquid Extraction (LLE)

LLE has been used successfully as a means of extracting different drugs from biological specimens for many years, and is still the preferred method in many laboratories.

De Gier and 't Hart[39] reported recoveries of 97 ± 8% for Diazepam and 100 ± 12% for Desmethyldiazepam extracted from plasma into a toluene - heptane mixture at pH9. Prazepam was used as the Internal Standard (IS). Before analysis of the extract, derivatisation of N-desmethyldiazepam was carried out to give the N-butyl derivative which along with Diazepam was detected using Electron-Capture Gas Chromatography (EC-GC).

Al-Hadidi and Oliver[40] utilised LLE in order to study the stability of Temazepam in Blood, while Laakkonen and Heiskanen[41] developed a procedure for screening serum samples for benzodiazepines using LLE in conjunction with Gas Chromatography connected to a Nitrogen-Phosphorus Detector (GC-NPD) and with High Performance Liquid Chromatography (HPLC).

LLE has also found applications in screening for a large number of unrelated drugs[42,43,44,45] using a variety of detection techniques, including, Immunoassay[14,46,47], GC-NPD[48] and High Performance Liquid Chromatography using a Diode Array Detector (HPLC-DAD)[49].

Hughes and Osselton[15] compared nine Liquid-Liquid and two Solid-Phase extraction procedures which were evaluated on the basis of sample cleanliness, extraction efficiency and reproducibilty. HPLC was used for the identification and quantification of Tricyclic Antidepressants from whole blood samples. LLE extraction methods were reported as being the preferred method, as they were simpler, less time consuming and more suitable for forensic casework. Solid-Phase extractions were performed on Analytichem Bond-Elut™ cyanopropyl (CN) extraction cartridges (1mL) and involved no sample pretreatment prior to extraction.

LLE involves the use of large solvent volumes, which are expensive and require complex and lengthy sample handling steps. SPE does not suffer from either of these problems and with the advent of better sample preparation techniques, the extraction of drugs from whole blood using SPE has been made possible[4].

2.3 Solid-Phase Extraction

2.3.1 Introduction

The first materials to be used for the Solid-Phase extraction of urine samples were first reported in 1970 with the introduction of Amberlite® XAD-2 resins (cross-linked polystyrene - divinyl benzene by Rohm and Haas; Philadelphia, PA)[18]. Use of

these resins included screening for drugs of abuse in urine[50] and more recently, the extraction of cocaine and benzoylecgonine from postmortem blood[19].

The need for large volumes of resin, multiple stepped analysis and the time required to complete the extraction made these phases expensive and difficult to use. Sep-Pak C_{18} cartridges were introduced in 1978 by Waters Associates (Milford, MA)[18]. These smaller columns decreased the analysis time and costs associated with drug analysis and proved useful for the extraction of tricyclic antidepressants from vitreous humor[31] and benzodiazepines from urine[16]. To obtain similar recoveries from plasma, a deproteinization step was required.

New methods reported for SPE, outnumber those reported for LLE, demonstrating the shift from LLE to SPE in both clinical and forensic toxicological laboratories. One other type of solid-phase used, is Extrelut (Merck, France) which has found applications in the analysis of propoxyphene and related compounds[51] and Benzodiazepines[52] in blood. Extrelut is available as a prepacked column or in granular form.

Several single-mode columns have been introduced for the analysis of urine and plasma samples including Bond Elut C_2[53,54], Bond Elut C_{18}[53,55] and Detectabuse GC/MS grade columns[56]. These columns contain non-polar sorbent materials (C_2, C_{18}) and methods have been reported for analysis of individual drugs and groups of structurally related drugs. Problems with low recoveries arose when screening samples for a wide range of drugs using a single-mode column. Lillsunde and Korte[5] used a Chem Elut™ (Analytichem International) column to extract 300 drugs from urine and reported recoveries ranging from 10 to 90%.

Cosbey *et al*[57], investigated the use of a 2-stage process involving two cartridges with different phases, **1.** Supelclean-ENVIcarb (hydrophobic extraction using non-porous carbon) and **2.** Bondelut PRS cartridge (ionic extraction on a strong cation exchanger), to extract basic drugs from whole blood. Recoveries for the drugs studied were high, e.g. 89.9% for Quinine. However, the problems encountered with single-mode columns remained with this 2 stage method as the second extraction can only separate the drugs extracted from the first column.

Logan *et al*[6], reported the use of a Bond Elut strong cation exchange (SCX) column (Analytichem), which contains silica particles with alkyl-bonded benzene-sulphonylpropyl, which combined both cation exchange and non-polar properties. This column was found to extract efficiently, over 100 basic drugs from urine, as well as certain drugs which proved difficult with LLE, e.g. morphine, benzoylecgonine, temazepam and oxazepam.

2.3.2 Mixed-Mode SPE Columns

Improvements continued with the introduction of mixed-mode SPE columns: Clean Screen™ DAU (Worldwide Monitoring Corporation: Horsham, PA), Bond Elut Certify™ (Varian Sample Preparation Products: Harbor City, CA) and narc2™spe (J.T. Baker: Phillipsburg, PA). These SPE columns have provided the analyst with the means of selectively extracting a broad range of drugs from various biological matrices. The sorbent consists of both hydrophobic and cation exchange groups.

Clean Screen™ DAU columns have been used for the extraction of cocaine and its metabolites[17,38] from a variety of matrices and for screening plasma and urine for

drugs of different physicochemical properties[24]. Bond Elut Certify™ has been used for the analysis of single or groups of related drugs in plasma[30], whole blood[31,58] and urine[29,59] samples.

The advantages of using a mixed-mode column as opposed to a single-mode column include, cleaner extracts and the ability to extract a wider range of drugs with different physicochemical properties. As these mixed-mode sorbents are contained in just one column, they have the added advantage of requiring less extraction steps and so are easier to use, as well as being more cost effective than the dual column procedure.

Chen *et al*[26], investigated the extraction of a broad range of acidic, neutral and basic drugs from plasma and urine using Bond Elut Certify columns. Prazepam was used as the Internal Standard and GC-FID was used for the analysis.

Their procedure used 2mL of sample (plasma), which was spiked with the appropriate drugs at 10 μg/mL. This concentration was chosen for analytical convenience and to represent toxicologically relevant levels for most of the drugs. The spiked sample was then diluted with 6mL of 0.1M phosphate buffer and vortexed to homogenise the resultant mixture.

The column was then conditioned with 2mL Methanol and 2mL of phosphate buffer at a flow rate of 2mL/min. The extraction procedure began with the application of the diluted sample to the column at a flow rate of 1.5mL/min. 1mL of deionised water was used as a wash to remove any interferences from the column. This amount was found to be sufficient to remove any interferences without affecting the drug recoveries.

In order to selectively elute the drugs from the column into two distinct groups, 0.5mL of 0.01M Acetic Acid (pH3.3) was added to the column at a flow rate of

1.5mL/min. At this pH, the acidic and neutral drugs have non-polar character and are retained by the hydrophobic groups of the sorbent, while the basic drugs are retained by the cationic groups due to their ionic character. The column was then dried under vacuum for 4 minutes, before addition of 50µL of Methanol with no vacuum. After a final drying period of 1 minute under vacuum to ensure the column contains no water, the drugs were selectively eluted.

The first solvent used was 4mL of acetone-chloroform (1:1) to elute the acidic and neutral drugs (Fraction A). The basic fraction was eluted using 2mL of 2% ammoniated ethyl acetate (Fraction B), at flow rates of 0.8 and 0.5 mL/min respectively.

After adding 100µL of prazepam (200µg/mL), to each fraction and evaporating at 40°C under nitrogen until 100µL remained, 1 or 2µL of each extract was injected onto the GC.

For each of the solvent mixtures, the optimum conditions were investigated and for the acetone-chloroform mixture, 89% of each drug tested, was recovered using 30 to 70% acetone in chloroform and so 50% was chosen. At this concentration, recoveries were highest using between 3 and 5 mL of the solvent mixture, and so 4 mL was chosen for the elution of the acidic/neutral fraction.

If the ethyl acetate mixture contained more than 3% ammonia, it was found to contain too much water, which damaged the GC column. 2% was found to be adequate for the elution of the basic drugs. The time required for the preconditioning and extraction steps was approximately 30 minutes when 10 columns were used simultaneously.

The authors noted that neither fraction should be evaporated to dryness as this would lead to losses of the more volatile drugs, e.g. methamphetamine. The two fractions should not be added together as this would result in loss of benzodiazepines due to the alkaline conditions.

The recoveries for the drugs ranged from 82.4 to 105.5%, with a relative standard deviation of less than 10%. Drugs which have a pKa close to the pH of the extraction system (3.3), e.g Diazepam, can be found in both fractions, A and B.

A modification of the above extraction procedure was reported by Chen *et al*[27], for the analysis of whole blood using both Bond Elut Certify and Clean Screen DAU columns. A Hewlett-Packard 5880 gas chromatograph with a flame ionization detector (FID) was used for analysis. The oven was fitted with a 30m, HP-1 fused-silica wide-bore capillary column (0.53mm i.d, film thickness 0.88μm). The temperature program was as follows:

Initial temp. - 80 °C	Initial time - 2 mins.
Initial Rate - 20 °C/min.	Final temp. - 215 °C
Rate A - 5 °C/min.	Final temp. - 285 °C
Final time - 2 mins	Total time - 26.75 mins.
Injector temp. - 275 °C	Detector temp. - 310 °C.

The injection port was in the splitless mode and the helium carrier gas flow rate was 10mL/min. The temperature program and GC conditions were also used in the previous report.

Several sample pretreatment methods were investigated and the method which gave better recoveries involved, spiking 1mL of whole blood at a concentration of 2μg/mL and then sonicating the sample for 15 minutes at room temperature. Next the blood was diluted with 6mL of 0.1M phosphate buffer (pH6.0), vortexed for 30 seconds and centrifuged at 2000 rpm for 15 minutes and the pellet removed. The extraction procedure used was that reported by Chen *et al*[26], except the prazepam internal standard was at a concentration of 20μg/mL.

Sample pretreatment techniques are essential for the analysis of whole blood as the presence of red blood cells causes problems with solid-phase extraction. Direct application of whole blood onto an SPE column would result in the column becoming blocked and producing low and irreproducible recoveries. The problem associated with protein binding in plasma and serum, is of greater importance with whole blood. Sonication of the blood was needed to break up the red blood cells, in order to free the drugs which were bound to them, and the dilution step was utilised to reduce the viscosity of the sample and prevent blockage of the column.

This method (Method A), was compared to three precipitation methods which are commonly used for removal of proteins; zinc sulphate-methanol (Method B), acetonitrile (Method C), and Methanol (Method D). The recoveries from using Method A were significantly better than for the three precipitation methods and was used for blood pretreatment.

The removal of cholesterol which was retained on the column, was not possible without losing drugs. However, since the retention time did not interfere with the GC analysis of the drugs, no steps were taken to remove it .

The recoveries from whole blood ranged from 81.2% and 102.5%, with relative standard deviations less than 8.2%. The reproducibility of Bond Elut Certify columns was also investigated by testing six different lots for the extraction of five different drugs. The recoveries were higher than 86% with a lot-to-lot relative standard deviation of less than 3.9%.

This extraction procedure was applied to CleanScreen DAU mixed-mode columns and the results were found to be comparable to those of Bond Elut Certify. The authors concluded that the method they had developed could be applied to other commercial mixed-mode bonded silica SPE columns that contain similar functional groups to those of Bond Elut Certify.

The use of Gas Chromatography with a nitrogen-phosphorous detector (GC-NPD) has also been investigated by Chen *et al*[22], and Zweipfenning *et al*[60] using the same extraction procedure as previously reported. The only difference in the procedure used by Zweipfenning *et al*, was the use of 3mL of 2% ammoniated ethyl acetate, instead of 2mL.

GC-NPD was used to overcome the sensitivity limits of FID. An NPD can detect drugs in the ng/mL range and helps improve the detection limit of basic compounds, due to its enhanced sensitivity towards nitrogen-phosphorus containing compounds.

The authors reported that, the peak height ratios of the drugs tested to prazepam were not reproducible when the oven temperature was programmed over a large range or when the temperature was increased to a higher rate. To compensate the temperature program was changed from that reported earlier[26]. The recoveries from whole blood were comparable to those reported using GC-FID for concentrations of 2μg/mL and the sensitivity increase was indicated by the detection of basic drug concentrations of 100 - 200 ng/mL.

2.3.3 Automation of SPE Procedure

SPE has a number of advantages over LLE outlined previously and one of these is the possibility of automation of the procedure.

Automation of present manual SPE methods would ideally improve reproducibility, give higher throughput, reduce labour costs, and extractions could be carried out after working hours.

Automated SPE systems available include, AASP® (Varian Sample Preparation Products: Harbor City, CA), ASPEC (Gilson: Middleton, WI), Millilab™ (Bedford:

Milford, MA), Auto Spe-ed™ (Applied Separations: Allentown, PA) and Zymate™ (Zymark: Hopkinton, MA).

Krogh *et al*[21], described an alternative method for the analysis of opiates in plasma and whole blood using the automated sequential trace enrichment of dialysate (ASTED) system for automated sample preparation. On-line dialysis is used as a purification step prior to chromatographic analysis, and with slight modifications can be used for the analysis of other basic drugs. Approximately 100 plasma or whole blood samples can be analysed in 24 hours.

Automated methods based on the extraction procedures used in manual SPE methods, tend to concentrate on analysis of individual or groups of related drugs and very few methods were reported for screening body fluids[7,24].

Chen *et al*[24], reported a semi-automated SPE procedure for screening using CleanScreen DAU columns using the ASPEC system. The extraction method used was based on the method reported by Chen *et al*[26], and the procedure was semi-automated, as the collection tubes had to be changed manually to collect the two fractions (A and B).

The recoveries ranged from 73% for methamphetamine to 96.3% for methylphenobarbital, with relative standard deviations of less than 5%. The recoveries were comparable to those acheived by manual SPE methods, and the reproducibilities were better.

Chen *et al*[7], then reported an improved fully automated SPE procedure using the ASPEC system with Bond Elut Certify with recoveries ranging from 80% to 103%, with relative standard deviations less than 4.6%.

2.4 Conclusion

Based on the available literature, the manual SPE method reported by Chen *et al*[27], was selected for the purposes of this project along with GC-FID for analysis. Bond Elut Certify was selected to compare the extraction efficiencies to those obtained from the experimental mixed-mode sorbents investigated.

Since an automated SPE system was not available, the project was designed to develop a manual SPE method, that could be modified to the requirements of an automated system as part of further work.

Chapter Three - Experimental

3.1 Materials and Reagents

All nine drugs and the Internal Standard(IS) were supplied by the Department of Forensic Medicine and Science from their own store. Table 4 contains the list of drugs and their physical/chemical properties. Stock standards were prepared by appropriate dilution with a (1:1) solvent mixture of methanol and ethyl acetate to 1mg/mL. All standards were stored in the freezer below 0 ^{o}C.

All chemicals were A.C.S. grade and all solvents were HPLC grade obtained from BDH Laboratory, England.

Columns:

Isolute® Confirm HCX, HCX-2, HCX-3 and HCX-4 mixed-mode and Isolute® ENV+ Solid-Phase Extraction (SPE) columns manufactured by International Sorbent Technology were all supplied by Crawford Scientific (Holm Street, Strathaven, ML10 6NB) along with Bond Elut Certify™ SPE columns and a Vac Elut box with 10 points manufactured by Analytichem International.

The Isolute® Confirm HCX, HCX-2, HCX-3 and HCX-4 mixed-mode columns differ in the non-polar character of the mixed-mode sorbent and the Isolute® ENV+ columns consist of hypercrossed polystyrene based polymer technology which retain polar analytes. Bond Elut Certify™ is a bonded silica solid SPE column, and is used to extract acidic, neutral and basic drugs.

Solutions:

0.1M Phosphate buffer. pH 6.0(1 Litre)

This was prepared by adding 900mL Deionised Water (D.I.) to 13.61g of KH_2PO_4 (M.W 136.09). The pH was adjusted to 6.0 with potassium hydroxide while stirring. This was made up to 1 litre with D.I. water, then stored in the refrigerator at < 3°C and discarded after 30 days.

0.1M Phosphate Buffer. pH 7.4(1 litre)

This was prepared as above except the pH was adjusted to 7.4 before adding D.I water to a total volume of 1 litre.

1.0M Potassium Hydroxide(100mL)

This was prepared by adding 100mL D.I water to 5.6g of potassium hydroxide (MW 56.11) and then stored at room temperature up to 3 months.

0.01M Acetic Acid. pH 3.3(100mL)

This was prepared by pipetting 57.5µL glacial acetic acid into 50mL of D.I water. The solution was mixed thoroughly before bringing the total volume to 100mL with D.I water and was then stored at room temperature up to two months.

2% Ammonium Hydroxide in Ethyl Acetate(100mL)

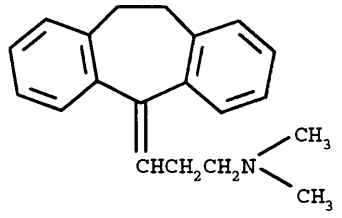
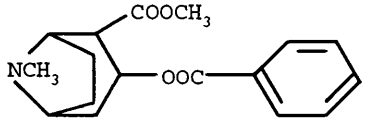
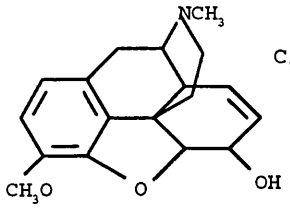
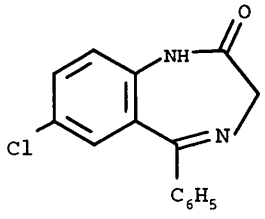
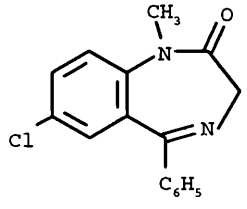
Prepared by adding 2mL of concentrated ammonium hydroxide to 98mL of ethyl acetate. This solution was sonicated for 5 minutes and was prepared fresh daily.

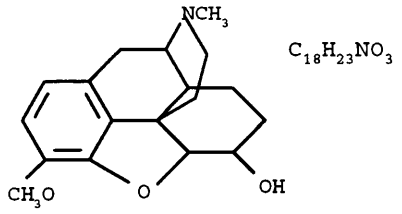
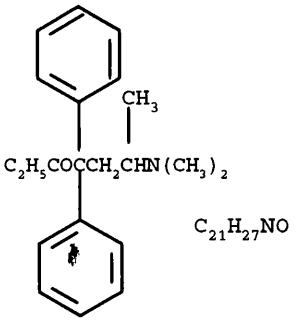
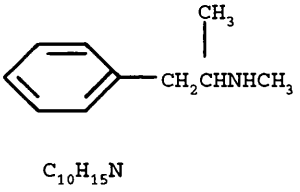
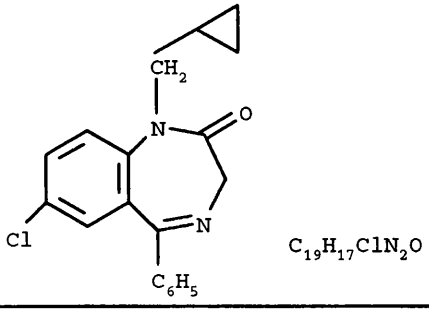
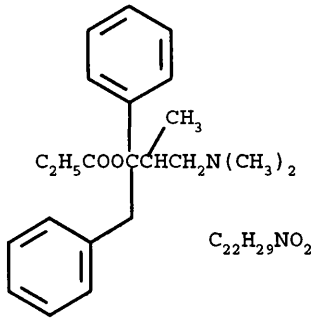
All glassware was cleaned in detergent, washed with water, dried, silanized and rinsed with methanol prior to use.

Blood:

Outdated whole blood was obtained from the Scottish Blood Transfusion Service

Table 4: Drug Information

Drug: **Amitriptyline** Mol. Wt.: 277.4 pKa: 9.4 Log P: 3.0 Protein Binding: 91 - 97%(plasma)	 $C_{20}H_{23}O$
Drug: **Cocaine** Mol. Wt.: 303.4 pKa: 8.6 Log P: - Protein Binding: -	 $C_{17}H_{21}NO_4$
Drug: **Codeine** Mol. Wt.: 317.4 pKa: 8.2 Log P: 0.6 Protein Binding: 7 - 25%(plasma)	 $C_{18}H_{21}NO_3 , H_2O$
Drug: **Desmethyldiazepam** Mol. Wt.: 285.3 pKa: 3.5, 12.0 Log P: - Protein Binding: 97%(plasma)	 $C_{15}H_{11}ClN_2O$
Drug: **Diazepam** Mol. Wt.: 284.7 pKa: 3.4 Log P: 2.7 Protein Binding: 98%(plasma)	 $C_{16}H_{13}ClN_2O$

Drug: **Dihydrocodeine** Mol. Wt.: 301.4 pKa: 8.8 Log P: -1.5 Protein Binding: -	
Drug: **Methadone** Mol. Wt.: 309.5 pKa: 8.3 Log P: 2.1 Protein Binding: 90%(plasma)	
Drug: **Methamphetamine** Mol. Wt.: 149.2 pKa: 10.1 Log P: - Protein Binding: -	
Drug: **Prazepam** Mol. Wt.: 324.8 pKa: 2.7 Log P: 3.7 Protein Binding: 97%(plasma)	
Drug: **Propoxyphene** Mol. Wt.: 339.5 pKa: 6.3 Log P: - Protein Binding: 70 - 80%(plasma)	

(Carluke). The blood was screened and was found to contain none of the drugs of interest in this project.

3.2 Instrumentation

A Hewlett-Packard 5890 series II Gas Chromatogram equipped with an FID was used for the screening and analysis. A 30m HP-1 fused-silica wide-bore capillary column (0.53mm i.d., film thickness 0.88μm) was installed in the GC. The injection port was in the splitless mode and helium was used as the carrier gas at a flow rate of 13mL/min.

The temperature program[26] was as follows:

Initial temp. - 80 °C	Initial time - 2 minutes
Initial rate - 20 °C/min.	Final temp. - 215 °C
Rate A - 5 °C/min.	Final temp. - 285 °C
Final time - 2 minutes	Total time - 26.75 mins.
Injector Temp. - 275 °C	Detector Temp. - 310 °C

Data acquisition used an HP3365 Series II ChemStation.

3.3 Experimental

The following experiments were designed to find the optimum operating conditions for the complete separation and identification of each of the basic drugs and the internal standard (IS), using Gas Chromatography(GC).

A stock standard mixture of all nine drugs and prazepam(IS) was prepared by dissolving weighed aliquots of each drug in a (1:1) mixture of methanol and ethyl acetate. The resultant solution was diluted with the solvent mixture to obtain a working standard solution containing each drug at an approximate concentration of 30 μg/ml.

3.3.1 Optimisation of GLC Drug Separation

Two different columns and two different temperature programs were used to investigate the optimum separation of the nine drugs.

Column 1: HP-5 fused-silica capillary column (30m × 0.32mm i.d. × 0.25μm film thickness, cross-linked 5% phenyl methyl silicone).

Column 2: HP-1 fused-silica wide-bore capillary column (30m × 0.53mm i.d., film thickness 0.88μm).

Temperature program No.1:

Initial temp. - 120 °C	Initial time - 2 minutes
Initial rate - 8 °C	Final temp. - 300
Final time - 5 minutes	Injector temp. - 275 °C
Detector temp. - 310 °C	

Temperature program No.2:

Initial temp. - 80 °C	Initial time - 2 minutes
Initial rate - 20 °C/min.	Final temp. - 215 °C
Rate A - 5 °C/min.	Final temp. - 285 °C
Final time - 2 minutes	Total time - 26.75 mins.
Injector Temp. - 275 °C	Detector Temp. - 310 °C

Procedure:

2μL of the working standard solution was injected onto Column 1, firstly using temperature program No.1, and then temperature program No.2. Column 2 was then fitted into the GC oven and 2μL of the working standard solution was injected at temperature program No.2.

Results and Discussion:

Figure 3.1 represents the separation of the nine drugs, using temperature program No.1. The numbering of the chromatogram for each drug, corresponds as follows; methamphetamine(1), methadone(2), amitriptyline(3), cocaine(3), Propoxyphene(3), codeine(4), dihydrocodeine(4), diazepam(5), desmethyldiazepam(6), prazepam(7).
It can be seen that there was co-elution between amitriptyline, cocaine and propoxyphene(3) and between codeine and dihydrocodeine(4).

In order to improve the separation between the drugs, the temperature program was changed to No.2, and the results are presented in Figure 3.2. The analysis time was shortened without any loss of separation, but none of the drugs coeluting at peaks (3) and (4) showed any sign of separating.

The next step was to use a variety of temperature programs to try to separate the two peaks with coeluting drugs. Rate A was increased, but this only resulted in a longer analysis time and no better separation. A second rate, Rate B was introduced, to try and separate the peaks, but did not produce any better separation.

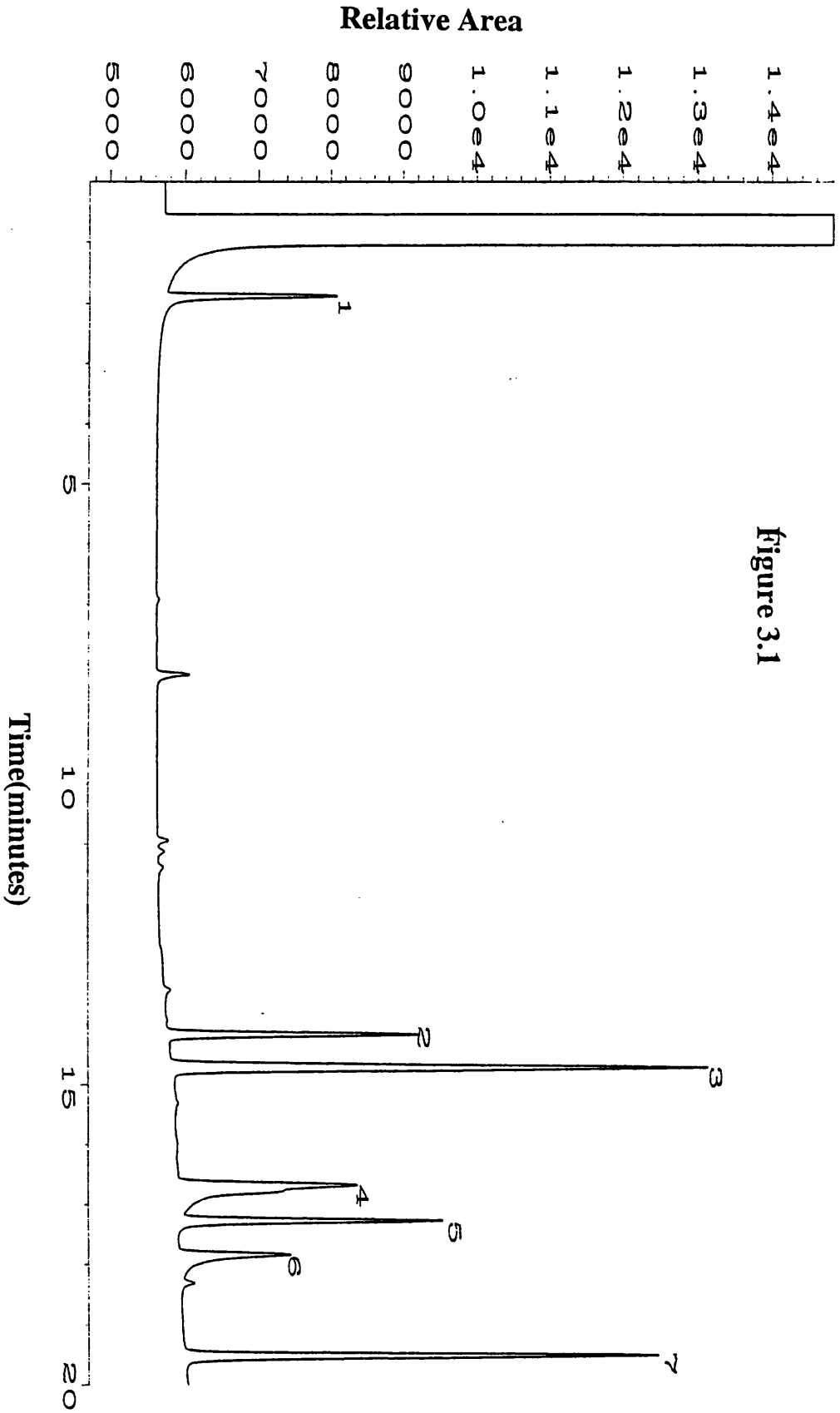

Relative Area
5000
6000
7000
8000
9000
1.0e4
1.1e4
1.2e4
1.3e4
1.4e4
1
2
3
4
5
6
7
5
10
15
20
Time(minutes)

Figure 3.1

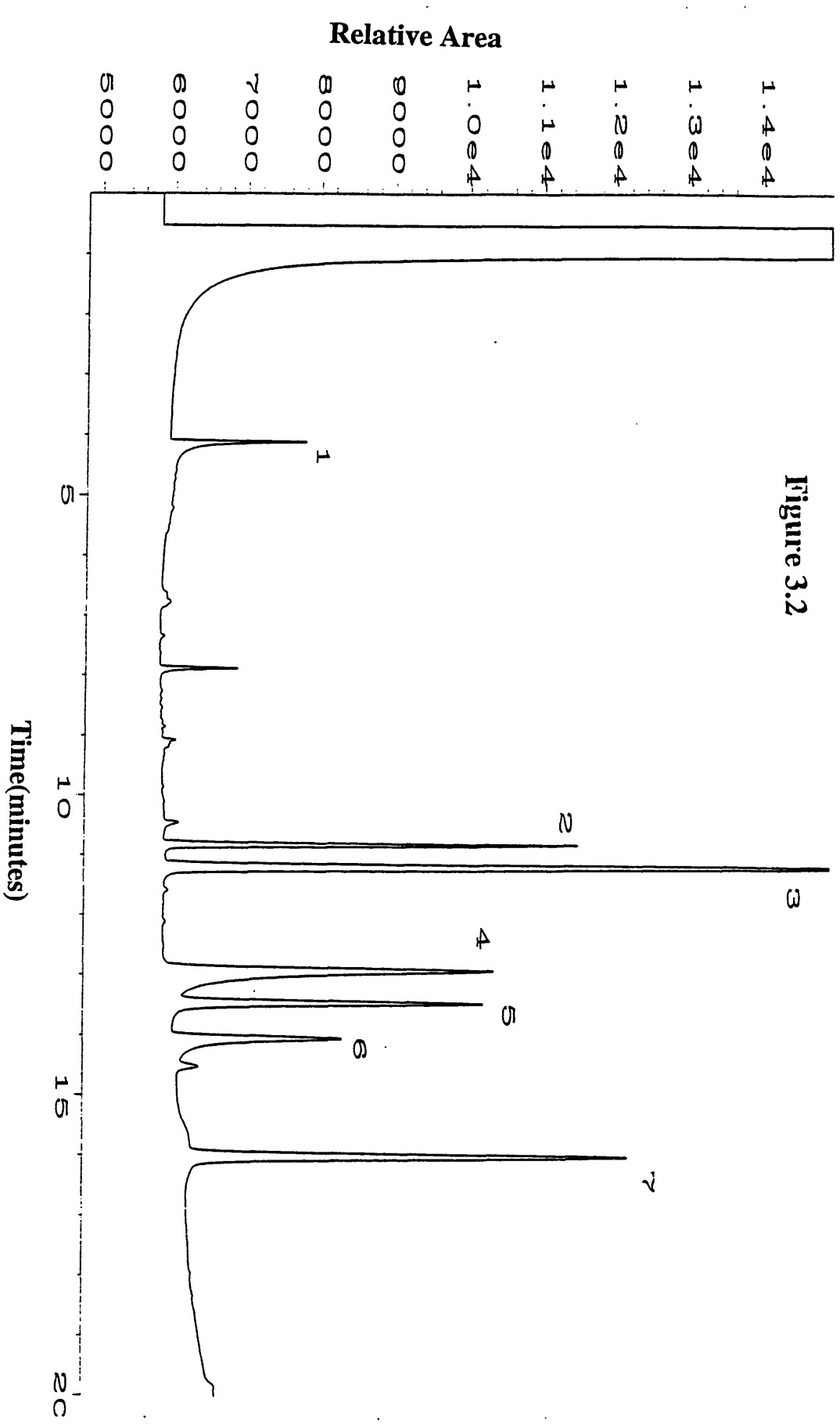

Relative Area
5000
6000
7000
8000
9000
1.0e4
1.1e4
1.2e4
1.3e4
1.4e4
Time(minutes)
5
10
15
20
1
2
3
4
5
6
7

Figure 3.2

Figure 3.3 represents the separation of the drugs on Column 2, at temperature program No.2., which are the conditions used by Chen et al[27]. There is no greater separation of the drugs than that produced using column 1, however, the peak height and shape have improved. At this point it was assumed that improving the separation would be too difficult due to the close proximity of the retention indices of the coeluting drugs.

Conclusion

Although both Columns 1 and 2 separated the drugs to the same extent, Column 2 gave better peak height and shape and was therefore used with the temperature program and conditions used by Chen et al[27]. Choosing these conditions also allowed the comparison of the results obtained from their study with the results obtained from this project.
The drugs were separated into three groups to allow the analysis of all nine groups.

A modification of the GC conditions used by Chen et al[27] was necessary for the optimum separation of the drugs. The Helium carrier gas flow rate was changed to 13mL/min. from 10mL/min to give an analysis time of 20 minutes.

Identification was possible by calculation of the Retention Indices of the drugs represented in Table 5.

Retention Index (RI):

In 1985, Kovats developed the retention index system for partition chromatography based on n-alkanes as reference substances in order to identify solutes from

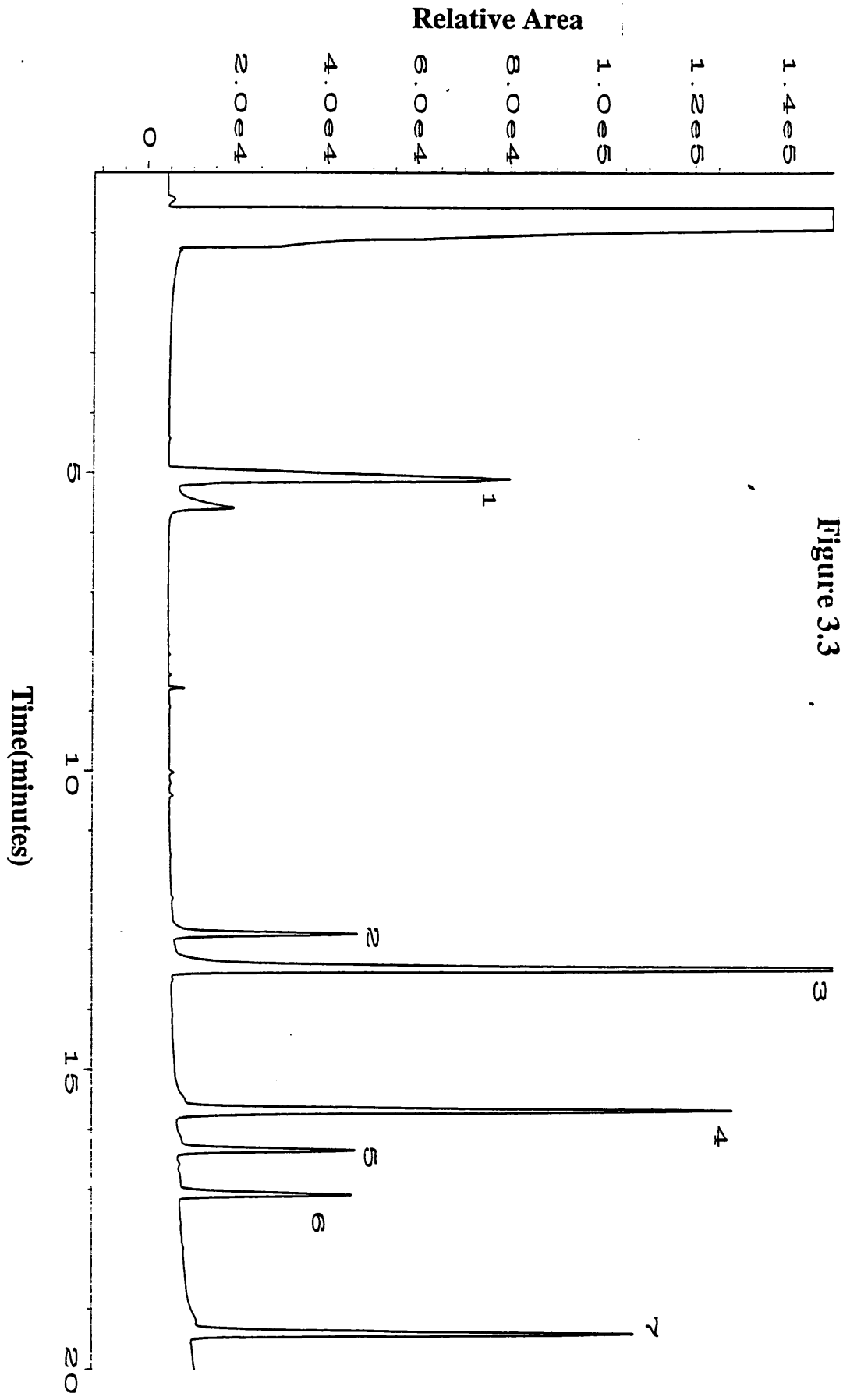

Relative Area
0
2.0e4
4.0e4
6.0e4
8.0e4
1.0e5
1.2e5
1.4e5
Time(minutes)
5
10
15
20
1
2
3
4
5
6
7

Figure 3.3

chromatograms. The RI is related to the retention time, but is more reliable and more useful for comparibility and reproducibility of retention data.

By definition, the RI for a normal alkane is equal to 100 times the number of Carbon atoms in the compound regardless of the column packing, temperature or other chromatographic conditions[61].

Due to the linear relationship between the logarithm of the net retention times of n-alkanes and the number of carbon atoms in the molecules, it is possible to relate the retention time of the unknown compound to that of the n-alkanes eluting before and after it.

Calculation of Retention Indices:

A mixture of ten alkanes (C_9, C_{10}, C_{11}, C_{12}, C_{15}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}) was prepared in hexane and 2μL of the resultant solution was injected into the GC at the conditions stated to produce Figure 3.4.

The retention index RI(A) of a compound A can be calculated using the following equation:

$$RI(A) = [RI(Y) - RI(X)] \frac{\log[t(A) \div t(X)]}{\log[t(Y) \div t(X)]} + RI(X) \quad .....(1)$$

where,

t(A) = net retention time of substance A

t(X) = net retention time of the n-alkane C_xH_{2x+2} eluting immediately before A

t(Y) = net retention time of the n-alkane C_yH_{2y+2} eluting immediately after A

RI(A), RI(X), etc. = retention index of substances A, X, etc...

Figure 3.5, represents the graphical means by which the RI of a compound can be determined.

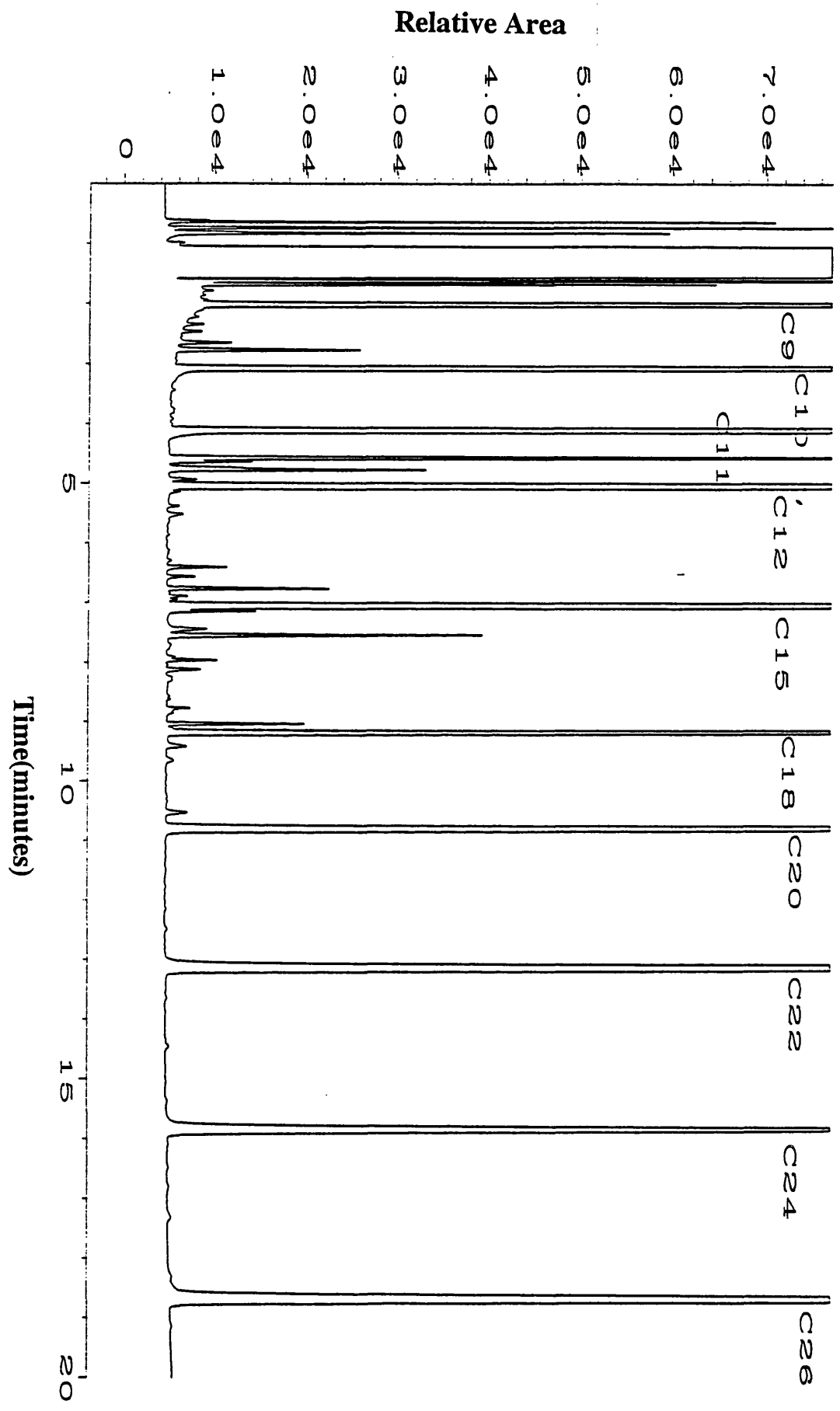

Relative Area
0
1.0e4
2.0e4
3.0e4
4.0e4
5.0e4
6.0e4
7.0e4
C9
C10
C11
C12
C15
C18
C20
C22
C24
C26
5
10
15
20
Time(minutes)

Figure 3.4

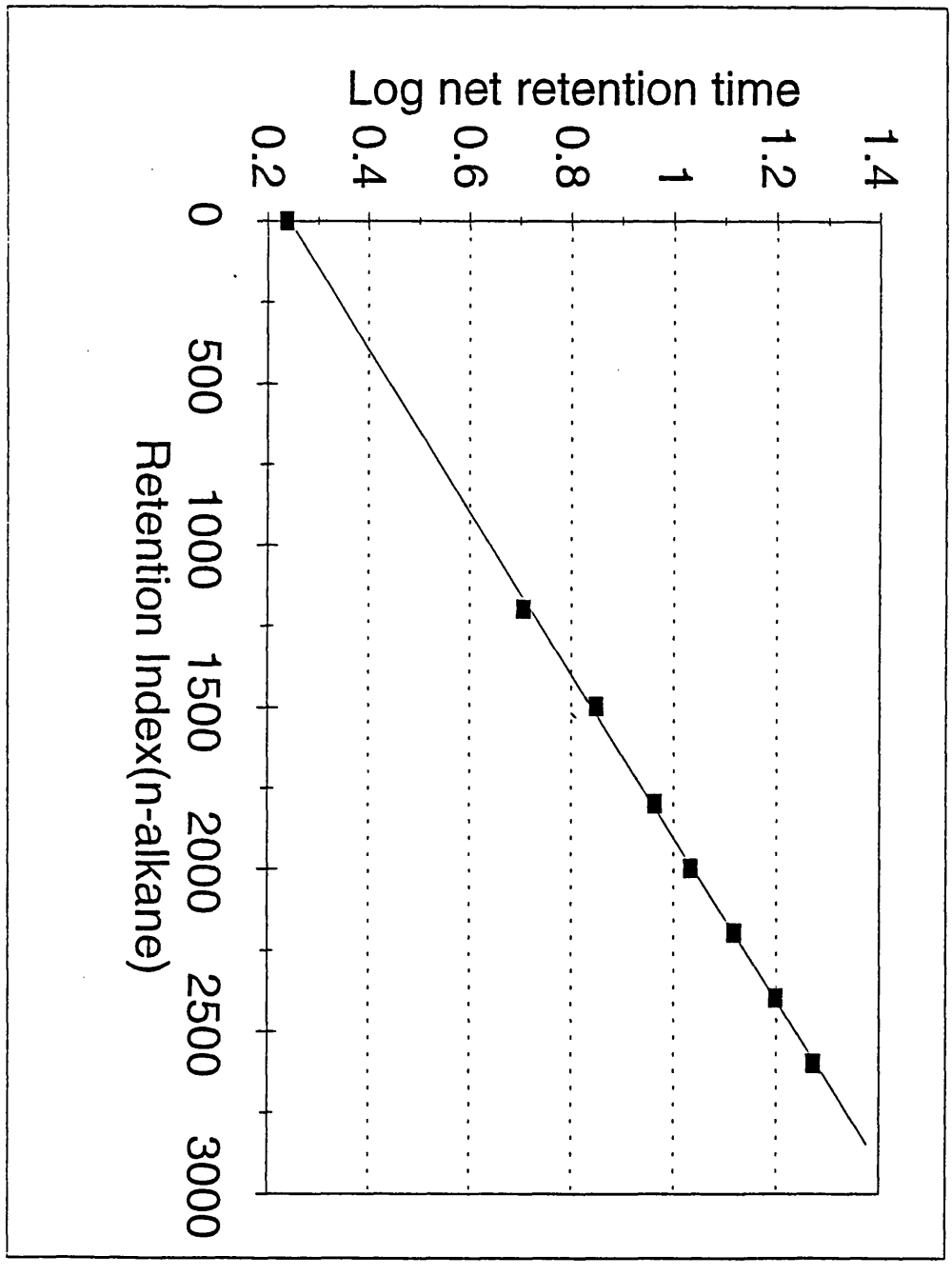

Log net retention time
0.2
0.4
0.6
0.8
1
1.2
1.4
0
500
1000
1500
2000
2500
3000
Retention Index(n-alkane)

Figure 3.5

Table 5: Retention Indices

Elution Order of Drugs	Retention Index[61]	Calculated RI (Eqn. 1)	Retention Time (mins)
Methamphetamine	1175	1165	4.719
Methadone	2145	2146	12.458
Cocaine	2187	2188	12.991
Propoxyphene	2190	2192	13.040
Amitriptyline	2194	2194	13.057
Codeine	2375	2390	15.688
Dihydrocodeine	2390	2390	15.822
Diazepam	2428	2417	16.049
Desmethyldiazepam	2490	2467	16.736

3.3.2 Reproducibility of Retention times

For the purposes of identification of drugs, an assessment of the reproducibility of retention times(RT) is required. This was done as follows:

Procedure:

The retention times of all nine drugs and the IS were tested for within and between-day run variability. 2μL of the working solutions were injected onto the GC ten times. The results are represented in Table 6.

Table 6: Reproducibility of Retention Times

Drug	Within Day Runs			Between Day Runs		
	Mean	Std. Dev	C.V%	Mean	Std. Dev.	C.V%
Methamphetamine	4.719	7.4E-4	0.0156	4.720	1.8E-3	0.0376
Methadone	12.458	7.4E-4	0.0059	12.463	3.2E-3	0.0259
Amitriptyline	13.057	1.7E-4	0.0013	13.057	4.1E-3	0.0315
Codeine	15.688	9.5E-4	0.0061	15.683	2.1E-3	0.0135
Diazepam	16.049	6.7E-4	0.0042	16.048	7.2E-3	0.0450
Desmethyldiazepam	16.736	6.8E-4	0.0040	16.738	5.4E-3	0.0325
Cocaine	12.991	6.3E-4	0.0049	12.990	2.7E-3	0.0217
Propoxyphene	13.040	3.0E-4	0.0023	13.039	2.7E-3	0.0204
Dihydrocodeine	15.822	4.2E-4	0.0027	15.822	9.3E-4	0.0059
Prazepam	19.091	5.1E-4	0.0042	19.094	6.8E-3	0.0325

Results:

The results for the within-day runs shows higher reproducibility with standard deviations ranging from 1.7×10^{-4} to 9.5×10^{-4}, with the coefficient of variance (%) ranging from 0.0013 to 0.0156. The between-day runs do not show as high reproducibility as the within-day runs, with standard deviations ranging from 9.3×10^{-4} to 7.2×10^{-3}, and the coefficient of variance (%) ranging from 0.0059 to 0.0450. However the use of the HP-1 column at the temperature program and conditions stated was found to be suitable for the separation of the basic drugs and for the purpose of this research.

3.3.3 Linearity in Detector Response

Linearity in the detector response is necessary for accurate quantification. For this purpose and also for finding the lower limit of detection, the detector response for each of the drugs was investigated.

The Limit of Detection is defined as the minimum level of analyte detectable under the assay conditions, while the Limit of Determination is the minimum level of analyte which can be reliably determined. Both these factors are dependent on the sensitivity of the analyte and the baseline noise levels at the time of determination, which are also dependent on the performance of the operating system.

The parameters can be calculated as follows:

Limit of Detection = Weight which produces a response which is double the amplitude of the noise.

Limit of Determination = weight which produces a response which is six times the amplitude of the noise or three times the Limits of Detection.

Procedure:

Serial dilutions of the stock standards were obtained to give two ranges of concentrations which were prepared with two different fixed amounts of internal standard (IS). The use of two different concentrations of IS was required to produce a peak height response similar to that produced by the drugs. Each concentration was injected (2μL) five times. The detector response ratio can be calculated using the ratio of the peak height or area of the drug standard to that of the IS, but for the

purposes of this project the peak height ratios proved to be more accurate and were calculated as follows:

$$\text{Response Ratio} = \frac{\text{Peak Height of Drug Std}}{\text{Peak Height of IS}}$$

Results:

The two sets of calibration curves for each of the nine unextracted standards are presented in Appendix 1 and were constructed by plotting the equivalent concentration of the drug in blood, versus the response ratio. The integration results for these graphs are shown in Table 7, where,

$$\mathbf{Y = Ax + C,}$$

and, y = response ratio, x = drug concentration.

Table 7: Calibration Curve Data

Drug	level	A	C	R^2
Methamphetamine	µg ng	0.37063 0.00231	-0.00719 -0.02526	0.99984 0.99958
Methadone	µg ng	0.38905 0.00439	0.01757 -0.00607	0.99917 0.99987
Amitriptyline	µg ng	0.72505 0.00500	-0.028 0.00139	0.99994 0.99982
Codeine	µg ng	0.23351 0.00074	-0.02816 -0.00355	0.99992 0.99965
Diazepam	µg ng	0.06024 0.00058	-0.00038 -0.00453	0.99990 099926
Desmethyldiazepam	µg ng	0.37208 0.00290	-0.00285 -0.06421	0.99952 0.99953
Cocaine	µg ng	0.40084 0.00221	-0.06098 -0.00946	0.99959 0.99968
Propoxyphene	µg ng	0.51278 0.00124	-0.04939 0.009189	0.99970 0.99943
Dihydrocodeine	µg ng	0.09959 0.00061	-0.01842 0.002777	0.99936 0.99959

The results show high linearity and reproducibility, for all the drugs at concentrations ranging from therapeutic levels (ng/mL) to fatal levels (μg/mL).

Table 8, includes the theoretical limits of detection for each of the nine basic drugs and the limit of determination, which were determined by serial dilution of the stock standard solutions.

Table 8: Limits of Detection and Limits of Determination(ng/mL)

Drug	Limit of Detection	Limit of Determination
Methamphetamine	7.5	22.5
Methadone	< 8.0	24.0
Amitriptyline	< 8.5	25.5
Codeine	9.0	27.0
Diazepam	16	48.0
Desmethyldiazepam	8.2	24.6
Cocaine	8.0	24.0
Propoxyphene	8.5	25.5
Dihydrocodeine	8.0	24.0

3.4 Development of Extraction Procedure

The following experiments were used to optimise the extraction procedure for efficient recovery of the nine drugs, using the three experimental mixed-mode SPE columns. The three extraction methods were carried out on all six SPE columns and the percentage recoveries for each drug and column combination were compared. The methods differed in the pH of the buffer used and the volume of the eluting solvent.

3.4.1 Application of Blood to SPE columns

Procedure: Method A

Sample Preparation - 100μL of the appropriate working standard solution was added to a glass vial and then diluted with 1mL of outdated blood, to give approximate drug concentrations of 3μg/mL. The sample was then sonicated for 15 minutes at room temperature in a sonic bath before dilution with 6mL of 0.1M phosphate buffer (pH6.0). The diluted blood was vortexed for 30 seconds then centrifuged at 2000 rpm for 15 minutes and the pellet discarded.

Extraction procedure - The extraction was carried out on each of the six SPE columns, which were installed on a 10 point Vac Elut box. The nine extraction steps are described below and are summarised in a schematic diagram in Figure 3.6.

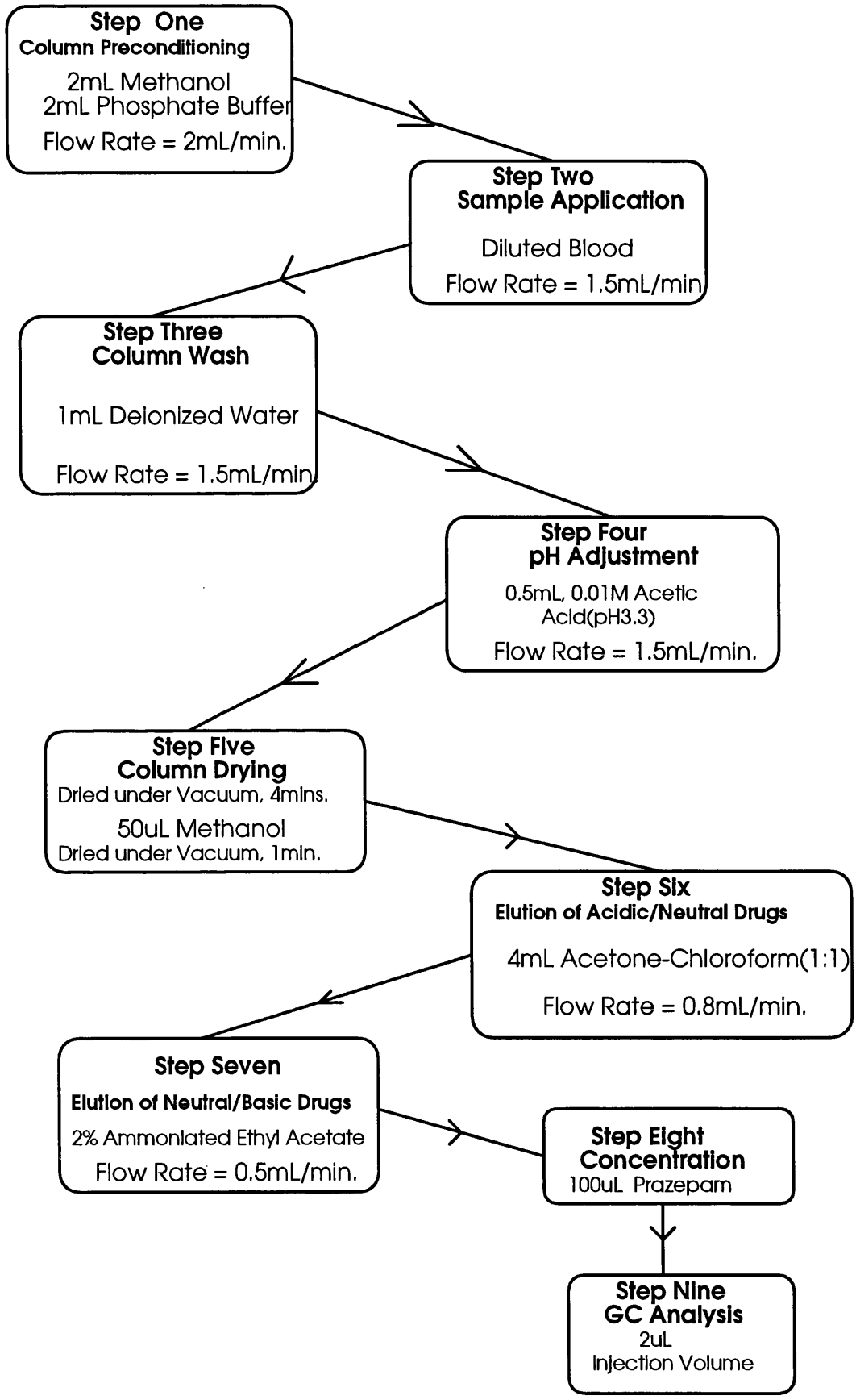


Figure 3.6: Schematic Diagram of Extraction Procedure

Step 1:

2mL of Methanol was introduced onto the column, followed by 2mL of phosphate buffer (pH6.0) at a flow rate of 2mL/min as a preconditioning step.

Step 2:

The blood sample was applied to the column at a flowrate of 1.5mL/min.

Step 3:

The column was then washed with 1mL of deionized water at a flowrate of 1.5mL/min.

Step 4:

The pH was adjusted by passing 0.5mL of 0.01M Acetic Acid (pH3.3) at a flowrate of 1.5mL/min.

Step 5:

The column was dried under a vacuum for 4 minutes before addition of 50μL of methanol, followed by a further 1 minute of drying under vacuum.

Step 6:

The column outlet and manifold basin were wiped clean with a tissue before adding 4mL of acetone-chloroform(1:1). The extract (Fraction A) was collected in a labelled evaporation tube at a flowrate of 0.8mL/min.

Step 7:

2mL of 2% ammoniated ethyl acetate was drawn through the column at a rate of 0.5mL/min and collected in a separate labelled evaporation tube (Fraction B).

Step 8:

100μL of Prazepam (33μg/mL) was added to each fraction, before both were evaporated at 40°C in a water bath under a nitrogen stream until approximately 100 μL of solvent remained in each tube.

Step 9:

2μL of each fraction was injected into the GC.

Results and Discussion:

The recoveries for the six columns using Method A are shown in Table 9 and were found to vary depending on the drug extracted and the column used. Each percentile given in the table is the mean value of three separate extractions, none having a Standard deviation in excess of 9.6. Columns HCX-2, HCX-3, HCX-4 and Bond Elut Certify(BEC) gave recoveries of greater than 60% for all of the drugs.

Addition of the internal standard to the blood sample before the extraction would require taking into account the extraction recovery of prazepam(IS).

Table 9: Percentage Recoveries - Method A (pH6.0)

Drugs	HCX	HCX-2	HCX-3	HCX-4	ENV+	BEC
Methamphetamine	46.8	75.8	79.1	75.0	46.5	103
Methadone	89.1	84.8	88.3	86.9	82.7	83.3
Amitriptyline	79.4	60.0	71.6	75.3	57.4	76.5
Codeine	68.3	81.8	70.2	68.5	104	83.5
Diazepam	73.3	66.4	67.7	70.4	66.7	70.3
Desmethyldiazepam	72.1	68.6	70.0	70.6	63.2	71.2
Cocaine	99.5	80.4	101	105	112	90.0
Propoxyphene	84.5	93.6	91.2	83.6	88.8	89.8
Dihydrocodeine	73.0	88.4	63.5	74.2	77.3	85.4

3.4.2 Effect of pH on Recovery

Procedure: Method B

The sample preparation and extraction procedure for Method B was the same as for Method A, except that the 0.1M phosphate buffer was changed from pH6.0 to pH7.4.

Results and Discussion:

Table 10 contains the data for both Methods A and B. Method B gave better or comparable results with the odd exception. Each percentile given in the table is the

mean value of three separate extractions, none having a standard deviation in excess of 9.6 for Method A and 7.5 for Method B. Column HCX gave recoveries greater than 66.4%, and HCX-2 (> 64.2%), HCX-3 (> 62.8%) and HCX-4 (> 60.4%).

Table 10: Percentage Recoveries - Methods A and B

Drugs	HCX	HCX-2	HCX-3	HCX-4	ENV+	BEC
Methamphetamine(A)	46.8	75.8	79.1	75.0	46.5	103
(B)	83.9	87.9	79.2	80.9	24.0	89.3
Methadone(A)	89.1	84.8	88.3	86.9	82.7	83.3
(B)	98.4	102	84.7	60.4	68.8	77.7
Amitriptyline(A)	79.4	60.0	71.6	75.3	57.4	76.5
(B)	67.5	67.9	62.8	62.0	65.3	55.5
Codeine(A)	68.3	81.8	70.2	68.5	104	83.5
(B)	70.9	85.1	80.2	86.6	108	85.8
Diazepam(A)	73.3	66.4	67.7	70.4	66.7	70.3
(B)	74.9	69.1	74.8	62.8	72.3	75.4
Desmethyldiazepam(A)	72.1	68.6	70.0	70.6	63.2	71.2
(B)	66.4	64.2	71.6	90.1	64.6	73.7

3.4.3 Effect of pH and Increased Solvent Volume

Procedure: Method C

Using the 0.1M phosphate buffer (pH7.4) as in Method B, the sample preparation and extraction steps were the same as Methods A and B except that, 3mL of 2% ammoniated ethyl acetate was used to elute the basic drugs in Step 7 instead of 2mL.

Results:

The results for Method C shown in Table 11 were very disappointing, with the majority of drug recoveries having decreased in comparison to Method B. As in each case before, the percentile given in the table is the mean value of three separate extractions, none having a standard deviation in excess of 9.6 for Method A, 7.5 for Method B and 11.4 for Method C.

Columns HCX and HCX-3 had recoveries greater than 51.2 and 50.8% respectively.

Table 11: Percentage Recoveries - Methods A, B and C

Drugs	HCX	HCX-2	HCX-3	HCX-4	ENV+	BEC
Methamphetamine(A)	46.8	75.8	79.1	75.0	46.5	103
(B)	83.9	87.9	79.2	80.9	24.0	89.3
(C)	103	97.3	90.0	57.7	19.9	84.3
Methadone(A)	89.1	84.8	88.3	86.9	82.7	83.3
(B)	98.4	102	84.7	60.4	68.8	77.7
(C)	81.1	74.7	63.1	82.1	59.5	72.1
Amitriptyline(A)	79.4	60.0	71.6	75.3	57.4	76.5
(B)	67.5	67.9	62.8	62.0	65.3	55.5
(C)	51.2	49.6	64.8	49.3	54.8	45.6
Codeine(A)	68.3	81.8	70.2	68.5	104	83.5
(B)	70.9	85.1	80.2	86.6	108	85.8
(C)	102	82.8	80.6	79.5	103	99.5
Diazepam(A)	73.3	66.4	67.7	70.4	66.7	70.3
(B)	74.9	69.1	74.8	62.8	72.3	75.4
(C)	59.9	60.5	62.2	60.5	59.1	55.7
Desmethyldiazepam(A)	72.1	68.6	70.0	70.6	63.2	71.2
(B)	66.4	64.2	71.6	90.1	64.6	73.7
(C)	54.5	50.9	50.8	41.4	48.8	59.2

3.4.4 Discussion and Summary of Results

The peak height ratios of each drug to that of Prazepam was used for quantitation. The % recoveries from whole blood shown in Tables 9, 10 and 11 were calculated by comparison of peak height ratios (P.H.R) of the extract with the calibration graph produced from drug standards.

Figures 3.7, 3.8 and 3.9 represent the extracts produced from Fractions A and B using phosphate buffers at pH 6.0 and 7.4 on Column HCX-3. The chromatogram produced using pH6.0 buffer was cleaner than that of the pH7.4 extract. However, there was no problem with co-extracting materials in either case.

The results shown in Table 11, show good recoveries for quantification purposes. Using method A, columns HCX-2, -3, -4 and BEC gave recoveries of greater than 60% for all drugs, while Method B gave better or comparable results with the odd exception. Method C was excluded as a greater solvent volume was required and this did not improve the drug recoveries.

The standard deviations from the three extraction methods, were higher than those reported in the literature from similar experiments[26,27]. This may be due to the use of water flow to create the required pressure for regulating the flow through the SPE columns. The water pressure varied throughout the day and so it was very difficult to maintain the required flow rates. As a consequence of this, a greater variation in the recoveries was observed and this in turn contributed to the higher standard deviations.

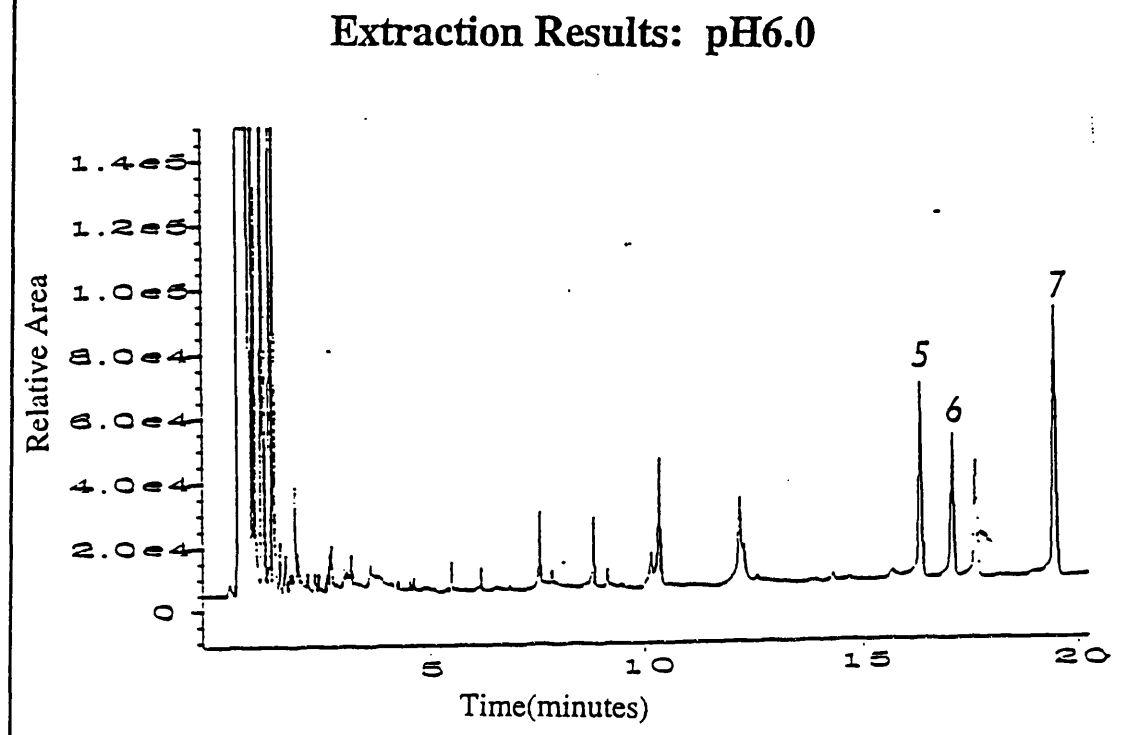


Figure 3.7A

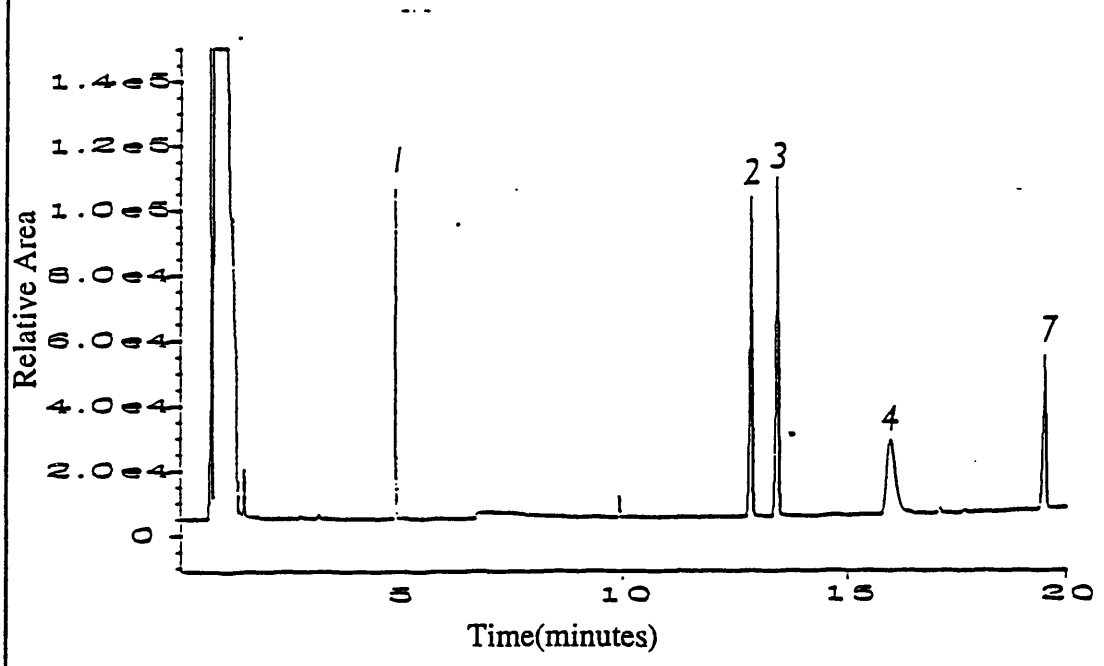


Figure 3.7B

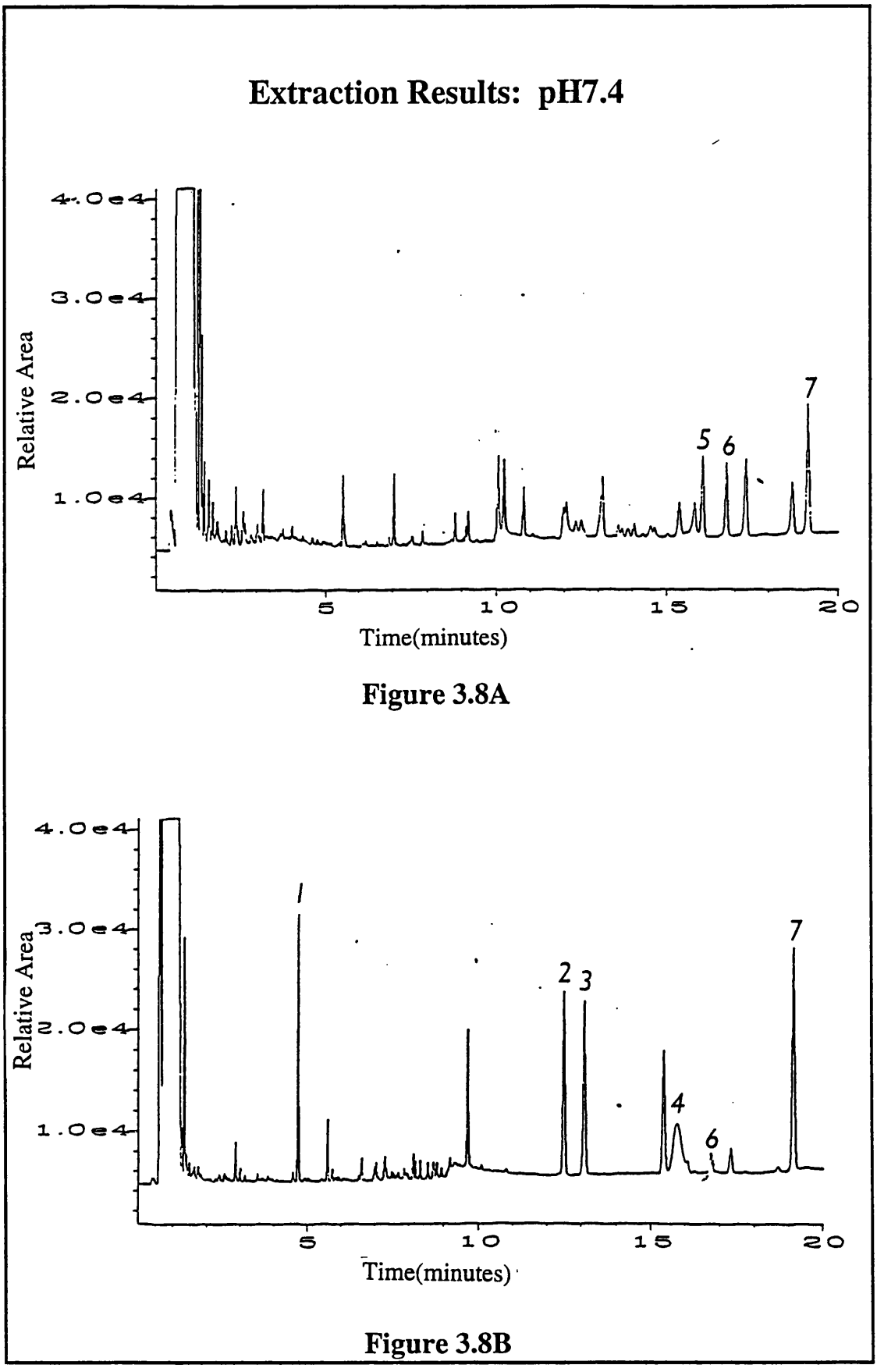


Figure 3.8A

Figure 3.8B

Extraction Results: pH7.4

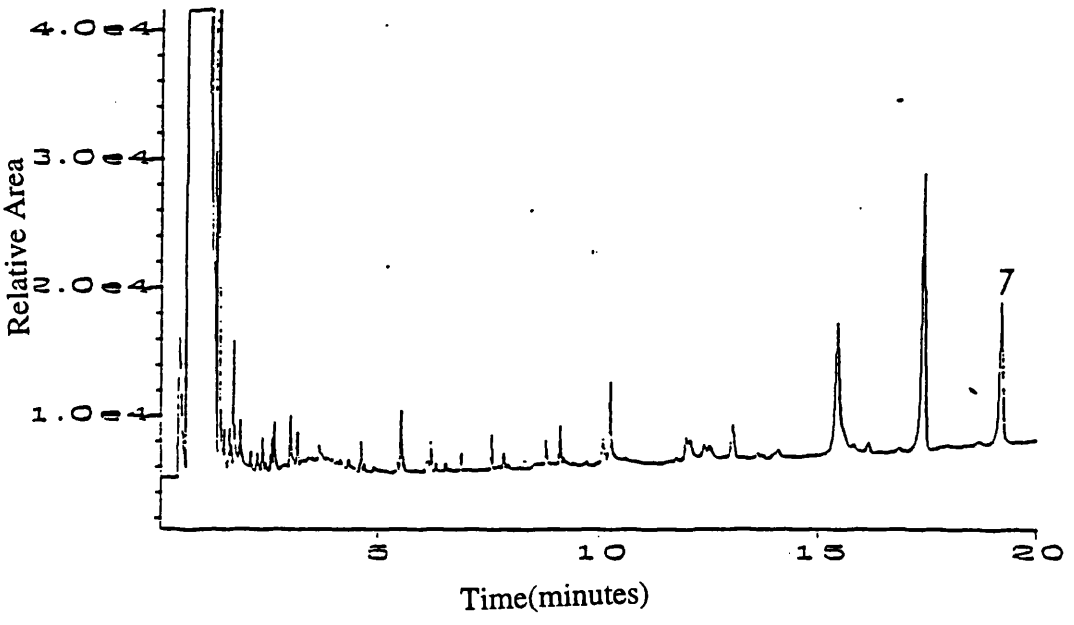


Figure 3.9A, blank

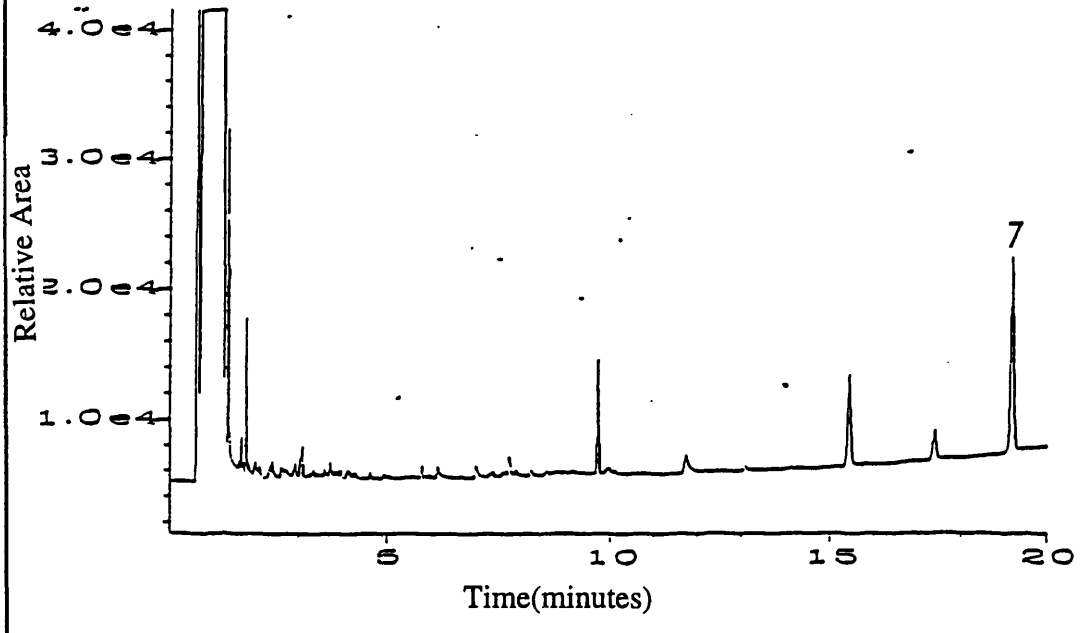


Figure 3.9B, blank

3.5 Establishment of Extraction Procedure

3.5.1 Introduction

It was necessary to demonstrate a linear response for the procedure to enable quantitative analysis for the basic drugs used from sub-therapeutic levels to fatal levels.

Due to time restrictions, only the HCX-3 column was used for this experiment, using the extraction procedure, Method B.

3.5.2 Procedure

Calibration curves were constructed for each drug by plotting the response ratio of the peak height of the drug/IS against their corresponding spiked concentrations in blood.

Standards were spiked in the range of 0µg/mL to 30µg/mL. The blood samples were extracted on the HCX-3 column using Method B.

3.5.3 Results

The calibration curves for all of the drugs are presented in Appendix 2, and were constructed by plotting the concentration of the drug in blood, versus the response ratio. The graphs are all linear over the specified range. The correlation coefficients are presented in Table 12 and range from 0.99937 to 0.99985.

Table 12: Correlation Coefficients for Extraction Procedure

Drug	R^2
Methamphetamine	0.99978
Methadone	0.99985
Amitriptyline	0.99953
Codeine	0.99978
Diazepam	0.99952
Desmethyldiazepam	0.99970
Cocaine	0.99985
Propoxyphene	0.99937
Dihydrocodeine	0.99976

These results indicate that, it is possible to extract basic drugs from HCX-3 from blood using method B and therefore the extraction procedure can be utilised as a preliminary step for further analysis.

Prazepam(IS) was included in the extraction of the sample to give a quantitative assay. This ensures that any loss of the drug during the extraction or analysis is accounted for.

Prazepam was chosen as the internal standard as it satisfied the following requirements;

(a) Internal standard (IS), should be resolved completely from all other peaks.

(b) IS should elute close to the peaks of interest or in the middle of the range if many peaks are involved.

(c) The concentration of IS should be approximately similar to the drug of interest.

(d) It should have physical and chemical properties similar to the drug of interest.

Chapter Four: Conclusion

4.1 Aim of Study

Basic drugs are frequently encountered in society, whether in a therapeutic capacity or increasingly as drugs of abuse. Forsyth[62], reported polydrug use amongst participants in the Glasgow dance scene. It was found that 70% of the group had used stimulants and hallucinogens (cocaine, amphetamine, psilocybin and ecstacy). The mean number of drugs used was 10.7, with a range of 3 - 18, with a total of 51 discrete drugs having been used.

The nine basic drugs in this study were chosen to represent drugs commonly abused in Scotland and for their differing physico-chemical properties. Many different methods are available for the analysis of body fluids, especially for urine and plasma. Whole blood samples proved difficult to analyse due to interference from co-extracting lipids and the binding power of the blood proteins.

Solid-phase extraction (SPE) was introduced as a simpler alternative to liquid-liquid extraction (LLE), but whole blood tended to block the columns and prevent accurate and reproducible recoveries. Sample pretreatment methods such as dilution and sonication, overcame these difficulties and resulted in a less viscous sample which allowed the flow of whole blood through the SPE column.

In the present study, the extraction efficiencies of three experimental mixed-mode SPE columns was investigated for the recovery of basic drugs from whole blood. These experimental columns were then compared to three mixed-mode SPE columns

which are routinely used in clinical and forensic laboratories. Gas Chromatography with a flame ionisation detector (GC-FID) was used for the analysis of the drugs in conjunction with retention indices.

The GC oven was fitted with an HP-1 fused silica wide-bore capillary column, which was found to give greater separation and resolution than the HP-5 capillary column. The temperature program stated by Chen et al[27], was found to be sufficient with a slight modification to the Helium carrier gas flow rate, from 10mL/min. to 13mL/min. The conditions used were found to be suitable for the determination of all nine drugs in whole blood at therapeutic levels.

In order to compare the columns, three different extraction methods were investigated, differing in the pH of the buffer and the volume of solvent used to extract the basic drugs. Using a modification of the method stated by Chen et al[27], (Method A), involving a buffer pH of 6.0, columns HCX-2, HCX-3, HCX-4 and Bond Elut Certify (BEC) gave the best recoveries for all nine drugs, under these conditions.

When the pH of the extraction system is approximately 6.0, the weakly basic drugs (diazepam and desmethyldiazepam) were in their un-ionised forms and were absorbed by the non-polar functional groups of the mixed-mode sorbent. The basic drugs (methamphetamine, methadone, amitriptyline, cocaine, propoxyphene, dihydrocodeine and codeine) were retained by both the polar and non-polar retention characteristics of the column.

The pH of the extraction system was adjusted to 3.3 using 0.01M acetic acid, in order to selectively elute the drugs from the column. The weakly basic drugs with pKa's

close to 3.3 were in their non-ionised form and so were eluted using 4mL of acetone:chloroform (1:1).

The basic drugs with pKa values higher than pH3.3 and the remaining weakly basic drugs were in their ionised forms and were retained on the column until, the addition of 2mL, 2% ammoniated ethyl acetate which firstly discharged and then eluted the drugs by ethyl acetate.

When the pH was changed to 7.4, (Method B), columns HCX, HCX-2, HCX-3 and HCX-4 gave comparable or better recoveries than Bond Elut Certify or ENV+ columns for each of the drugs under these conditions. Method B also gave better or comparable recoveries in comparison to Method A, for all six columns and each of the nine drugs.

Changing the pH from 6.0 to 7.4, increased the recoveries of the majority of the basic drugs and this is most probably a result of the increase in the number of drugs in their un-ionised form, which leads to a greater retention of the drugs by both the non-polar and polar retention characteristics of the column.

Method C involved the use of an increased elution volume, but this resulted in a reduction in the drug recoveries for each of the six SPE columns. One reason for this could be due to the loss of drugs during the increased evaporation time.

In conclusion, both methods A and B can be used to successfully extract basic drugs from whole blood. Both methods are simple, rapid and reproducible, ideal for routine analysis in a forensic laboratory.

4.2 Future Work

In order to establish the extraction procedure for each of the columns, a linear response needs to be demonstrated, for the procedure, and this in turn enables quantitative analysis of the basic drugs. Due to time restrictions, only the HCX-3 column was used to estalish the extraction procedure, Method B and so this procedure should also be carried out for each of the remaining five columns.

In addition, further studies would be of great interest, using the experimental columns for the extraction of body fluids other than whole blood, e.g. urine, vitreous humor and hair. The use of alternative detection methods would also provide information on the sensitivity of each method for the drugs analysed. A comparison between Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) is one such case.

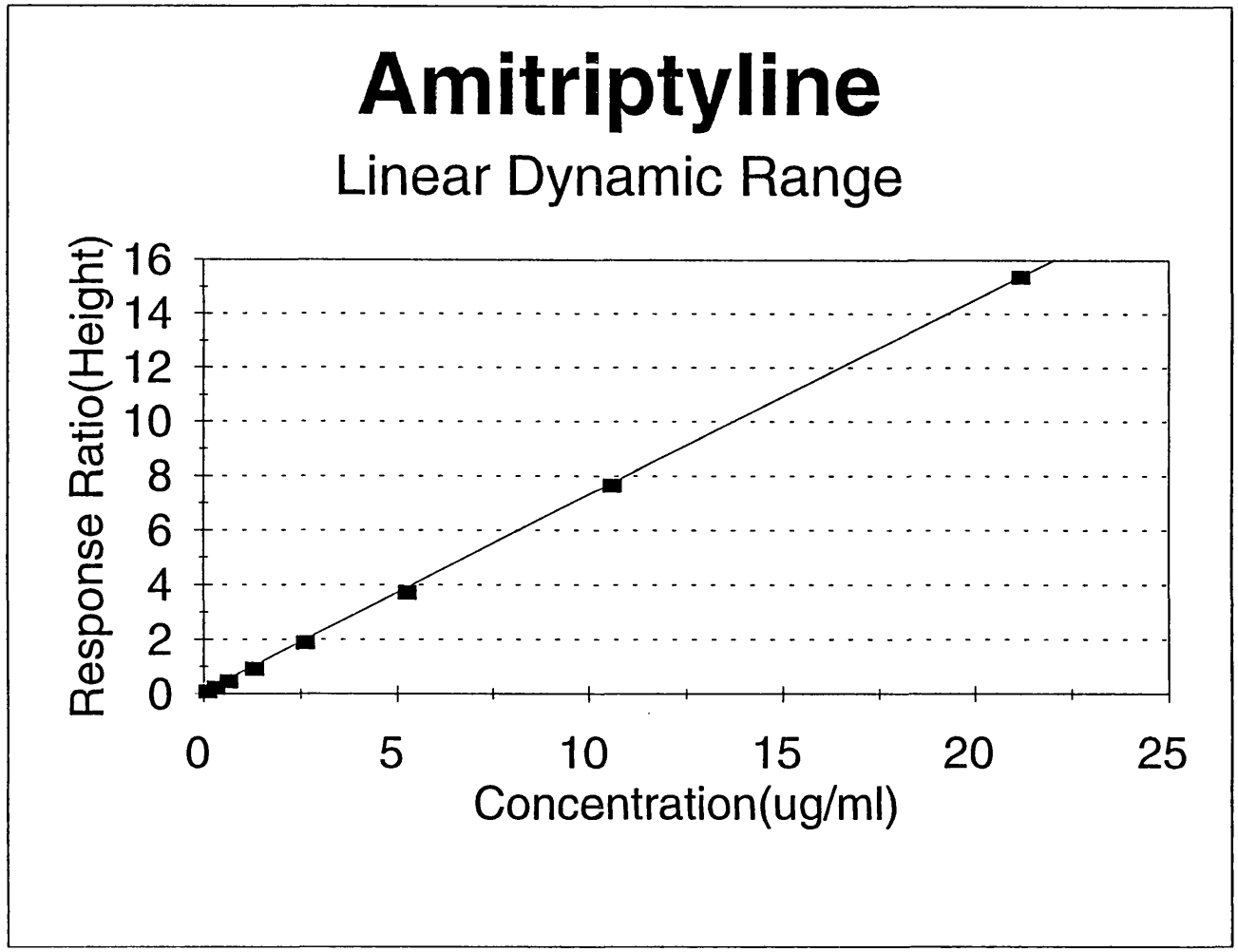
Greater optimisation of the extraction procedure for each of the columns would also prove beneficial and could be adapted for the analysis of other toxicologically relevant drugs. Improvements in recoveries could also result from the use of a better elution solvent, greater control of the vacuum and flow rates, and the use of different buffer pH's.

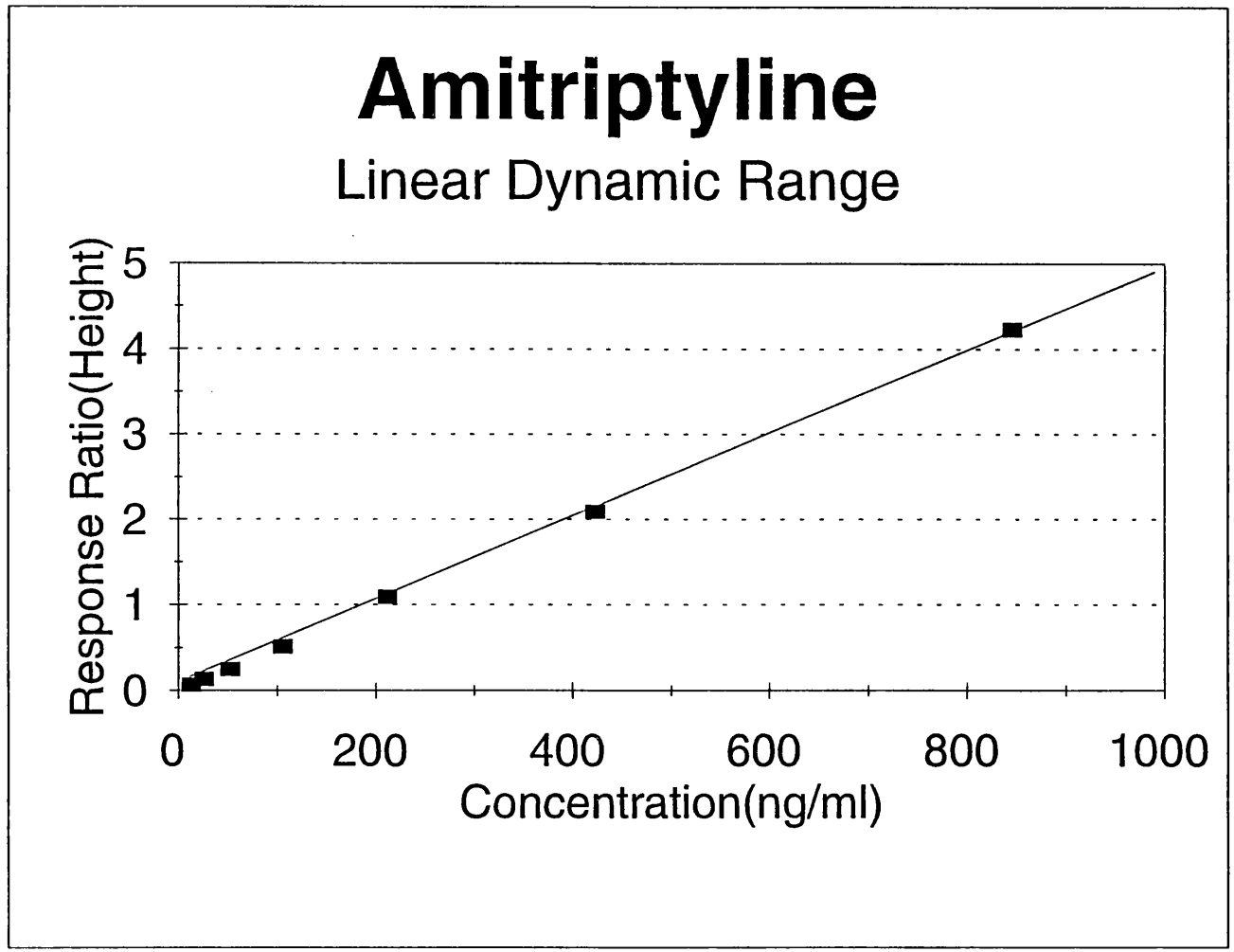
A more in depth analysis of the exact mechanisms at work within the experimental SPE columns would be advantageous for the optimisation of a mixed-mode sorbent which could be used to selectively extract a wide range of drugs from a variety of different biological fluids and this is the subject of further work currently being undertaken in the laboratory.

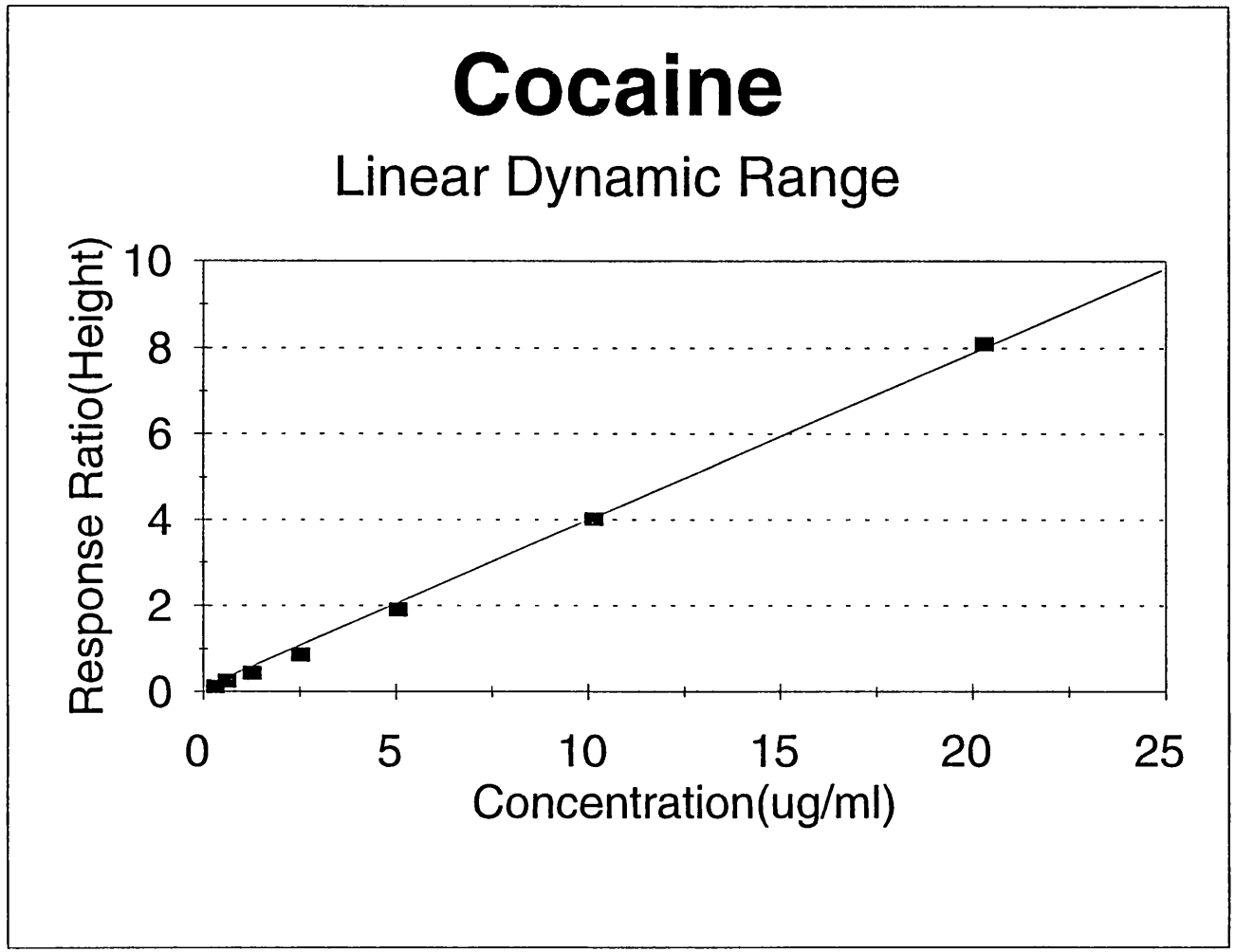
The analysis of blood samples from authentic cases is also necessary in order to evaluate the method for routine analysis.

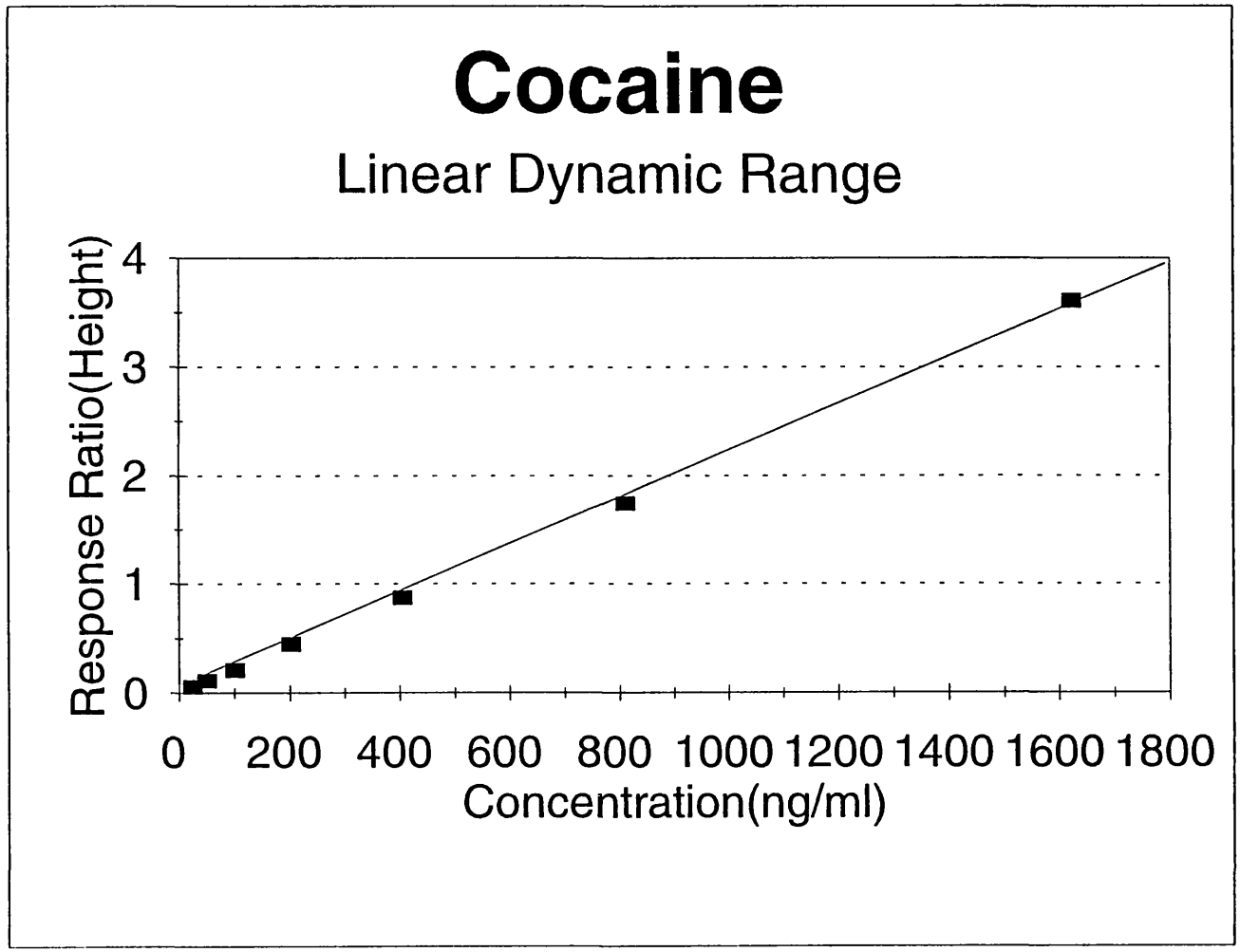
Appendix 1

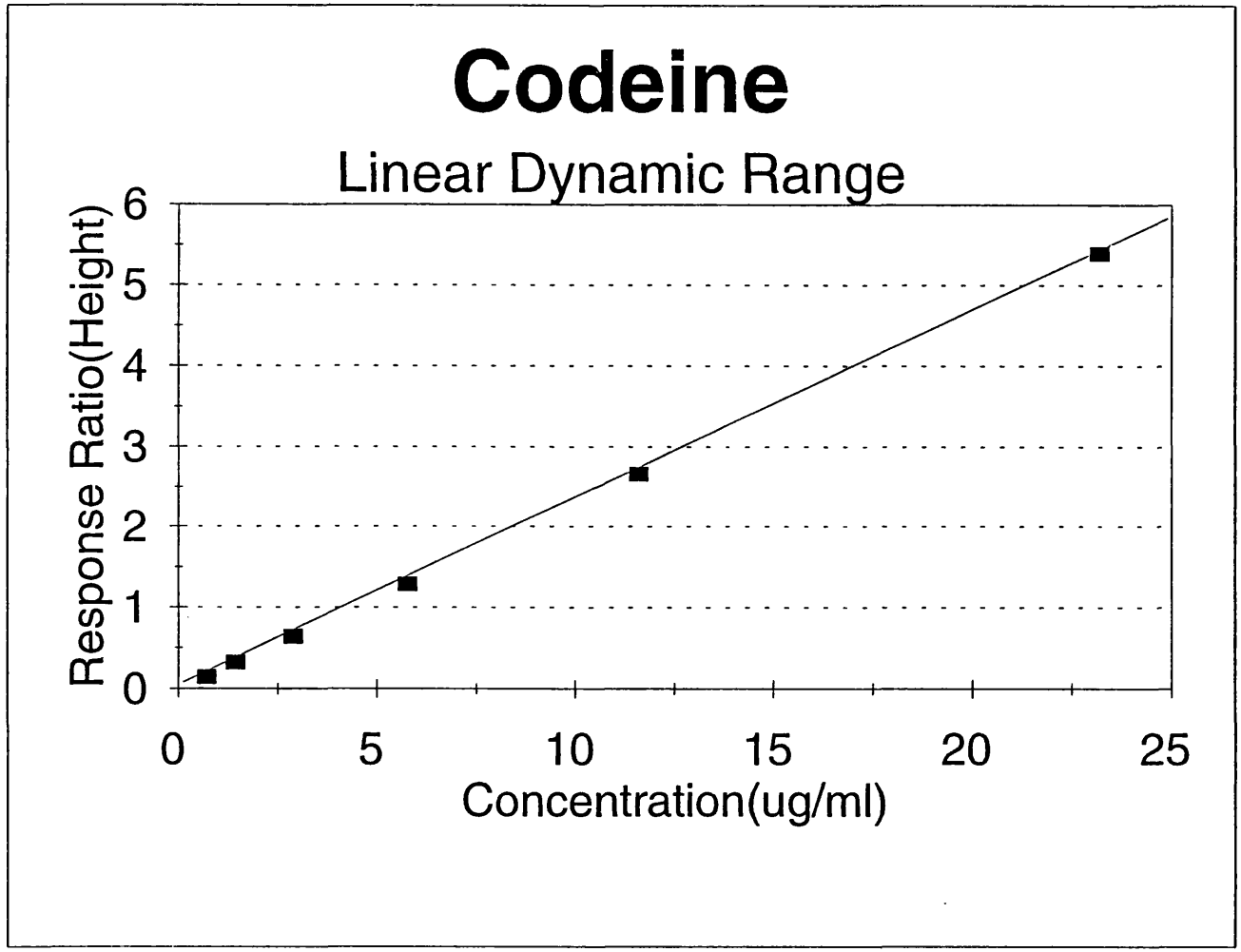
Calibration Curves (18) of Unextracted Standards
Concentration = ng/mL and μg/mL.

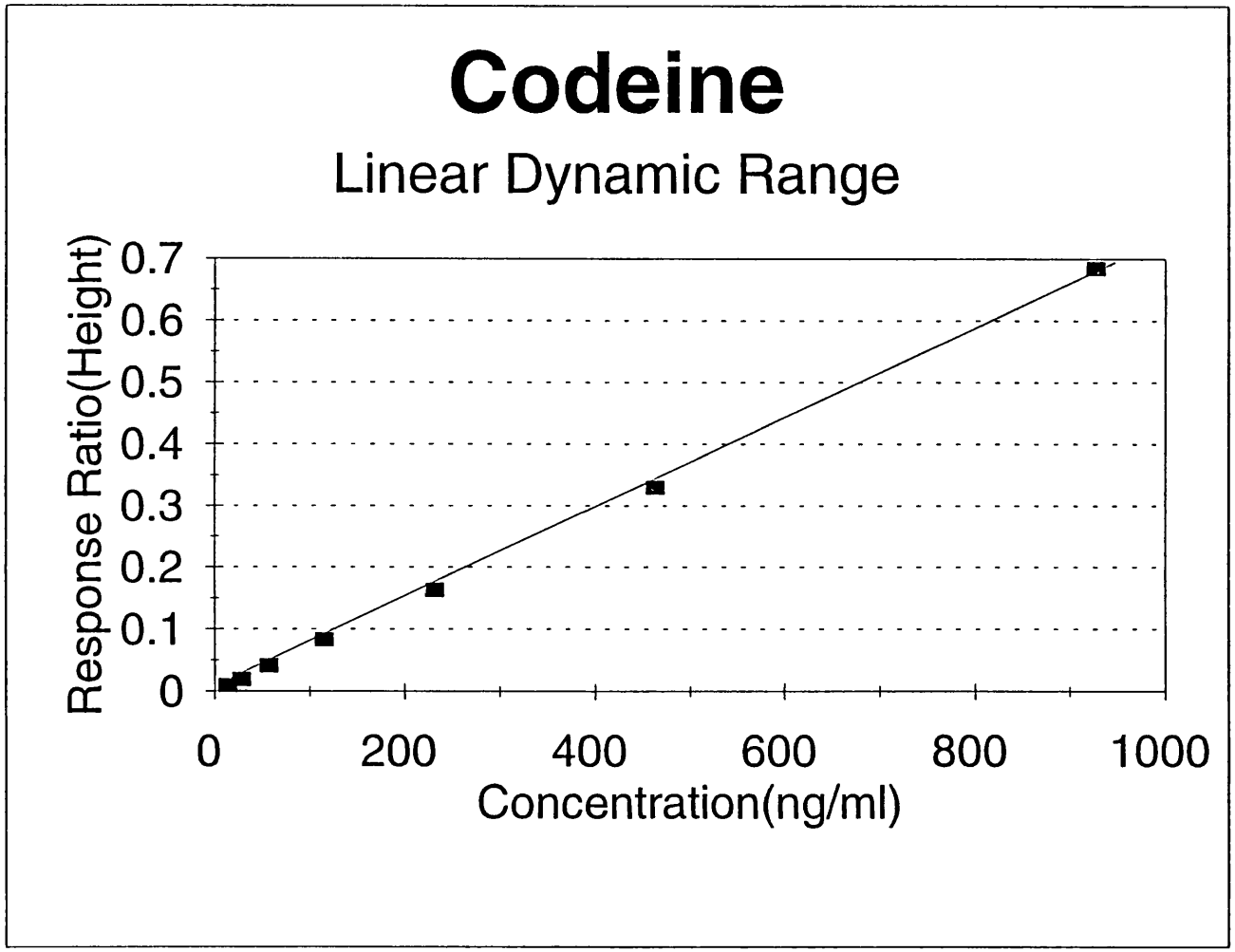

Amitriptyline
Linear Dynamic Range
Response Ratio(Height)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
Concentration(ug/ml)


Amitriptyline
Linear Dynamic Range
Response Ratio(Height)
0
1
2
3
4
5
0
200
400
600
800
1000
Concentration(ng/ml)


Cocaine
Linear Dynamic Range
Response Ratio(Height)
10
8
6
4
2
0
0
5
10
15
20
25
Concentration(ug/ml)

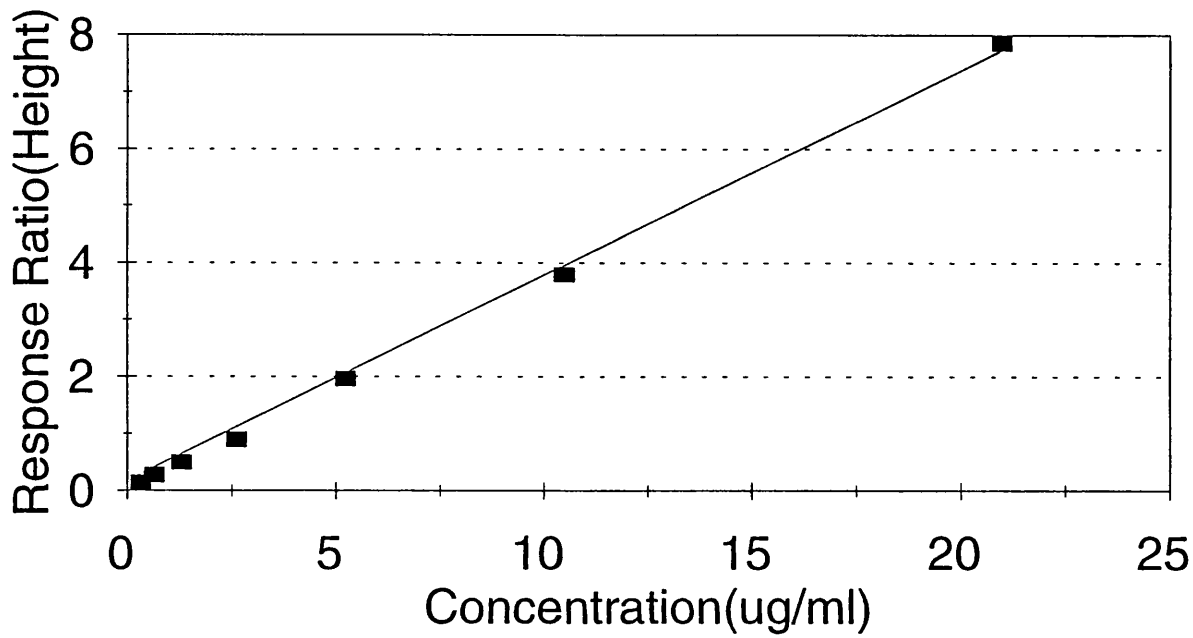

Cocaine
Linear Dynamic Range
Response Ratio(Height)
0
1
2
3
4
0
200
400
600
800
1000
1200
1400
1600
1800
Concentration(ng/ml)


Codeine
Linear Dynamic Range
Response Ratio(Height)
0
1
2
3
4
5
6
0
5
10
15
20
25
Concentration(ug/ml)


Codeine
Linear Dynamic Range
Response Ratio(Height)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
200
400
600
800
1000
Concentration(ng/ml)

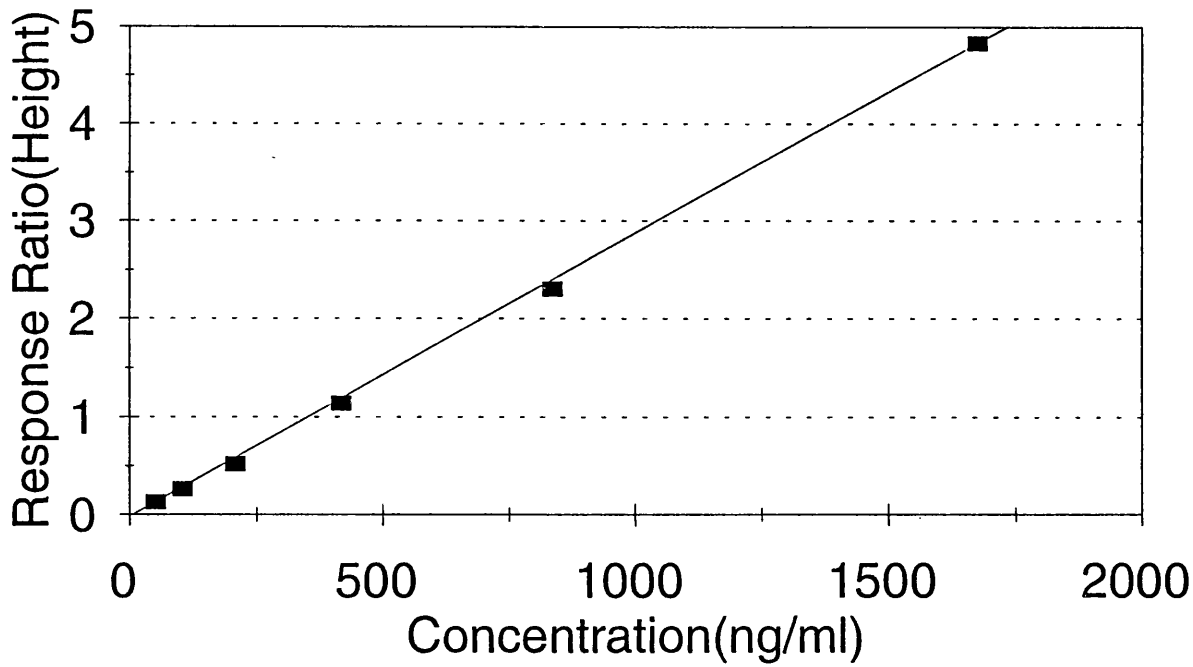
Desmethyldiazepam

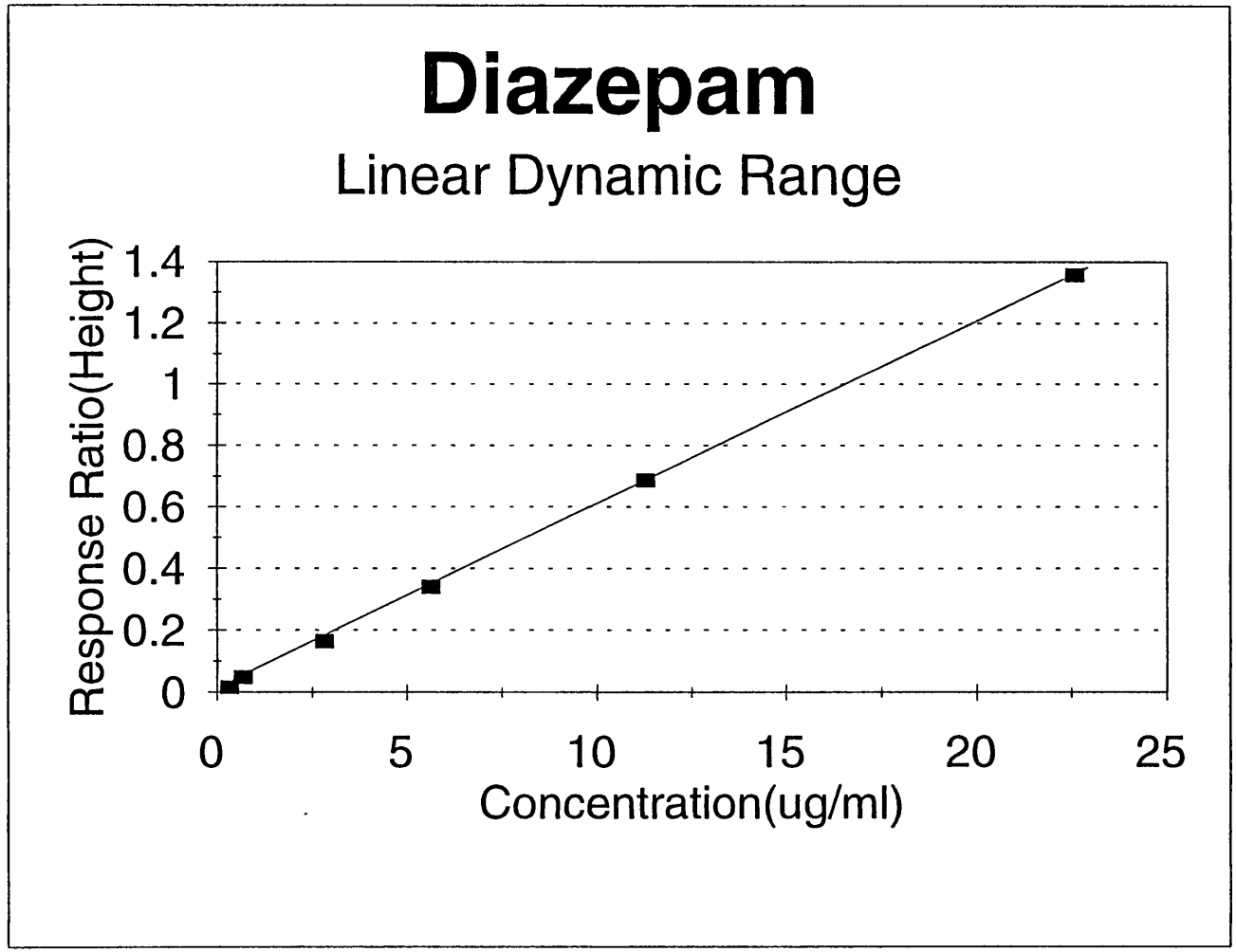
Linear Dynamic Range

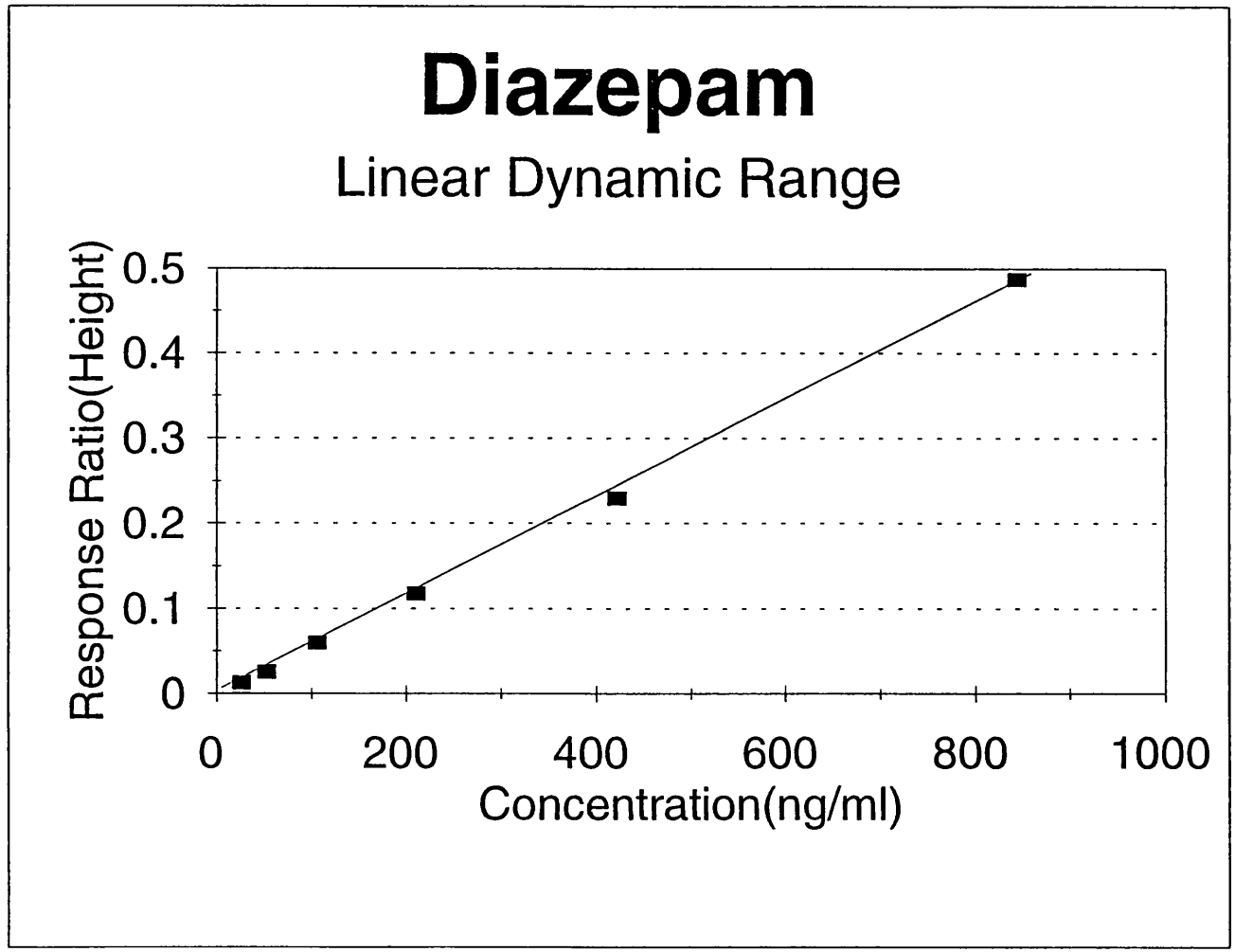


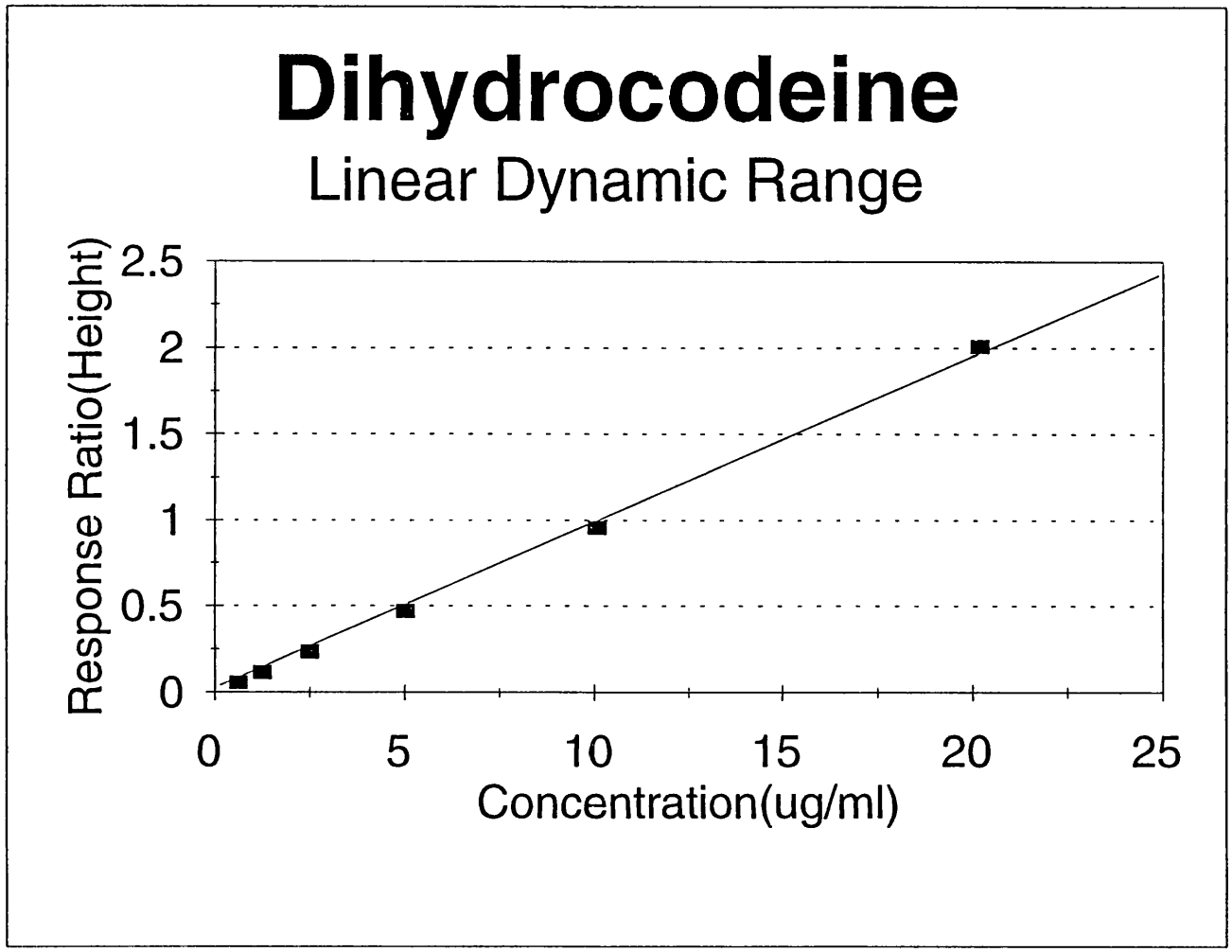
Desmethyldiazepam

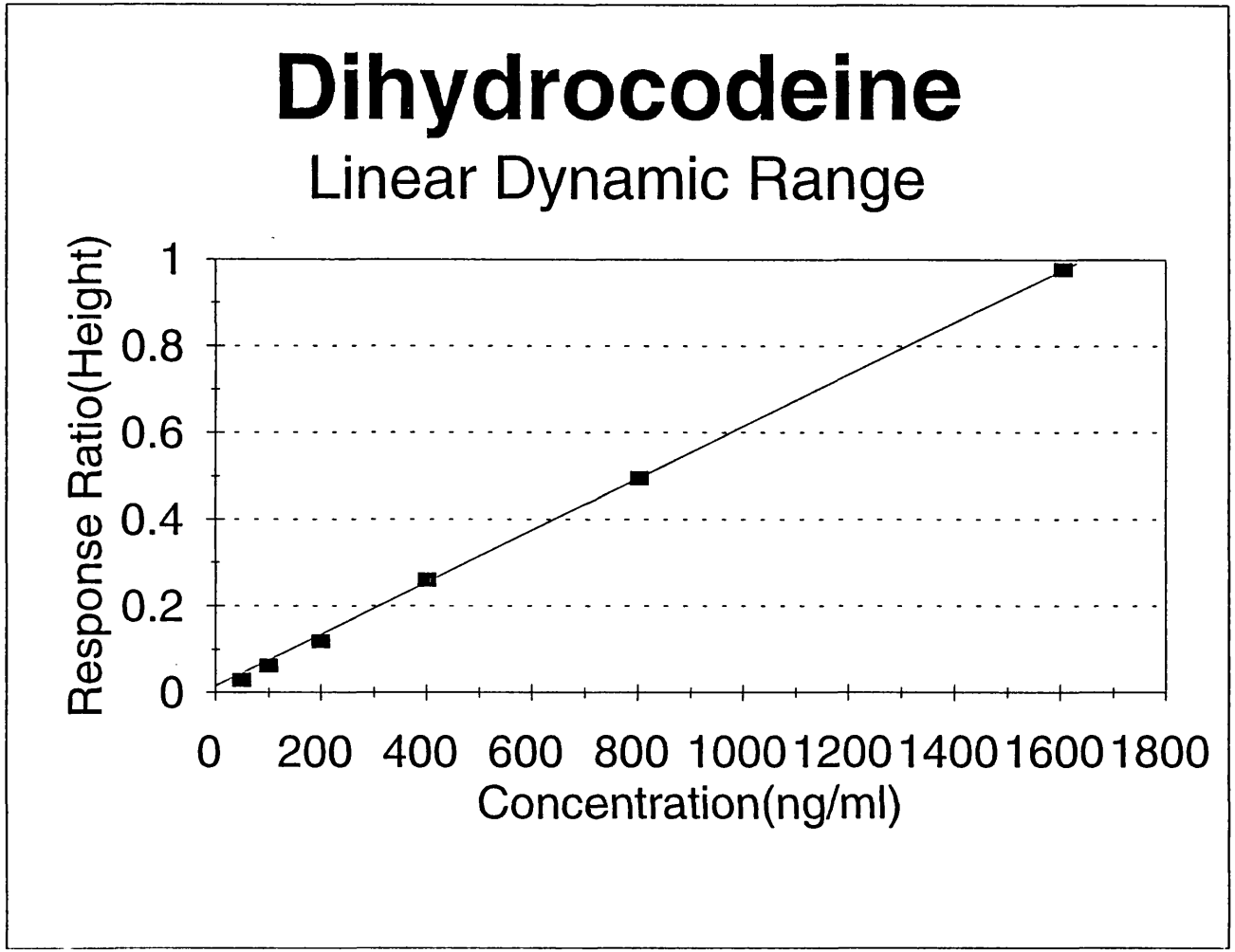
Linear Dynamic Range

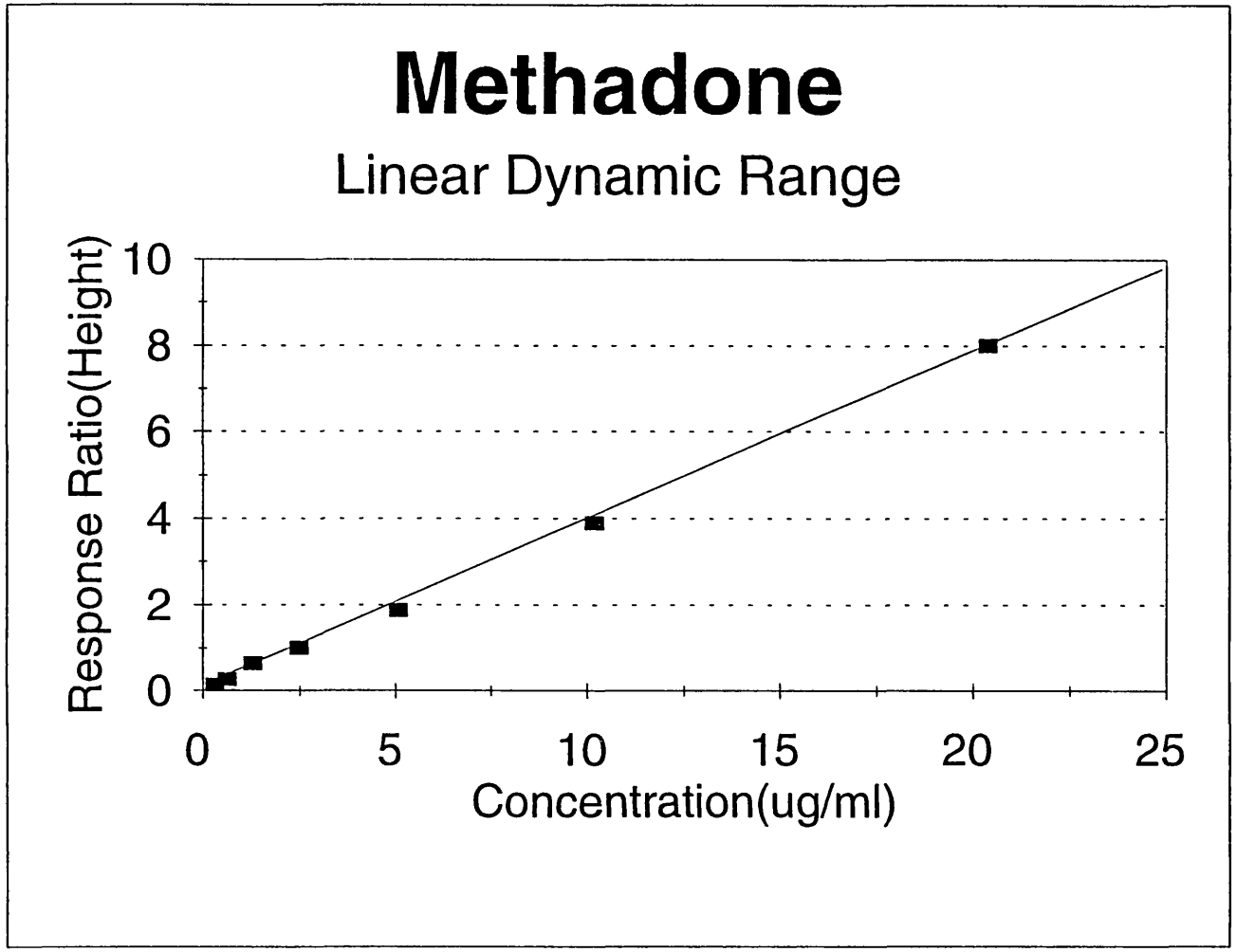


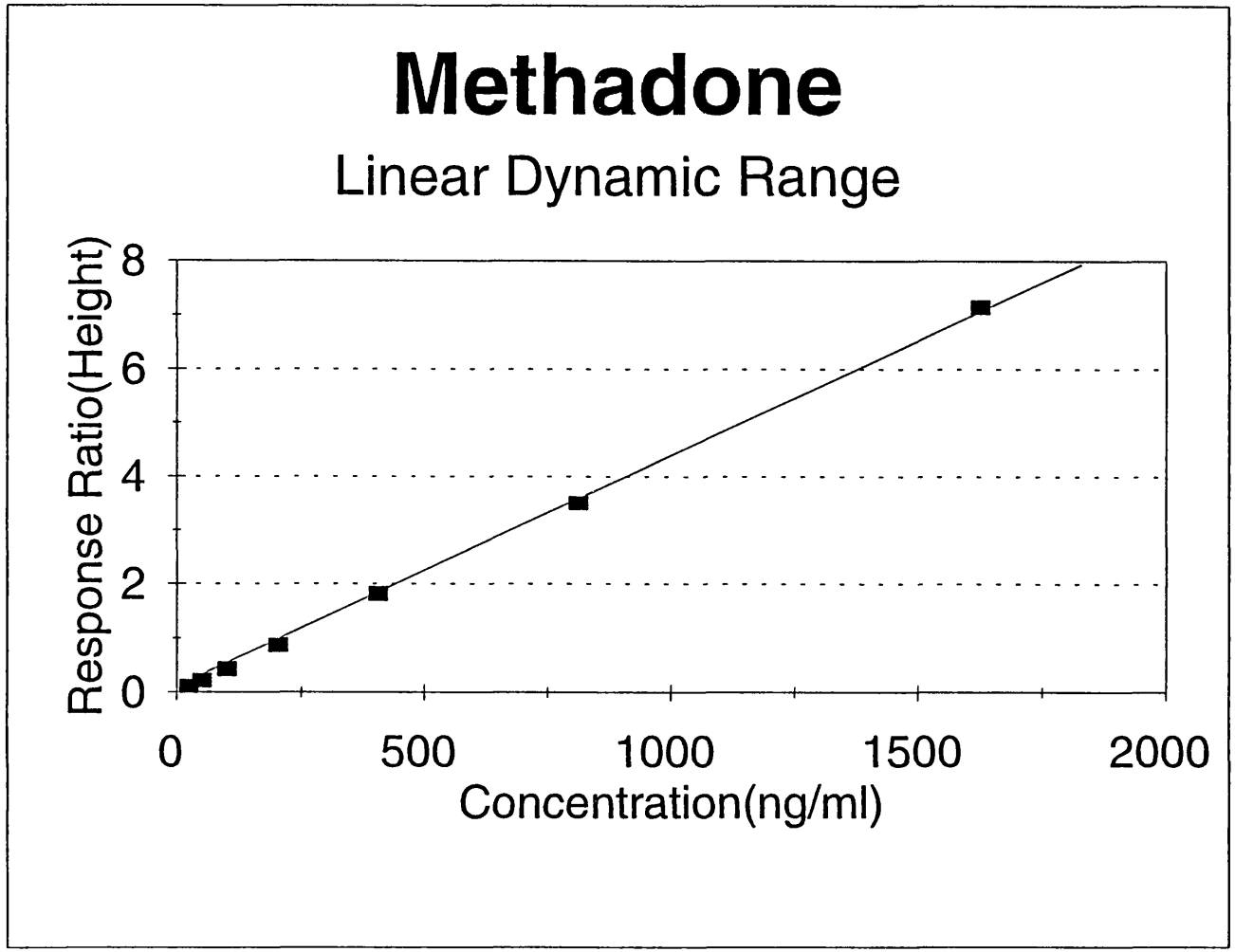

Diazepam
Linear Dynamic Range
Response Ratio(Height)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Concentration(ug/ml)

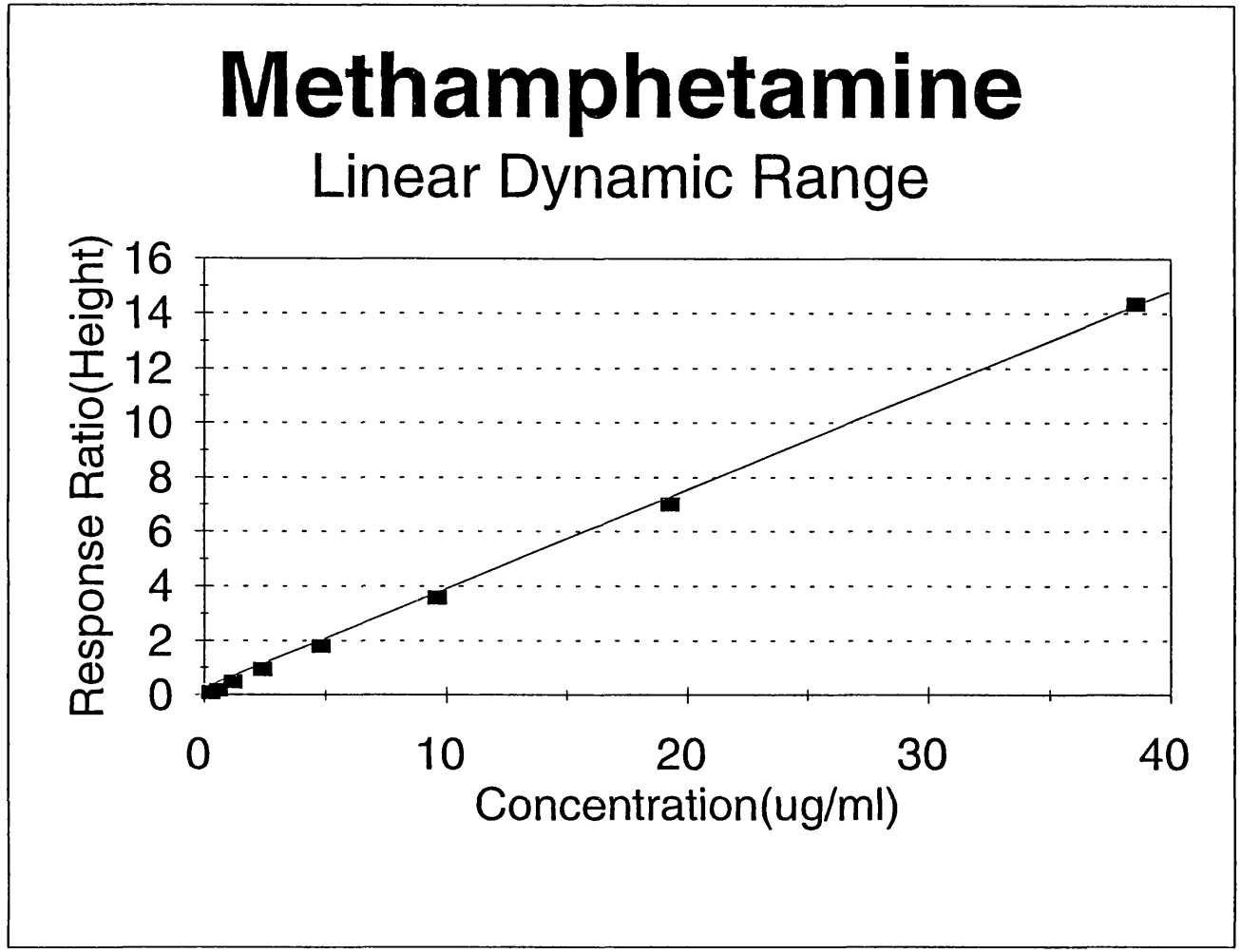

Diazepam
Linear Dynamic Range
Response Ratio(Height)
0.5
0.4
0.3
0.2
0.1
0
0
200
400
600
800
1000
Concentration(ng/ml)

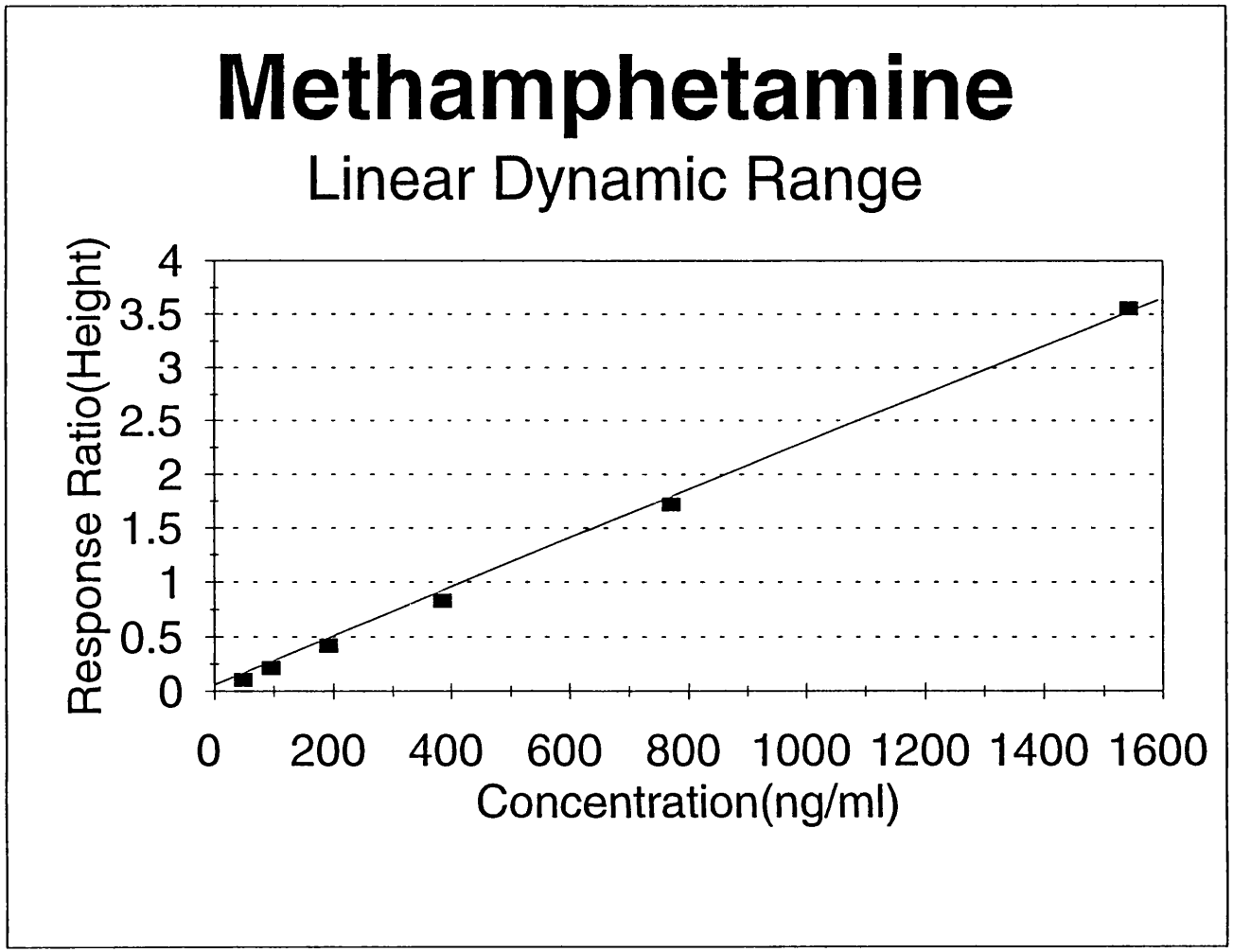

Dihydrocodeine
Linear Dynamic Range
Response Ratio(Height)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Concentration(ug/ml)

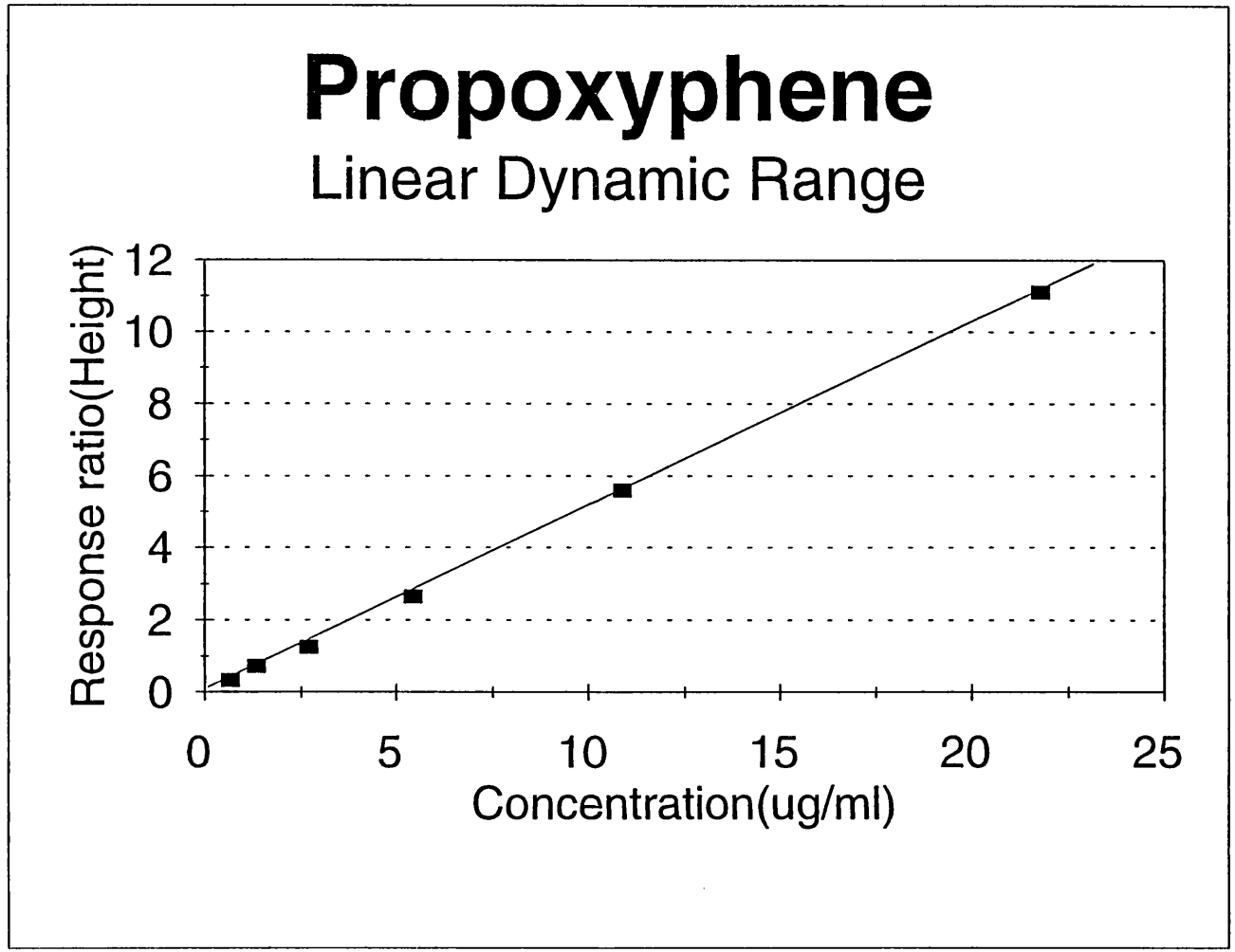

Dihydrocodeine
Linear Dynamic Range
Response Ratio(Height)
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
1200
1400
1600
1800
Concentration(ng/ml)

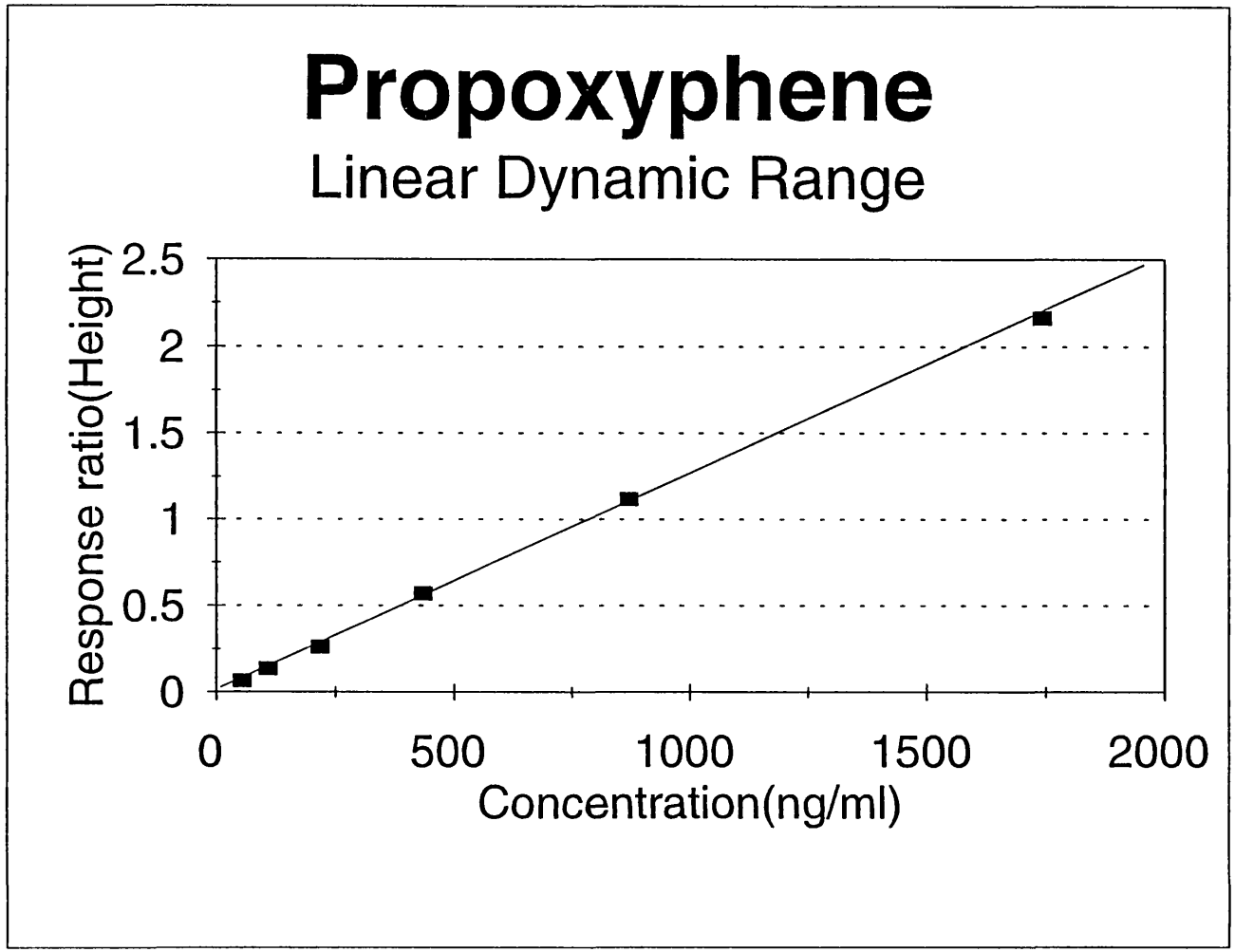

Methadone
Linear Dynamic Range
Response Ratio(Height)
10
8
6
4
2
0
0
5
10
15
20
25
Concentration(ug/ml)


Methadone
Linear Dynamic Range
Response Ratio(Height)
8
6
4
2
0
0
500
1000
1500
2000
Concentration(ng/ml)


Methamphetamine
Linear Dynamic Range
Response Ratio(Height)
16
14
12
10
8
6
4
2
0
0
10
20
30
40
Concentration(ug/ml)

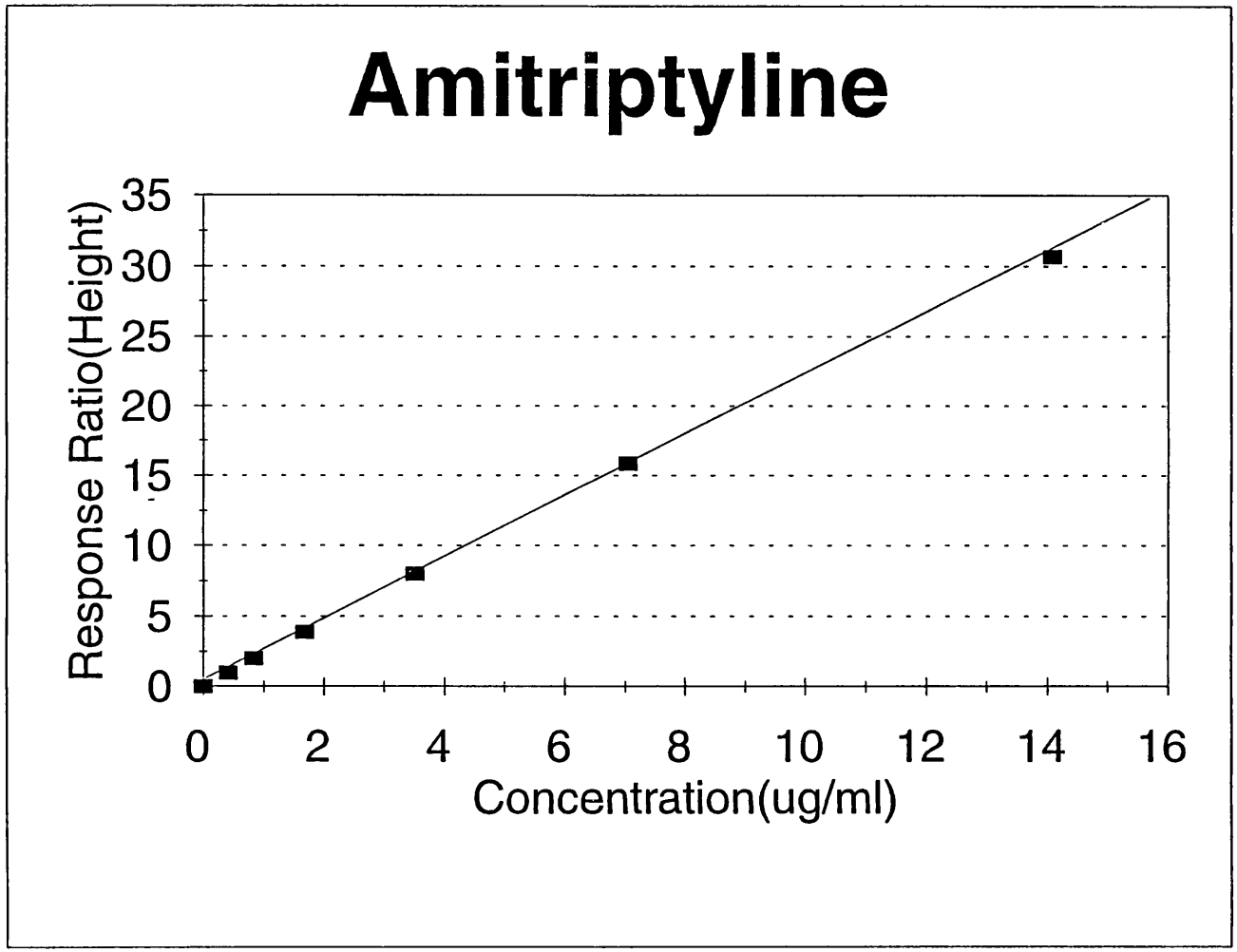

Methamphetamine
Linear Dynamic Range
Response Ratio(Height)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
200
400
600
800
1000
1200
1400
1600
Concentration(ng/ml)

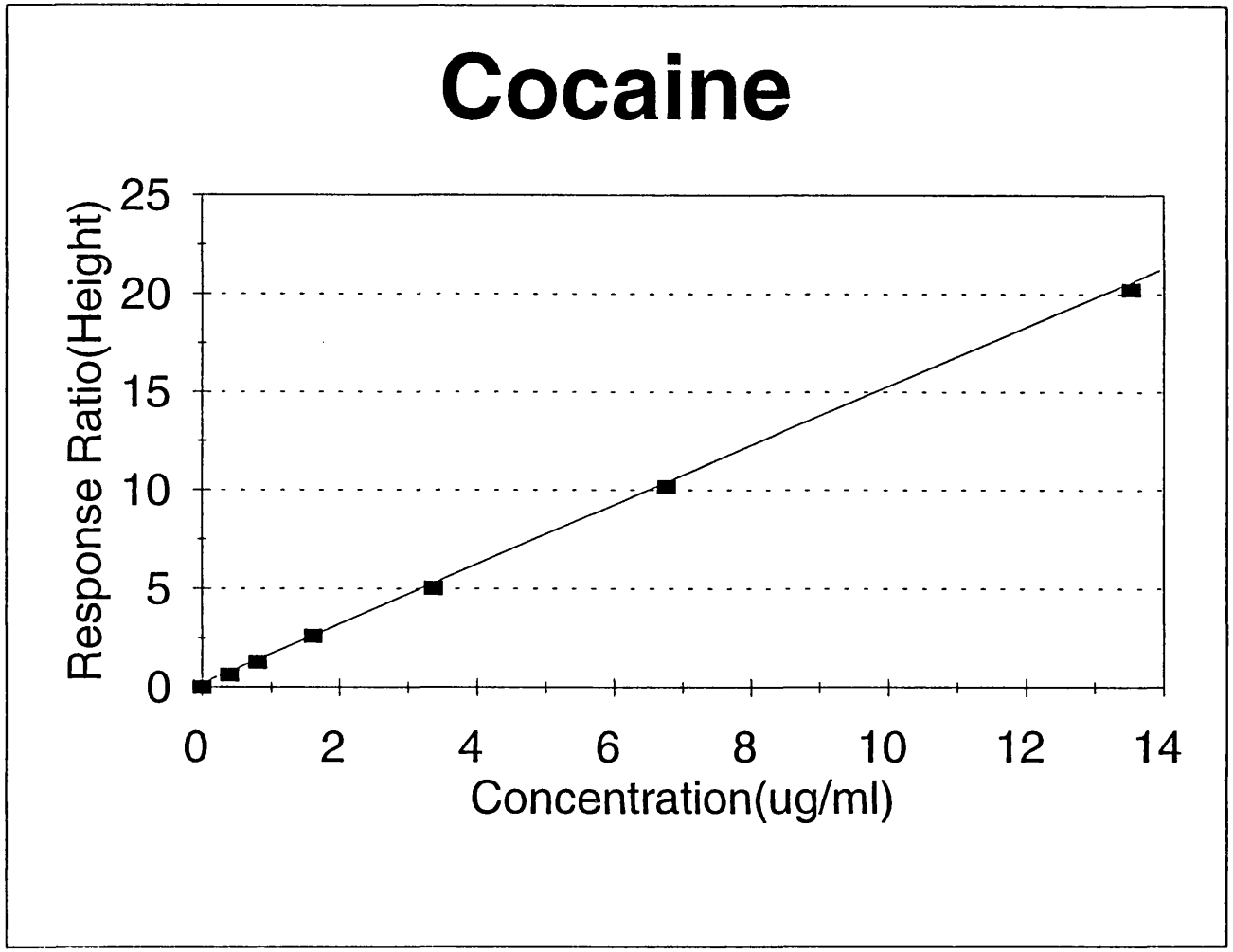

Propoxyphene
Linear Dynamic Range
Response ratio(Height)
12
10
8
6
4
2
0
0
5
10
15
20
25
Concentration(ug/ml)

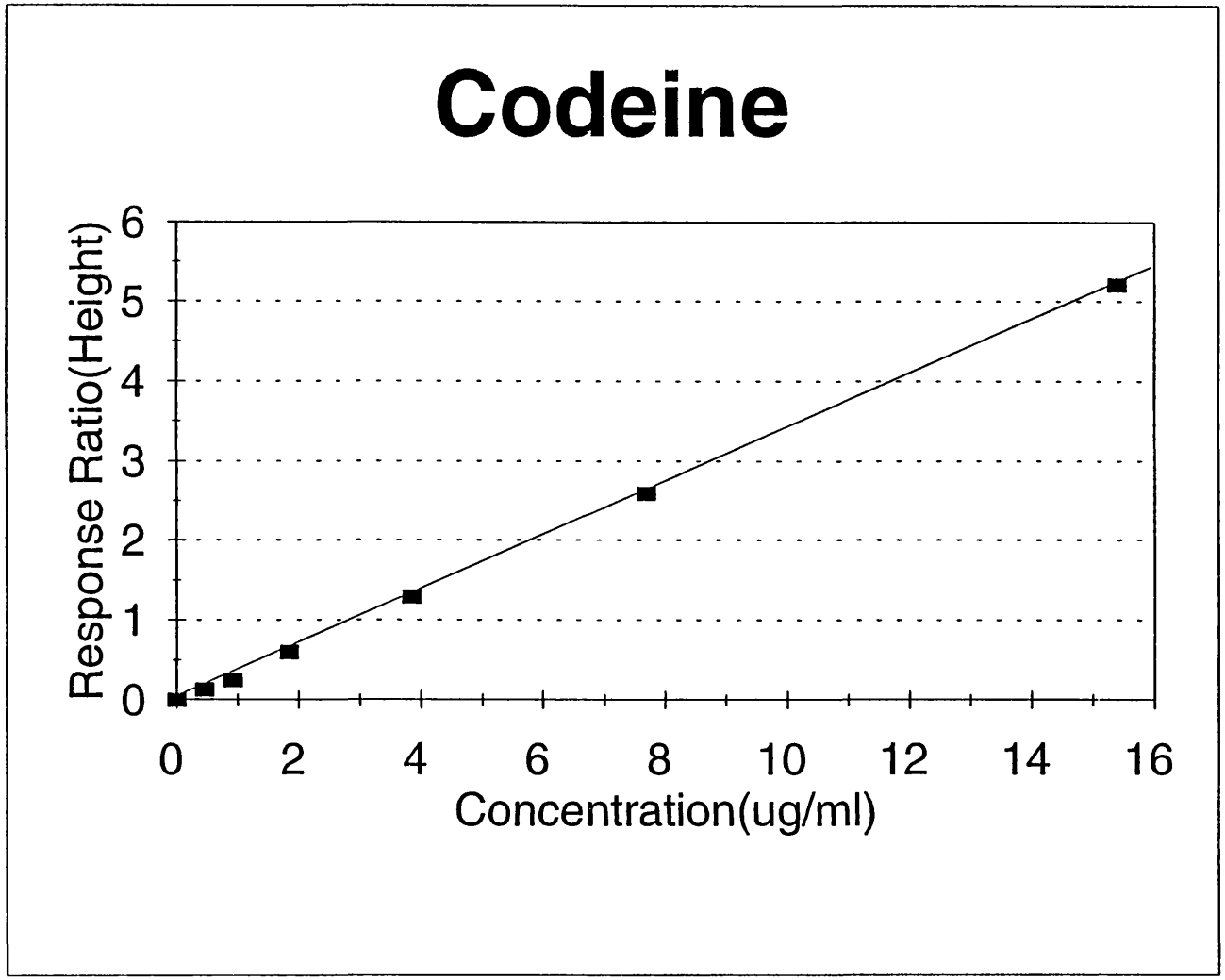

Propoxyphene
Linear Dynamic Range
Response ratio(Height)
2.5
2
1.5
1
0.5
0
0
500
1000
1500
2000
Concentration(ng/ml)

Appendix 2

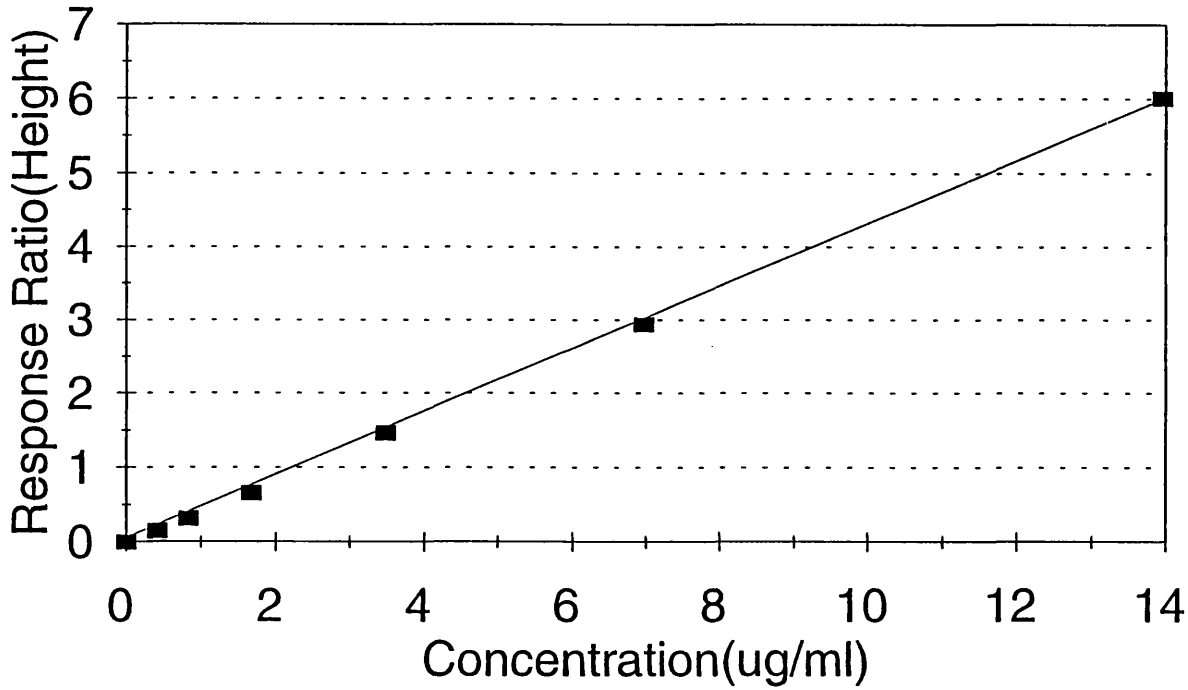
Calibration Curves (9) of Basic Drugs Recovery From Blood Concentration = ng/mL to μg/mL.

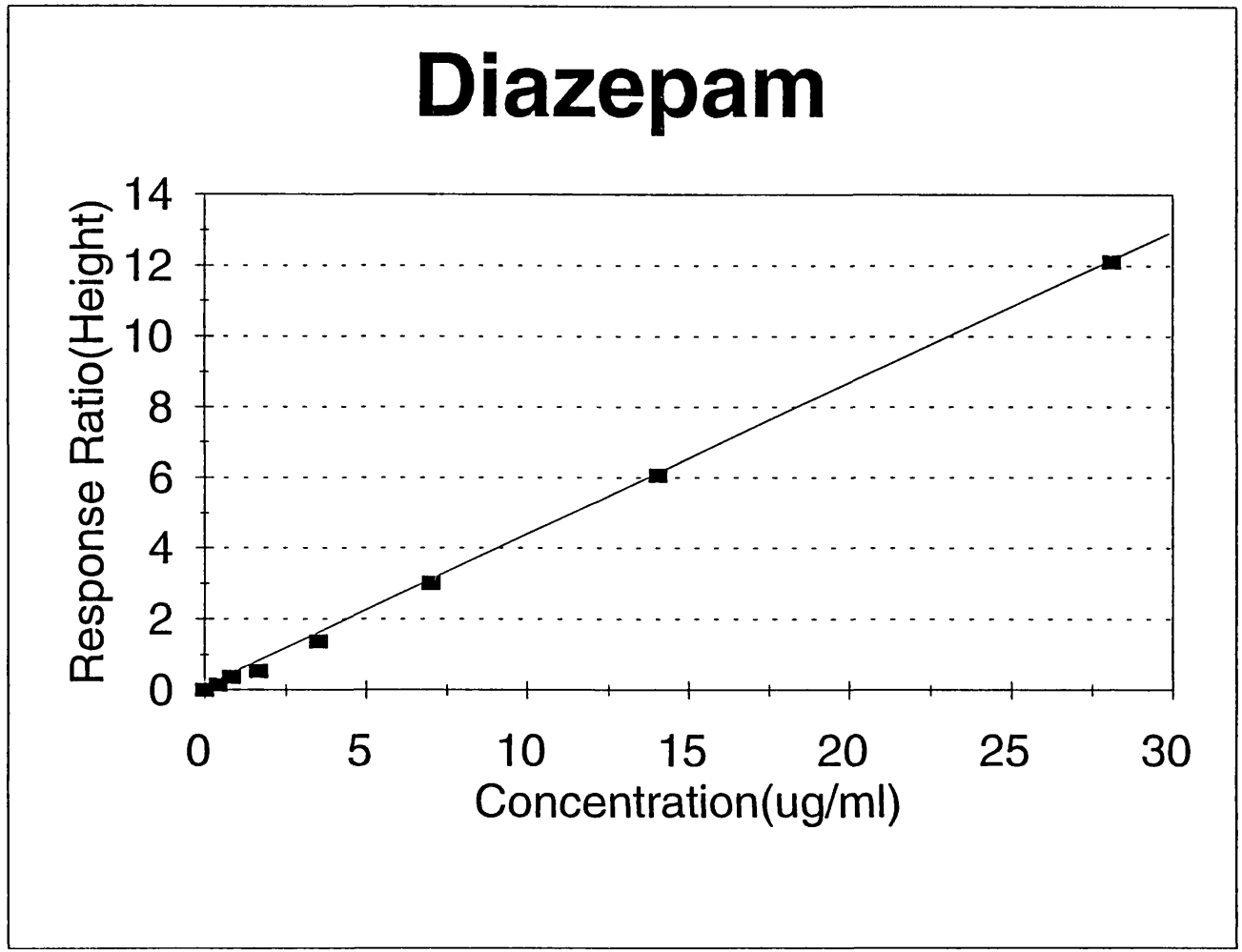

Amitriptyline
Response Ratio(Height)
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Concentration(ug/ml)

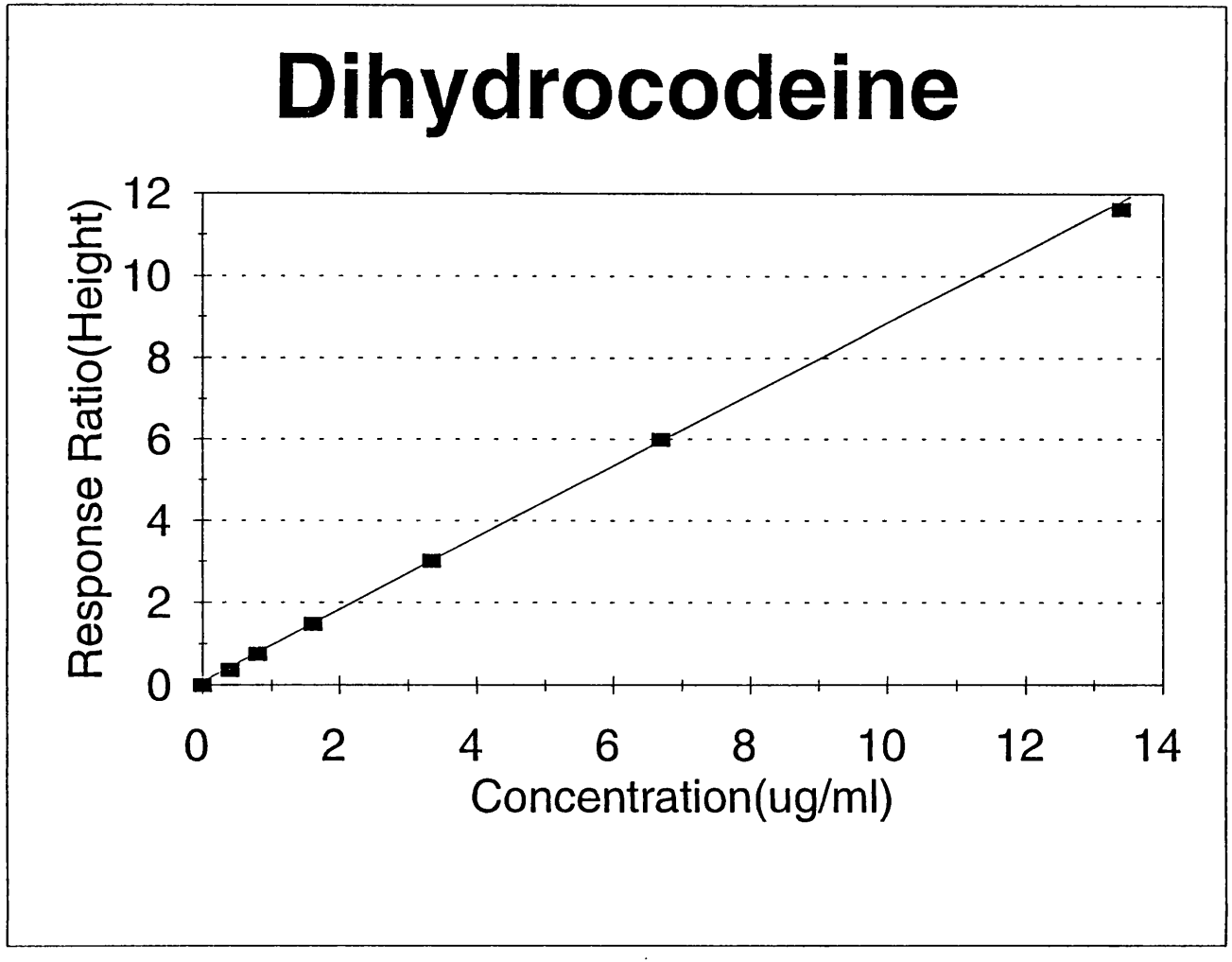

Cocaine
Response Ratio(Height)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
Concentration(ug/ml)

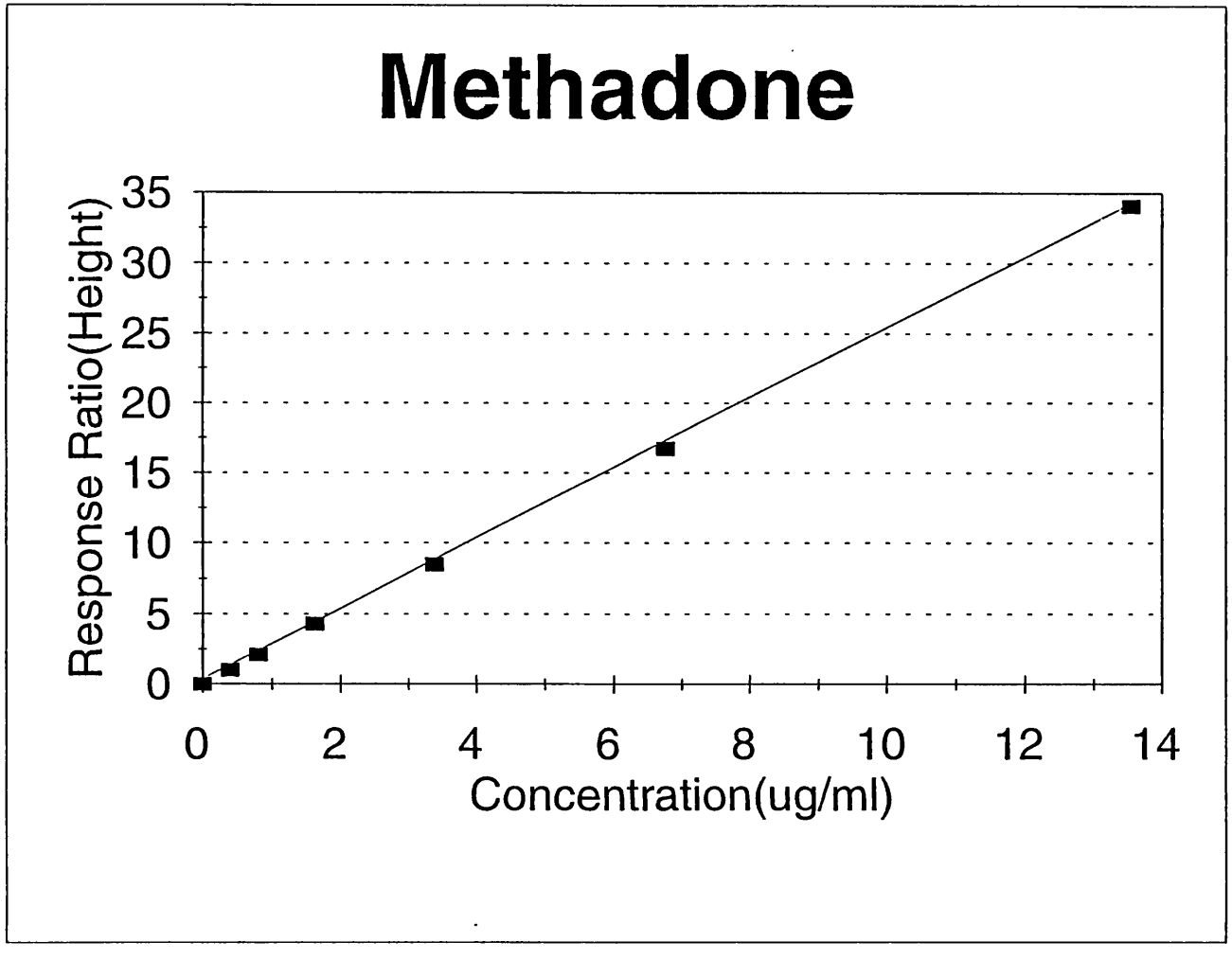

Codeine
Response Ratio(Height)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
Concentration(ug/ml)

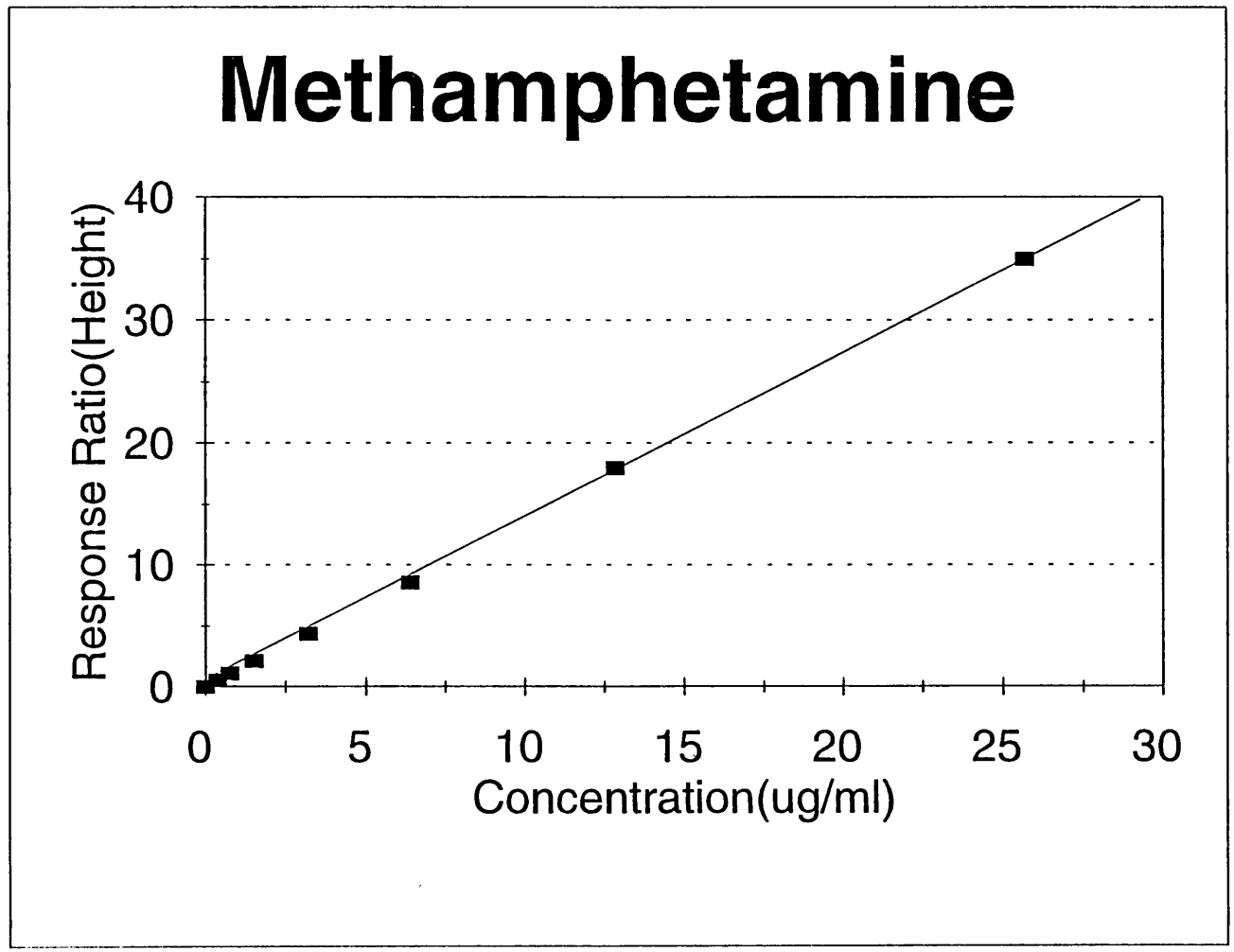
Desmethyldiazepam

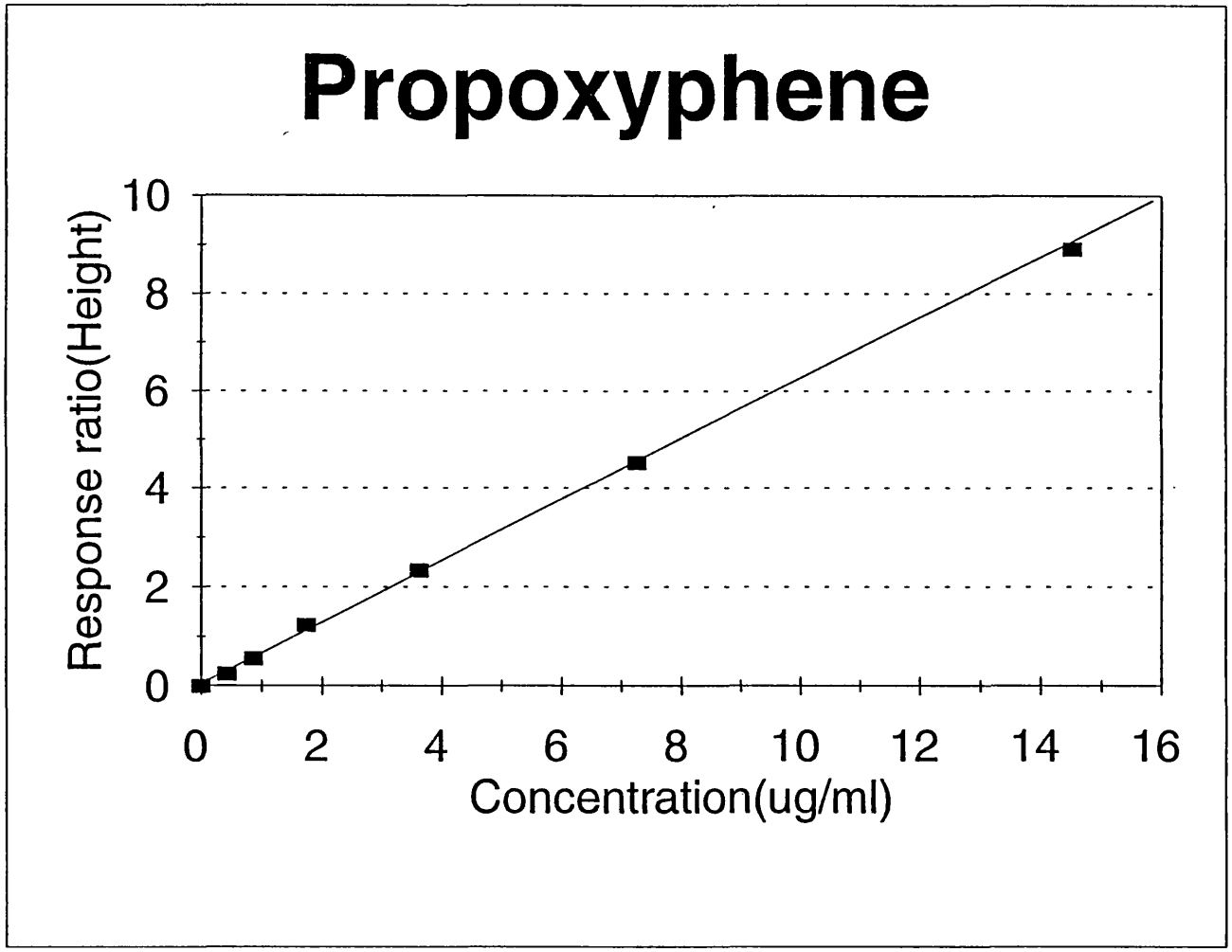



Diazepam
Response Ratio(Height)
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Concentration(ug/ml)


Dihydrocodeine
Response Ratio(Height)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
Concentration(ug/ml)


Methadone
Response Ratio(Height)
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
Concentration(ug/ml)


Methamphetamine
Response Ratio(Height)
40
30
20
10
0
0
5
10
15
20
25
30
Concentration(ug/ml)


Propoxyphene
Response ratio(Height)
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
Concentration(ug/ml)

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