THE SOLID-PHASE EXTRACTION OF DRUGS FROM BIOLOGICAL FLUIDS.

Thesis submitted in accordance with the requirements of the University of Glasgow for the degree of Doctor of Philosophy by

Christine Mary Moore.
BSc (Hons.), MSc., C.Chem. M.R.S.C.

Department of Forensic Medicine and Science

June 1989

DEDICATION

For my father (R.I.P.)

ACKNOWLEDGEMENTS

I should like to thank the National Racing Greyhound Club for their financial support of this work, and the staff at the Veterinary School, Garscube, for their help in acquiring greyhound urine samples. I should also like to thank Dr. John Oliver for his supervision of this project and Professor Alan Watson for his encouragement during the last three years.

Grateful thanks are extended to all the technical and secretarial staff, without exception, at the Department of Forensic Medicine and Science, particularly Mrs. Margaret Harrison and Mrs. Elizabeth Doherty for their tireless explanation of word-processing techniques as well as emotional support. The help and support of other colleagues and friends within the department, especially Anita Quye, is also gratefully acknowledged.

I should particularly like to thank Dr. Ian Tebbett at the University of Illinois at Chicago, Ms. Shelley Kalita (Director) and Ms.Vicki Hoogervorst at the Illinois Racing Board, Elgin, and Dr. Barry Logan at the University of Tennessee, Memphis, for their co-operation in the final stages of this work.

Finally, I should like to thank all my friends, particularly Jennifer Chalmers and Rosemary Porter, for their unwavering confidence in my ability.

The most thanks, however, go to all the members of my family, especially my sister, Anne, but most of all, to my mother, without whose unfailing emotional and financial support none of this would have been possible. Thanks mum.

ABBREVIATIONS

Weights

pg	picogram
ng	nanogram
ug	microgram
mg	milligram
g	gram
kg	kilogram
w/w	weight/weight

Volumes

ul	microlitre
ml	milliltre
l	litre
v/v	volume/volume

Analytical Methods

HPLC	high pressure liquid chromatography
GC	gas chromatography
LCMS	liquid chromatography-mass spectrometry
GCMS	gas chromatography-mass spectrometry

Extraction Methods

L/L	liquid/liquid
SE	solvent extraction
SPE	solid-phase extraction
DE	diatomaceous earth

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CONTENTS

3. EXPERIMENTAL

4. RESULTS AND DISCUSSION

SUMMARY.

Solid-phase extraction has become an increasingly important technique over the last decade.

In this study, the properties of bonded silica sorbents for use in the solid-phase extraction of drugs from various biological fluids are investigated. Sorbents exhibiting predominantly non-polar, polar or ion-exchange interactions are all considered.

Initially, the extraction of some common benzodiazepines (diazepam, triazolam, flunitrazepam and their metabolites), from the urine of racing greyhounds is studied.

Further method development is described involving the extraction of xylazine (a veterinary tranquilliser), from greyhound urine, mazindol (a central nervous system stimulant), from racehorse urine, and basic drugs from human post-mortem urine samples.

Comparison of these solid-phase methods with existing solvent extraction procedures, from an efficiency point of view, is carried out for racing greyhound and human post-mortem samples.

Such comparisons are based on a number of criteria: the total time spent on extraction; solvent cost; glassware requirements; sample requirements; possible simultaneous extraction of a number of samples; potential automation of extraction; cleanliness of extracts, and necessity for evaporation and/or derivatisation steps prior to analysis.
The extraction methods presented are all reproducible and highly efficient as well as being economically viable for routine use in a toxicological laboratory.
Further applications of solid-phase extractions are investigated.

A novel high-pressure liquid chromatographic (HPLC) analysis method for benzodiazepines is described, which is compatible with liquid chromatography-mass spectrometry (LC-MS).
New HPLC methods for xylazine and mazindol analysis are developed.
Finally, a potentially fully automated basic drug screening solid-phase extraction method and analysis by HPLC with diode array detection for the determination of drugs from human post-mortem urine samples is described. The results of this method were confirmed by gas chromatography-mass spectrometry (GC-MS).

1. AIMS.

The aims of this project were:

1. To study the potential use of bonded phase silica sorbents for the extraction of drugs of abuse from biological fluids.

2. To develop a single solid-phase extraction method for the determination of benzodiazepines and their metabolites in the urine of racing greyhounds.

3. To develop a single solid-phase extraction method for the determination of basic drugs in human post-mortem samples.

4. To compare solid-phase extraction methods with existing solvent extraction methods in order to determine the most efficient and economical procedure.

5. To develop new HPLC analysis systems for xylazine and mazindol.

6. To develop a fully automated basic drug screening system for greyhound and human samples, incorporating solid-phase extraction followed by HPLC analysis with diode array detection.

2. INTRODUCTION

2.1. Drugs

2.1.1 Benzodiazepines

Benzodiazepines have been widely used since the early 1960's as tranquillisers, anti-anxietics, sleep inducers, hypnotics and anti-epileptics. Benzodiazepines can also show amnesiac actions, whose degree and duration are pharmacodynamically dose related and different for various benzodiazepine derivatives. Their wide availability has targeted them as potential drugs of abuse in sport and in everyday life.

All benzodiazepines undergo extensive biotransformation in the human body (Kaithsa, 1977) so the detection methods of benzodiazepines in biological fluids must include the identification of metabolites due to the lack of availability in sufficient amounts of the free unchanged drug.

Diazepam (Valium®)

Diazepam-(7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one) was approved for human use in 1963 and is still the most widely prescribed benzodiazepine for the treatment of anxiety in the young and the elderly (Bellantuono et al., 1980; Nolan and O'Malley, 1988).

It also displays anti-convulsant properties, so it is used in the treatment of epilepsy.

The recommended single therapeutic dose is 10mg for humans and 5mg for greyhounds (Baselt et al., 1977). Although diazepam elimination is very slow, with an elimination half-life of over 24 hours, the long half-life is not reflected in a long duration of clinical effect (Shader and Greenblatt, 1981).

Metabolic studies in man and in animals (Schwartz et al., 1965) have shown that the compound is biotransformed into three major metabolites (Figure 1). These metabolites are also pharmacologically active : N-desmethyldiazepam having been studied most extensively and shown to produce physical dependance in animals (McNicholas et al., 1985).

The presence and persistence in the body of both N-desmethyldiazepam and oxazepam is significant, since both possess anti-convulsant properties.

Diazepam's third major metabolite, temazepam, is used clinically for the treatment of insomnia (Fillingim, 1979).

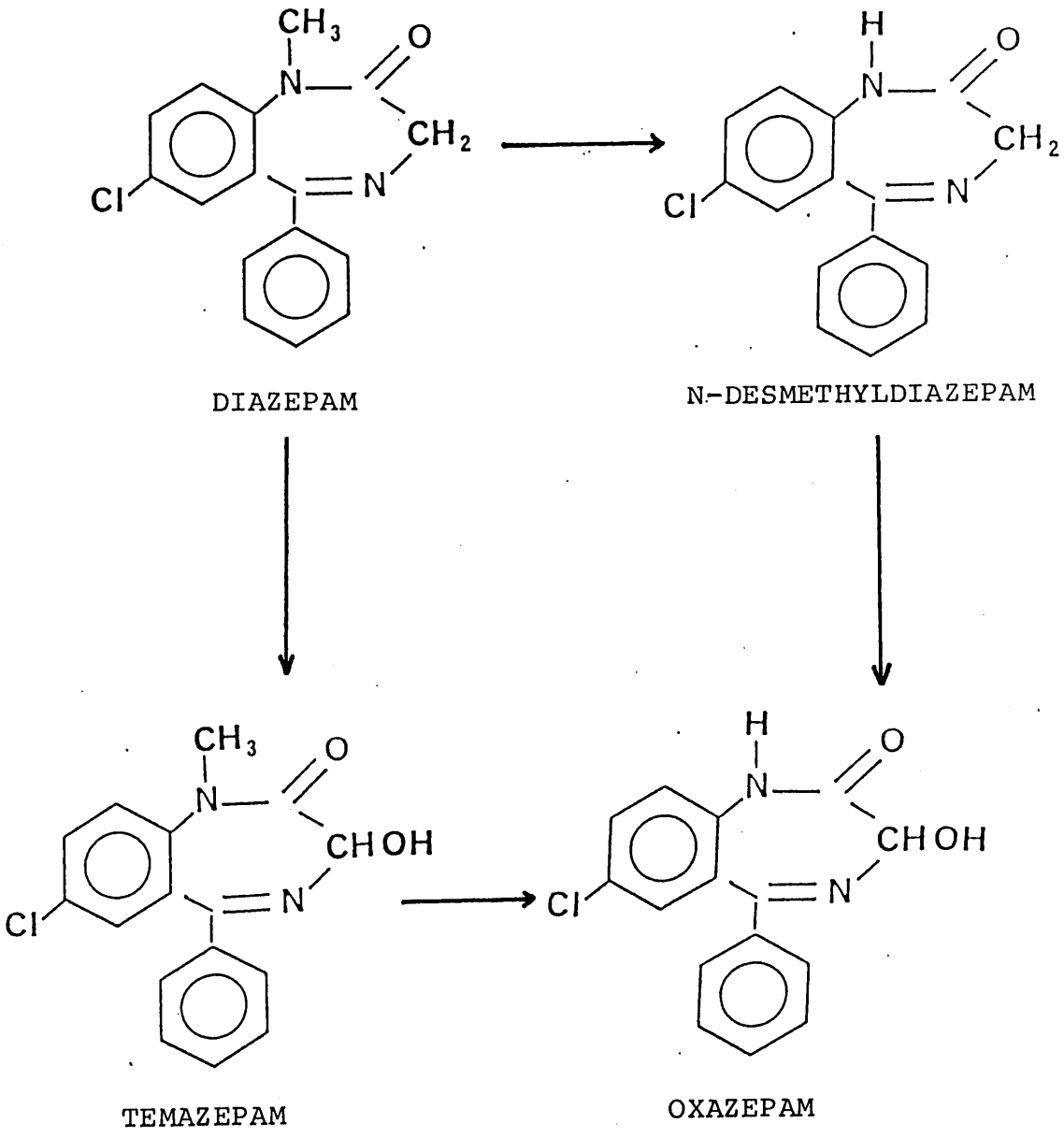
It is therefore important to monitor the concentration of all four compounds in the urine of humans and racing greyhounds in any analytical scheme designed to detect the misuse of diazepam.

Triazolam (Halcion®)

Triazolam-(8-chloro-6-(o-chlorophenyl)-1-methyl-4H-s-triazolo[4,3-a][1,4]-benzodiazepine) is a triazolo benzodiazepine derivative with sedative and hypnotic

FIGURE 1.

The metabolic routes of diazepam.



properties. In clinical comparison with nitrazepam (5mg) and oxazepam (50mg) for the treatment of insomnia, triazolam (0.5mg) gave superior results in terms of effect and reduced side-effects (Dordain et al., 1981). It is eight times more potent than diazepam as a hypnotic. Triazolam's high potency is chemically related to the presence of an orthochlorophenyl group and the triazolo ring fused to the 1,2 position of the B ring, which is known to potentiate the hypnotic properties of a benzodiazepine (Moffett, 1976). With excellent pharmacological activity and low toxicity, triazolam compares favourably with existing benzodiazepines and is considered highly promising as a sleep inducing agent (MacLeod, 1981). Clinical evaluation of this new hypnotic has indicated the compound is safe and effective (Purpura, 1981).

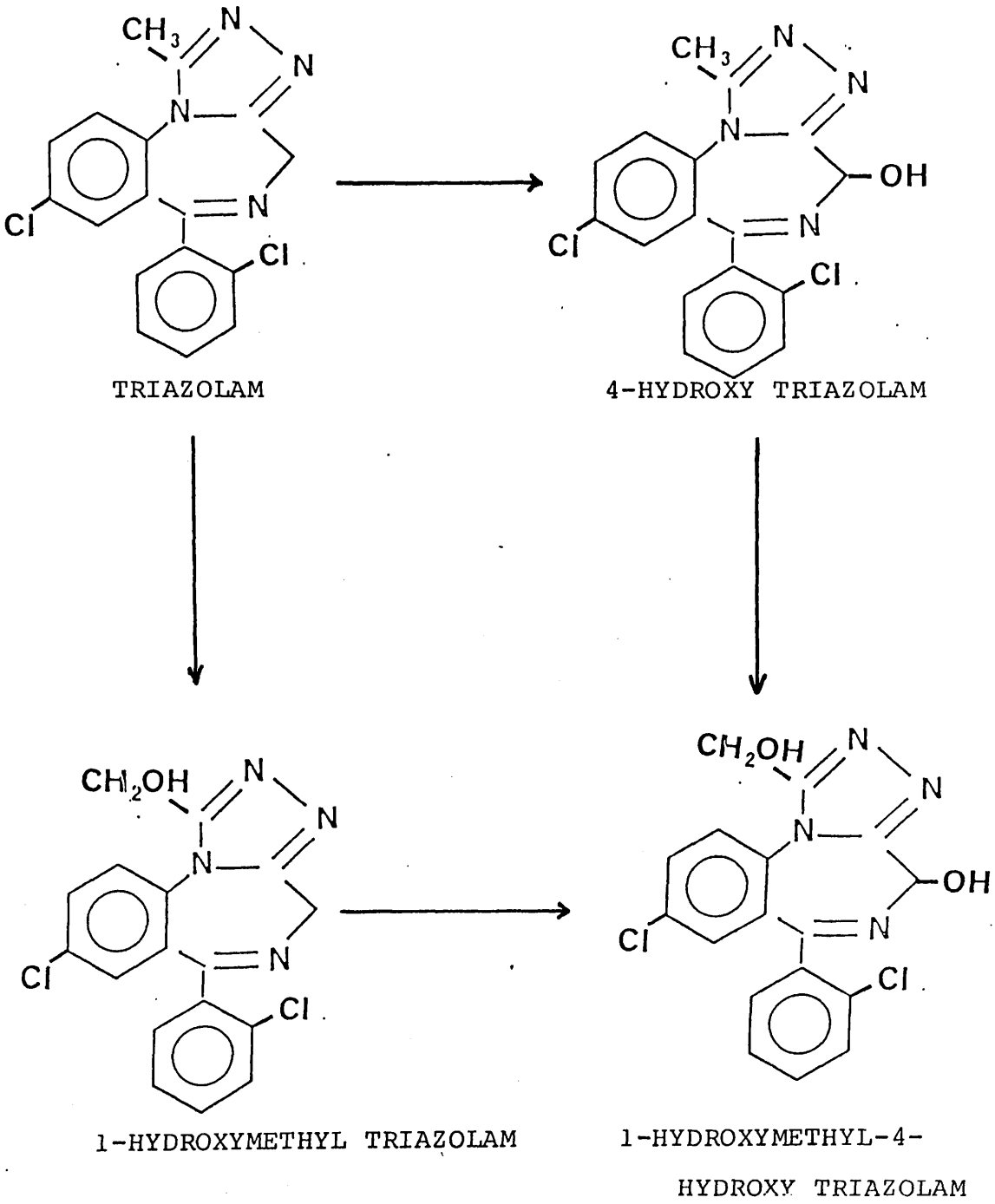
The recommended therapeutic dose for humans is 0.25-0.5mg (equivalent to 0.005-0.01mg/kg) as an oral hypnotic. (The therapeutic dose for greyhounds, on a weight basis was then calculated as 0.125-0.25mg).

The drug has a very short half-life of two to three hours making detection of the parent drug extremely difficult (Eberts, 1979; Greenblatt et al., 1981 and 1983; Smith et al., 1983).

It is extensively metabolised in man and in the dog, mainly by hydroxylation (Figure 2). The major metabolites are 1-hydroxymethyltriazolam and

FIGURE 2.

The metabolic routes of triazolam.



TRIAZOLAM

4-HYDROXY TRIAZOLAM

1-HYDROXYMETHYL TRIAZOLAM

1-HYDROXYMETHYL-4-HYDROXY TRIAZOLAM

4-hydroxytriazolam (Metzler et al., 1977) and the 1-hydroxymethyl metabolite is reported to have 50-100% of the pharmacological activity of the parent compound. These metabolites can be further converted to benzophenones.

In the urine of male beagle dogs, ten metabolites of triazolam were detected which were mostly conjugated (Eberts, 1977).

The absorption, distribution and excretion of triazolam in rats, dogs and monkeys is reported (Eberts, 1974; Kitagawa et al., 1979) as well as the pharmacological properties and therapeutic efficacy of triazolam in insomniatic patients (Pakes et al., 1981). There is also some temporal variation in pharmacokinetic and pharmacodynamic parameters (Smith et al., 1986).

The metabolites of triazolam must therefore be determined in any analytical procedure designed to detect drug misuse.

Flunitrazepam (Rohypnol®)

Flunitrazepam-(5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one) is used for premedication and induction of anaesthesia (0.015-0.03mg/kg) (Rizzi et al., 1975; George and Dundee, 1977; Richardson and Manford, 1979) and as a potent oral hypnotic (0.5-2mg) in humans, (1-2mg in greyhounds) (Nicholson and Stone, 1980).

The hypnotic effects of flunitrazepam predominate over the sedative, anxiolytic, muscle-relaxing and anti-

convulsant effects characteristic of benzodiazepines, due to the presence of the nitro-group and the fluorine atom which both increase hypnotic potency (Stovner et al.,1973). Thus, it is used as a night-time hypnotic and in anaesthesiology; due to the pronounced hypnotic effect it is not appropriate as a daytime sedative (Mattila and Larni, 1980).

However, it displays anti-convulsant properties in various animal species (Zbinden et al., 1967; Randall and Kappell, 1973).

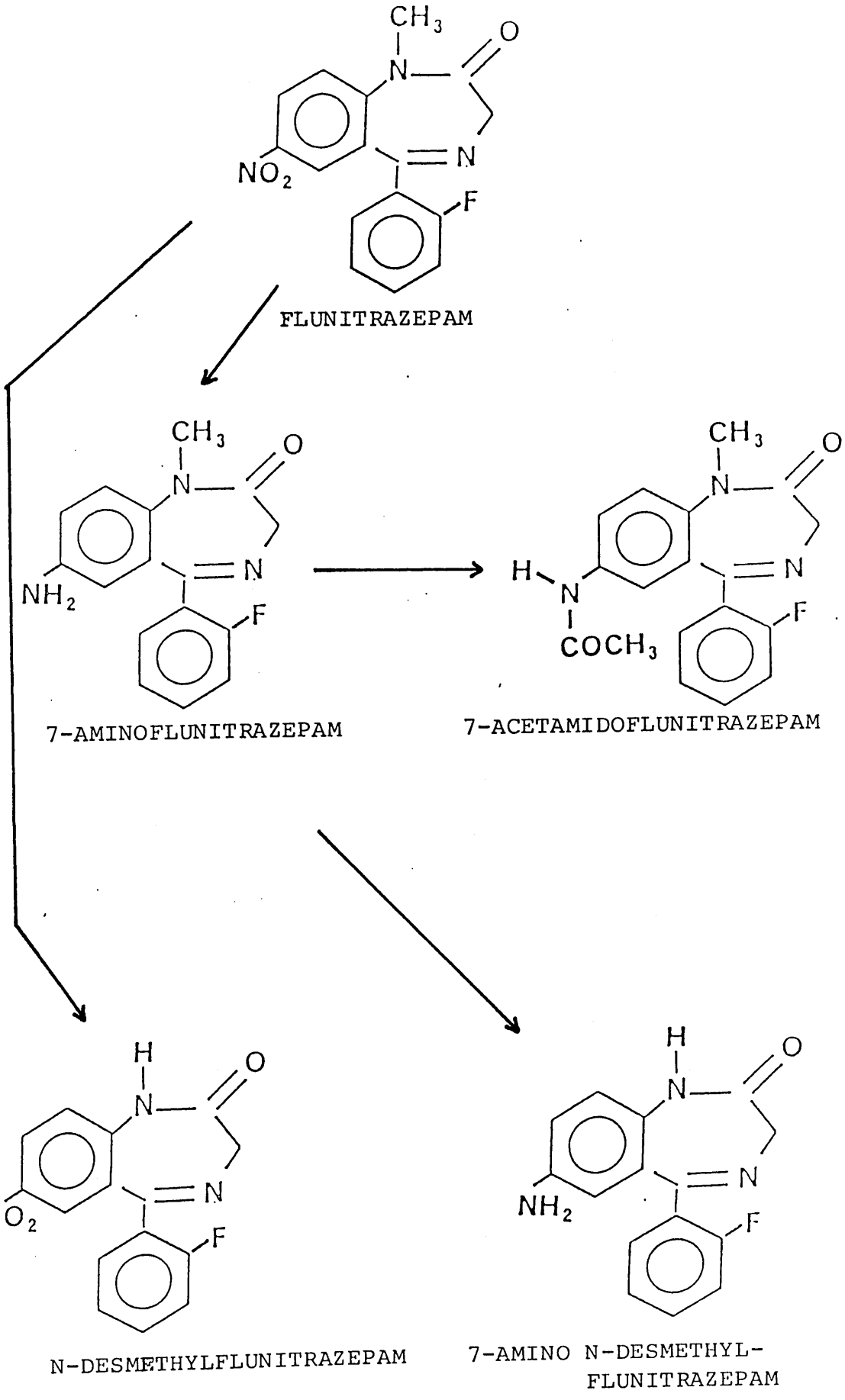
Flunitrazepam is extensively metabolised by reduction of a nitro group to an aromatic amine group, followed by acetylation; by hydroxylation at the 3-position, followed by conjugation with glucuronic acid; and by desmethylation at the N-1 position (Figure 3).

Flunitrazepam in the dog is rapidly eliminated and exhibits a "first pass" metabolism effect following oral administration, whereby the N-desmethyl metabolite is the major detectable drug component in the blood. Neither the parent drug nor its N-desmethyl metabolite is detected in the urine, suggesting extensive and complete biotransformation (Kaplan et al., 1974).

The other main metabolite of flunitrazepam is 7-aminoflunitrazepam, which shows anaesthetic activity in animal studies. No measurable amounts of this metabolite or the N-desmethyl metabolite could be found in the plasma of dog or man after one single therapeutic intravenous injection (Vree et al., 1977).

FIGURE 3.

The metabolic routes of flunitrazepam.



The half-life of flunitrazepam has been reported as approximately 20 hours (Mattila and Larni, 1980) but as with diazepam, this is not reflected in a prolonged clinical effect. However, there is a report of biphasic elimination in the dog with a half-life of 15 minutes in the alpha phase and 150 minutes in the beta phase (Vree et al., 1977).

The extensive biotransformation of flunitrazepam therefore makes detection of the parent drug difficult, so determination of any metabolites is significant.

2.1.2. Xylazine (Rompun®)

Xylazine (5,6 -dihydro -2- (2,6-xylidino) -4H- 1,3 thiazine) (Figure 4) is a widely used veterinary drug. The compound's sedative, analgesic and muscle relaxant properties are due to its action on the autonomic and the central nervous system (Clarke and Hall, 1969).

Xylazine shares certain pharmacological properties with a number of structurally related drugs, for example phenothiazines (Gallenosa et al., 1981). Although it is intended as a veterinary drug, it's metabolism in racing greyhounds is not well documented.

A highly potent drug, it is used more extensively to sedate much larger animals. The pharmacokinetics of xylazine in the plasma of horses (Sams, 1979), cattle, sheep and dogs (Putter and Sagner, 1973), by both intravenous and intramuscular routes is reported.

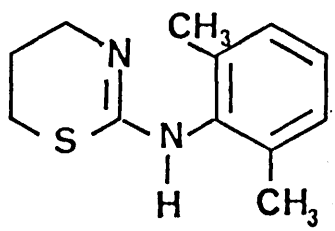
The peak level of drug concentration in the plasma is reached after 12-14 minutes in all species studied following intramuscular injection. The half-life in the alpha distribution phase is approximately 1.2 minutes (cattle) and 5.9 minutes (horses) (Garcia-Villar et al., 1981).

In dogs, the onset of sedative action is between ten and fifteen minutes after injection and the period of analgesia is said to be relatively short (15 to 30 minutes) (Newkirk and Miles, 1974). The recommended therapeutic single dose for greyhounds is 0.05 ml/kg.

Although xylazine is not intended for human use or

FIGURE 4.

The structure of xylazine.



XYLAZINE

abuse, cases of non-fatal poisonings (Carruthers et al., 1979; Gallanosa et al., 1981; Lewis and O'Calaghan, 1983;) and fatal poisonings (Poklis et al., 1985) have been reported.

None of the literature addresses metabolic pathways of xylazine, but rather concentrates on the levels of parent drug present after administration. No parent xylazine is found in the plasma of cattle following therapeutic doses (recommended dose: 0.2mg/kg), a point which could be explained by the low dosage used for this xylazine sensitive species (Garcia-Villar et al., 1981). The extensive metabolism of xylazine is supported further by the observed lack of unchanged xylazine in the urine of sheep (Putter and Sagner, 1973). In the dog, recommended dosage is higher than in other species (1-3mg/kg i.v.), and unchanged xylazine is detected at least up to two hours after dosing (Garcia-Villar, 1981).

No literature is available concerning metabolic pathways in racing greyhounds.

Therefore, the determination of parent xylazine in biological fluids is feasible to detect the misuse of this drug in racing greyhounds.

2.1.3. Mazindol (Sanorex®)

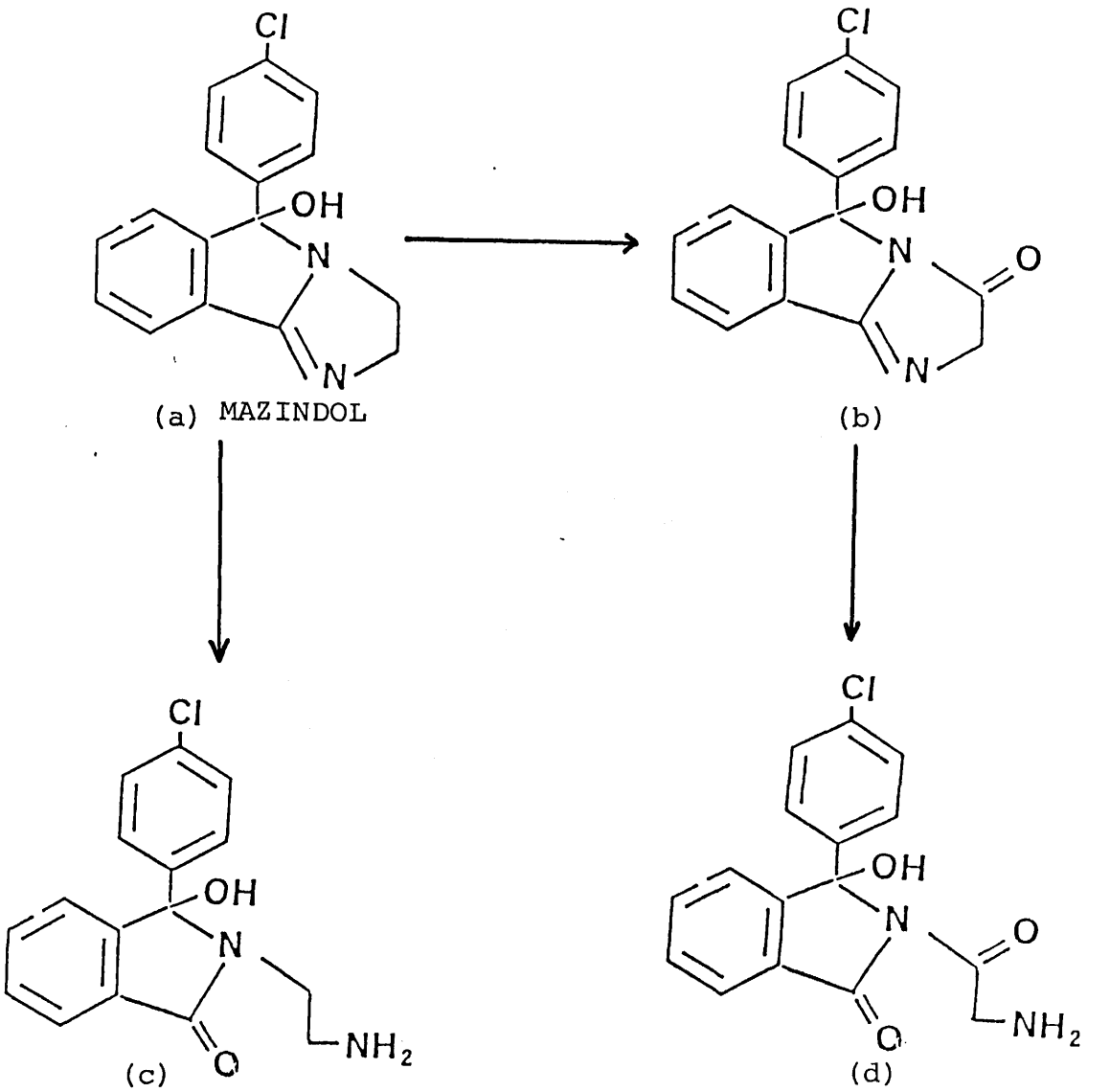
Mazindol (5-(p-chlorophenyl)-5-hydroxy-2,3-dihydro -5H-imidazo [2,1-a] isoindole) is used in the treatment of anorexia due to it's effect as a stimulant of the central nervous system (Sandoz Pharmaceuticals, 1980). However, it differs structurally and in mechanism of action from the phenethylamine drugs such as amphetamine. The pharmacology of mazindol has been studied extensively (Dugger et al. 1976). Metabolic studies in man, dog and rat have shown that the drug is considerably biotransformed into a number of metabolites (Dugger et al., 1979). The major metabolite in all three species was 5- (p-chlorophenyl) -2,5- dihydro -5- hydroxy -3H- imidazo [2,1-a] isoindol -3- one (Figure 5). Conjugation is the major pathway of excretion of mazindol metabolites via the urine in man and dog, but not the rat. Appreciable amounts of parent mazindol are excreted in the urine of man and dog, but again not in the rat. Both the rat and the dog excrete the drug faster than man (Dugger et al, 1979).

The action of mazindol on the central nervous system gives it potential as a pre-race stimulant for greyhounds and racehorses, and so it's use in racing animals is prohibited (International Association of Racing Analysts).

The metabolism of mazindol in horses has been studied recently (Timmings et al., 1985). This supports the

FIGURE 5.

The metabolic routes of mazindol.



(a) 5-(p-chlorophenyl)-5-hydroxy-2,3-dihydro-5H-imidazo(2,1-a)isoindole.

(b) 5-(p-chlorophenyl)-2,5-dihydro-5-hydroxy-3H-imidazo(2,1-a)isoindol-3-one.

(c) 2-(p-chlorobenzoyl)-N-2(aminoethyl)benzamide.

(d) 3-(p-chlorophenyl)-2-glycyl-3-hydroxy-1-isoindolinone.

findings that mazindol metabolites are present at much higher levels and are excreted much later than parent mazindol (Dugger et al., 1976-1979). The major metabolite is the same as in dog and man. Unchanged mazindol was detected up to 12 hours after administration in the urine of all eight horses tested, although peak mazindol levels differed between horses. Plasma mazindol concentrations differed widely in terms of peak concentration and length of time after dosing that the parent drug could be detected.

The presence of parent mazindol is then adequate to prove misuse of the drug in the racehorse up to 12 hours after administration.

2.2 Analysis

2.2.1 Benzodiazepines

Over the past twenty years, analysis methods for drugs have been improving. A reduction in analysis time, by eliminating derivatisation or evaporation steps has been of great importance, and the lowering of detection limits has been a priority due to the increased potency and therefore lower therapeutic doses of novel drugs.

The need for rapid, accurate drug separations has led to many attempts to reduce analysis time, for example, direct injection of serum and blood samples onto liquid chromatography columns (Wahlund, 1981; Szczerba et al. 1986; Koenigbauer et al., 1987).

Several quantitative analytical procedures for determining benzodiazepines and their metabolites in biofluids have been developed. These include colorimetry (Frings and Cohen, 1971), spectrophotometry (Jatlow, 1972), radioimmunoassay (Dixon et al., 1975) and thin layer chromatography (Kaithsa and Tadrus, 1978; Van der Merwe, 1978;); all of which lack specificity as analysis methods.

From a sensitivity point of view, immunochemical techniques generally employed as screening tests are able to identify the presence of benzodiazepines at nanogram levels but they do not discriminate between different commercial benzodiazepines; moreover, quantitative analysis is difficult because the response

of different benzodiazepines and their metabolites towards the antigen-antibody reaction is different.

Among all the methods available, gas chromatographic (GC) determinations of benzodiazepines in plasma, urine and cerebrospinal fluid (Greenblatt, 1978-1981), have provided adequate sensitivity, specificity and resolution for clinical investigation particularly when combined with electron capture detection (ECD).

Gas chromatography with nitrogen-phosphorus detection (NPD) can be used alternatively, but the sensitivity is much less than with ECD (Dhar and Kutt, 1978).

The disadvantages of GC analysis are most obvious when considering the analysis of metabolites. Often this requires a time consuming extraction, followed by derivatisation into more volatile compounds before application to the gas chromatographic column (de Silva and Bekersky, 1974; Horton-McCurdy et al., 1979; de-Gier and Hart, 1979; Higuchi et al., 1979; Masahura et al., 1982). Moreover, some benzodiazepines and their metabolites (for example, oxazepam and chlordiazepoxide) are highly thermolabile and so cannot be subjected to the high temperature needed for GC analysis.

High pressure liquid chromatography (HPLC) is potentially the most useful method to allow separation and simultaneous quantitation of the parent compound and it's metabolites. For forensic purposes, analysis of benzodiazepines usually involves detection at

relatively high concentrations. Often, though, HPLC methods are inadequate for situations following therapeutic administration of the drugs (Bugge, 1976). Because of it's milder working conditions, HPLC is a more suitable technique for the analysis of thermally labile, high molecular weight, hydrophilic and hydrophobic compounds.

A further advantage of HPLC for the analysis of benzodiazepines is that compounds may be analysed without initial derivatisation (Scott and Bommer, 1970; Hulshoff et al., 1976; Chiarotti et al., 1986), although increased sensitivity of HPLC methods can be achieved by hydrolysis of the benzodiazepines into benzophenones (Violon et al., 1980-1982).

HPLC has not received the same attention as GC methods because of the poor specificity of single wavelength UV detection, and also due to the highly selective nature of the separations which are usually designed to separate only a few compounds (Hirayama and Kasuya, 1983; Ferslew et al., 1989). While the selectivity of LC makes retention time on a system a good marker for identification, it also reduces the potential of the technique for broad spectrum screening purposes.

The difficulties of connecting HPLC to a mass spectrometer and the restrictions which LC-MS places on the chromatographic conditions make GC-MS a more favoured technique.

This problem has been addressed by the use of diode

array detection (DAD) which improves the range and specificity of HPLC and makes it a reliable and informative technique for application in drug screening studies (Bogusz et al., 1985; Minder et al., 1987; Mura et al, 1987).

Diazepam

Diazepam and it's metabolites have been extensively analysed by gas chromatography, (Steyn and Hundt, 1975), using electron-capture detection (Vessman, 1977; Lindley, 1979;) or by nitrogen sensitive detection (Horton McCurdy et al., 1979).

The disadvantages of GC when applied to metabolic studies have already been outlined.

HPLC methods can be sufficiently specific and sensitive when focused on one or a few benzodiazepines, and these methods are very suitable for use in drug monitoring or when the intoxicant is known, for example, diazepam and it's metabolites.

Most literature involving benzodiazepine analysis includes diazepam, since it is one of the oldest members of the benzodiazepine group of drugs.

Column

Reversed-phase HPLC systems have been reported extensively for diazepam analysis and it is included in benzodiazepine review articles (Hailey, 1974; Clifford and Franklin-Smyth, 1974; Chiarotti et al., 1986).

Most of these systems incorporate C18 columns of standard length, internal diameter and 5um packing material.

Overall, 5um packing material results in better selectivity and resolution for the determination of diazepam and its metabolites (Brodie et al., 1978). Disadvantages of the existing techniques using C18 columns included background interference in the oxazepam region from blood extracts (Kabra et al., 1978); the need to modify the mobile phase to separate all the metabolites (Skellern et al., 1978); inadequate sensitivity for human urine extracts (200ng/ml) (Cotler et al., 1981); the analysis of derivatised benzophenones rather than underivatised benzodiazepines (Violon et al., 1980-1082); and the use of gradient HPLC which is not a readily available technique in all laboratories (Mura et al.,1987).

The use of C8 reverse-phase columns has also been reported (Rao et al., 1982; Sohr and Buechel, 1982; Komiskey et al., 1985).

This approach allows less chance of retaining the drug (less non-polar interaction) but an easier elution step. However, the requirement for modified mobile phases and various detection wavelengths for the different benzodiazepines is a major disadvantage when the assay is required as a screening method (Sohr and Buechel, 1982). Enhanced sensitivity is achieved using radiolabelled samples and a reverse isotope dilution method, but radioactive samples are not always convenient to work with (Komiskey et al., 1985).

The use of a phenyl-bonded HPLC column was reported

(Wong, 1983), but using this, some benzodiazepines, including temazepam, did not chromatograph.

The determination of diazepam in its injectable form, with no information regarding metabolites, was described using a microparticulate hexyl bonded-phase column (Smith and Nuessle, 1982).

A number of liquid-solid systems consisting of mixtures of buffers using methyl silica as the stationary phase for the separation of a number of benzodiazepines and their metabolites was reported (Tjaden et al., 1980). In order to separate some of the metabolites, though, the flowrate had to be reduced to 2.9ul/second giving a very long retention time for diazepam.

Diazepam and it's metabolites have also been separated by normal-phase chromatography (Scott and Bommer, 1970; Gonnet and Rocca, 1976; Bugge, 1976), anion-exchange (Moore et al., 1977) and cation exchange resins (Twitchett et al., 1976), although these methods are not as widely applicable as reversed-phase systems.

Mobile phase

Mobile phases used in HPLC analysis of diazepam are invariably mixtures of methanol, acetonitrile and buffers (usually incorporating phosphate salts). While methanol:water mixtures act as good, selective eluents, salts are often added to improve the separation (Horvath and Melander, 1977). The addition of dibasic sodium or potassium salts is common (Peat and Kopjak, 1979). However, there are disadvantages to salt

addition. Eluents containing salts (especially phosphates), require column flushing after each use. This is a time consuming process and failure to carry it out can result in column blocking.

Further, column corrosion of the stainless steel tubing in the system is a possibility when salts are present.

Eluents containing ion-pairing agents have also been investigated (Sohr and Buechel, 1982; Minder et al., 1987).

In these acidic mobile phases, the counter-ion forms an ion-pair or association complex with the ionic form of the analyte which is then retained by the reverse-phase column. These eluents show good potential in terms of sensitivity, but are more useful for the determination of strongly basic substances rather than benzodiazepines which are only weakly basic and are easily determined using a neutral mobile phase. These ion-pairing agents were not as efficient in improving peak shapes as was the addition of amines to the mobile phase (Minder et al., 1987).

Detection Wavelength and Limits

The most widely used single detection wavelength is 254nm. In some methods, 240nm is employed (Kabra et al., 1978; Rao et al., 1982) and also 229nm (Komiskey, 1985). All benzodiazepines absorb in the ultraviolet region (220-260nm), so their detection has rarely been a problem.

However, detection limits have indeed been a problem, particularly in therapeutic analysis. Some HPLC systems have been shown to be sufficiently sensitive for the analysis of metabolites in urine, serum and saliva after single dose administration to humans (Tjaden et al., 1980), but widely differing detection limits have been reported.

HPLC assays particularly designed for small animal studies have been described but these normally require radiolabelled drugs (Komiskey et al., 1985), and do not refer to urine samples (Klockowski and Levy, 1987).

The most sensitive method so far reported allows the detection of 0.5ng of diazepam in post mortem blood (Wong, 1983), but more common detection levels are 15-50 ng/ml (Kabra et al., 1978; Cotler et al., 1981; Ratnaraji et al., 1981; Smith and Nuessle, 1982). These limits are sufficient for diazepam and it's metabolites after single dose administration, but would not be adequate for the detection of more potent benzodiazepines which are present at much lower levels, for example, triazolam and flunitrazepam.

A recent alternative to ultraviolet detection is the use of electrochemical methods (Lloyd and Parry, 1988). However, these systems are often difficult to use because of the requirement for a deoxygenated eluent if a low background current is to be obtained (therefore giving satisfactory sensitivity). This problem can be overcome somewhat by maintaining the

eluent reservoir under slow reflux while in use, but overall, this system is not as easy to use or as flexible as those incorporating single wavelength detectors.

Sample size

The problem of sample size has also been considered. In many cases, particularly with samples involving laboratory animals, sample size may often be limited. Most papers require the use of 1-2ml of blood or plasma and 1-10ml of urine. The microsample determination of diazepam is described (Lau et al., 1987) and this is definitely an improvement, in terms of sensitivity (0.25 ng for oxazepam, N-desmethyldiazepam and diazepam; 0.5 ng for temazepam), on other reported HPLC methods. Sample size, however, is unlikely to be a problem in this work.

Reduction in extraction time

The use of microbore HPLC for diazepam analysis shows similar sensitivity to that of conventional HPLC. An alternative approach to extraction was developed, involving the pretreatment of serum via ultrafiltration to remove serum proteins followed by injection of the ultrafiltrate onto a reversed-phase pre-column using water as the mobile phase. The components of interest are trapped at the head of the pre-column and subsequently back-flushed onto a microbore column using

a stronger analytical mobile phase (Koenigbauer et al., 1987).

At trace levels, trace contaminants were noted - this was a major disadvantage of the packed pre-column which had a limited sample capacity due to its' small size. The sensitivity of the method, however, (4 ng/ml) compared well to other reported diazepam levels. (Kabra et al., 1978; 10 ng/ml in plasma: Brodie et al., 1978; 40 ng/ml in whole blood: Haver et al., 1986; 20 ng/ml in serum).

Reverse-phase chromatography, then, is generally accepted as the most versatile technique for separation of most major benzodiazepines, and a typical analysis system for diazepam and it's metabolites would be based on a C18 column using methanol, acetonitrile and sodium/potassium phosphate or acetate buffer (pH acid) as an eluting solvent. The most common system for detection of the drugs uses single wavelength UV light at 254nm, although anywhere between 220 and 260nm can be employed.

Such a system was described which separated diazepam from it's three main metabolites within 12 minutes (Lau et al., 1987).

Triazolam

Triazolam is a relatively new sedative-hypnotic drug and its analysis is not as widely reported as that of diazepam.

Radioimmunoassay and radio receptor assays lack

specificity as far as the active metabolites are concerned (Ko et al., 1977), but a urinary screening method for alprazolam, triazolam and their metabolites was reported using EMIT (Fraser, 1987).

So far, gas chromatographic (GC) (Greenblatt et al., 1981; Jochemsen and Breimer, 1981;), and liquid chromatographic methods (HPLC) (Adams, 1979; Theis and Bowman, 1983; Wong, 1984) allow only the detection of triazolam itself.

Due to it's extensive metabolism and short half-life, the detection of parent triazolam in biological fluids is unlikely, so these analysis methods are not viable for determination of triazolam after administration. However, the existence of unchanged triazolam in post mortem samples has recently been reported (Koves and Wells, 1986; Hama et al., 1987).

Capillary GC methods addressing this problem have been published (Coassolo et al., 1983) although application to urine samples was not described.

Only one HPLC method for the specific determination of triazolam and it's metabolites in human urine was found (Inoue and Suzuki, 1987). This method employed methanol-10mM phosphate buffer, pH8 (65:35 v/v) as the mobile phase, a C18 HPLC column, ultraviolet detection at 220nm and a flowrate of 1ml/minute.

The method is a typical HPLC system and it is surprising that more analysis methods for triazolam are not available, although triazolam is included in

reported HPLC analysis methods for screening benzodiazepines (Mura et al., 1987).

Flunitrazepam

Published analytical methods for the determination of flunitrazepam and its metabolites are somewhat limited. For urine samples, the available immunoassays for benzodiazepines are unable to discriminate between the different drugs owing to the cross reactivity of the antibodies used in this type of assay.

Gas chromatographic methods (de Silva and Bekersky, 1974; Faber et al., 1977; Jochemsen and Breimer, 1982) usually involve extremely long extraction procedures and derivatisation of the sample prior to analysis. The detection limits are low (0.5-1ng/ml) but measurable levels of neither parent flunitrazepam nor its metabolites were found after single therapeutic dose administration in the urine of man nor dog, since flunitrazepam is extensively metabolised in the body.

Determination by column liquid chromatography with ultraviolet detection at 230nm, gave a detection limit of 1ng/ml (Vree et al.,1977), and fluorimetric detection gave a similar detection limit of 0.5ng/ml using a 4ml sample (Sumirtapura et al., 1982).

A more recent publication, (Weijers-Everhard et al., 1986), describes an HPLC system with fluorimetric detection for analysis of 7-aminoflunitrazepam since the N-desmethyl metabolite is not detected in the urine. The method is based on the formation of highly

fluorescent acridine derivatives, so the advantage of HPLC as a direct analysis method involving no derivatisation, is lost. The method described, consisted of a Hypersil ODS column (C18) (5um, 100mm x 4.6mm i.d.). The fluorimeter was equipped with a 25ul through-flow cell and the wavelengths were set at 396nm (excitation) and 445nm (emission). The mobile phase consisted of methanol-water (55:45) containing 0.05M acetic acid buffer (pH 4.7) and 0.05mM tetramethylammonium hydroxide.

2.2.2. Xylazine

Although not widely studied, an increasing amount of literature regarding the analysis of xylazine is becoming available. Analytical methods reported for the determination of xylazine in biological fluids include spectrophotometry and thin-layer chromatography (TLC) (Putter and Sagner, 1973), but these methods are slow and are not sufficiently sensitive or specific for therapeutic level analysis.

Gas chromatographic (GC) analysis has made use of sulphur specific detection (Laitem et al., 1978) giving a detection limit of 90ng/ml as well as the more common nitrogen sensitive detectors (Rogstad and Yndestad, 1981; Poklis et al., 1985) giving detection limits of 10ng/ml and 20ng/ml of serum respectively.

The properties of xylazine on a variety of stationary phases for GC analysis were studied (Rogstad and Yndestad, 1981).

Tailing, a common observation with underivatised amines, was the main problem. Overall, semi-polar phases gave the best separation although there was still some tailing of the peaks. Higher resolution obtained with the capillary columns made glass capillary GC a superior technique for the determination of low concentrations of xylazine and gave good separation from contaminants.

However, analysis with conventional packed columns was simpler and less time consuming, and therefore more

suitable for routine analysis.

High-performance liquid chromatography (HPLC) analysis methods for xylazine are not widespread. Apparently these were only developed because the low volatility and ionic character of xylazine do not permit it's direct quantitation by GC. This is true to some extent, but recent GC methods have solved this problem. The use of an ion-pairing agent (heptane sulphonic acid) in the mobile phase, a C18 column, single wavelength detection at 225nm and a detection limit of 20ng/ml using a sample size of more than 0.5ml is described (Alvinerie and Toutain, 1981).

A more recent HPLC method for xylazine determination (Akbari et al., 1988) makes use of a Bondapak C18 (30cm x 2.9mm i.d.) column operated at ambient temperature. The system is equipped with a fixed wavelength absorbance detector (254nm) and the degassed mobile phase, consisting of acetonitrile-10^{-3}M aqueous hydrochloric acid-methanol (60:30:5), is pumped through the column at a flow-rate of 1ml/minute.

2.2.3. Mazindol

A relatively new drug in the sporting field in terms of abuse, mazindol is usually analysed using gas chromatography-mass spectrometry (GC-MS), (Timmings et al., 1985) or by radiolabelled methods (Dugger et al., 1979). Serious difficulties in the analysis of mazindol were reported (Timmings et al., 1985). The standard compounds tended to breakdown during injection into the gas chromatograph and attempts to derivatise the primary amine group with trifluoroacetic anhydride (TFAA) were unsuccessful. GC with nitrogen-phosphorus detection gave a poor detection limit because of the urinary background remaining in the extracts, so the final approach was to incorporate a mass selective detector (MSD) operated in the selective ion monitoring (SIM) mode.

The mazindol extracts were analysed using a Hewlett-Packard 5890 GC interfaced with a Hewlett-Packard MSD. The GC conditions were :

Column:	30m x 0.25mm i.d., DB-5
Injector:	Splitless ($250^{o}C$;0.60min. delay)
Oven:	$75^{o}C$ for 1min; $15^{o}C$/min.
and:	$285^{o}C$; $285^{o}C$ for 11mins.
Transfer line:	$285^{o}C$

The MSD was operated in SIM mode to reduce background effects.

There are no published HPLC methods for the drug mazindol.

2.3 Extraction Methods.

Extraction and analysis of drugs from urine, whether for forensic purposes, doping control (greyhounds or racehorses), pre-employment screening or clinical analysis is an expanding area of commercial interest where rapid, reproducible extractions and analytical techniques are of great value.

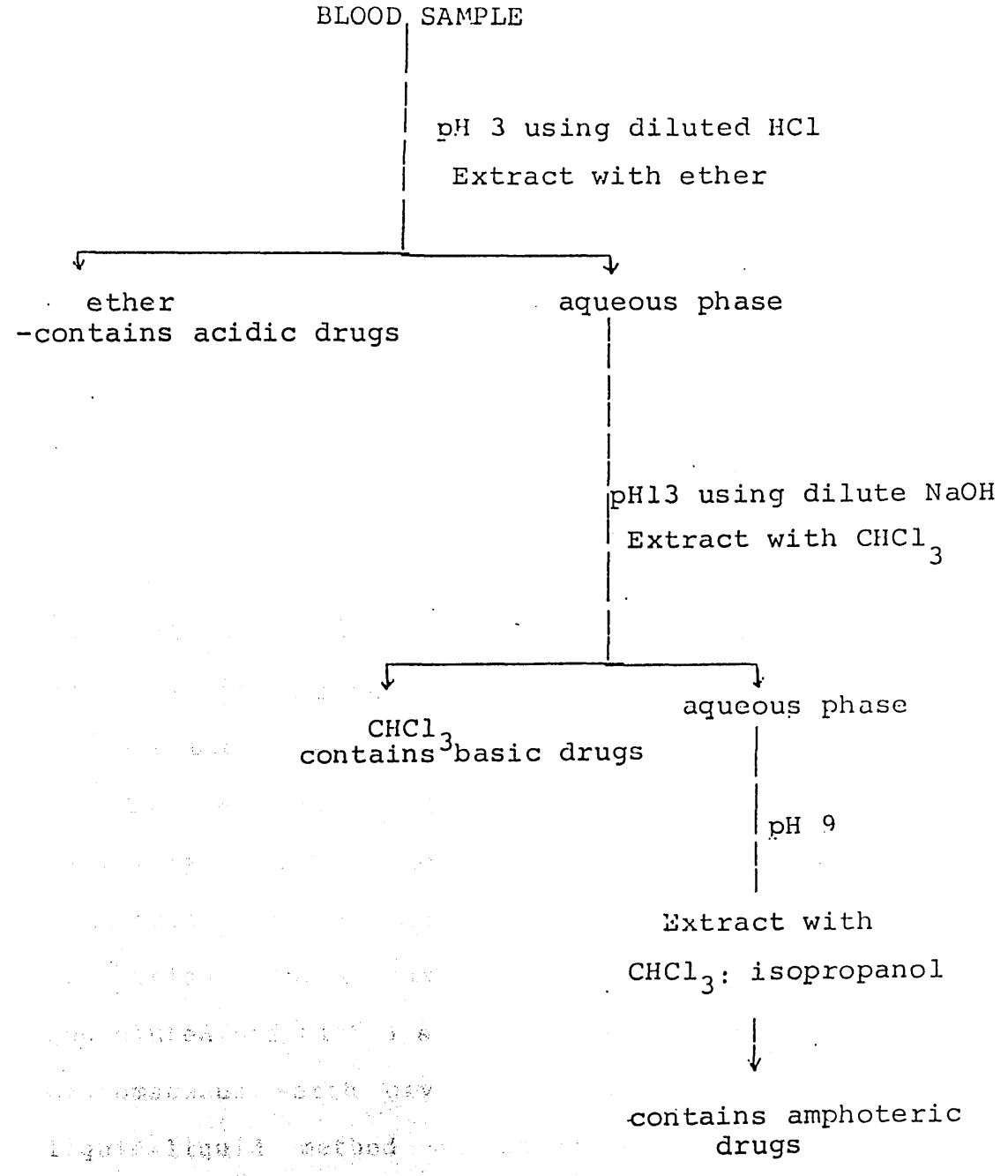
The efficiency of extraction, a reduction in extraction and analysis time and operator independance have all been recent priorities in the research world (Harkey and Stolowitz, 1984).

The majority of extractions from biological fluids are carried out using solvent extraction techniques. These methods are long and tedious, often requiring three or four extractions, purification and evaporation procedures, (Figure 6). Despite the number of stages involved, high drug recoveries have been reported (Horning et al., 1974; de-Gier and Hart, 1979). Such procedures are usually sufficiently efficient to extract therapeutic levels of drugs and/or their metabolites.

Far less of the literature has concerned itself with solid-phase extractions, which, over the past decade, have emerged as a powerful tool for chemical isolation and purification. The use of solid-phase columns as an alternative to liquid-liquid extraction, for the extraction of drugs in urine, has gained popularity

FIGURE 6.

Extraction of Drugs from a Blood Sample.



over recent years because of the reported excellent recoveries and ease of use (Good and Andrews, 1981; Stewart et al., 1984; Wilson, 1986). Further advantages include the simultaneous extraction of a number of samples, the prevention of emulsion formation as often occurs with solvent extraction, rapid sample preparation, removal of evaporation steps and potential automation.

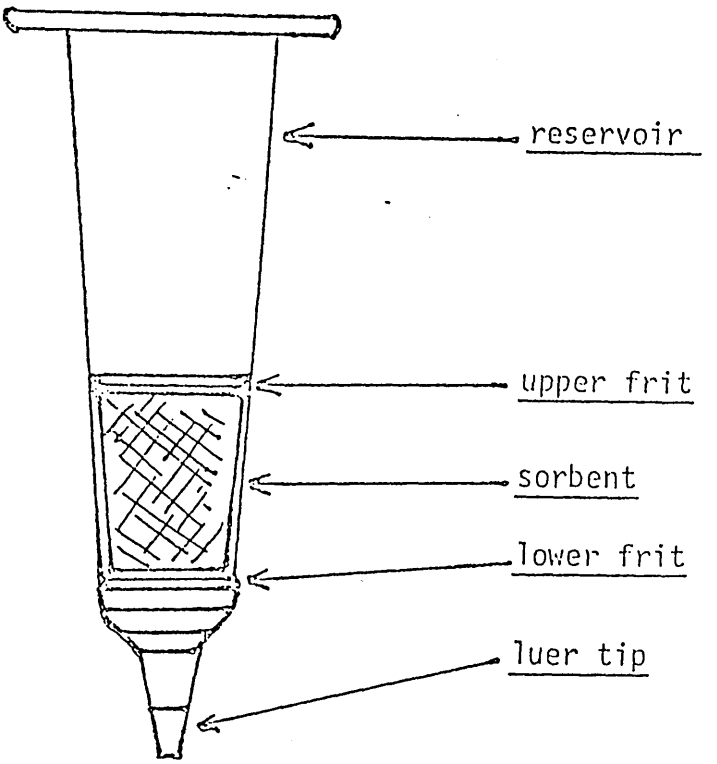
There are basically two approaches to the solid-phase extraction of drugs:- one in which drugs are separated from the biological matrix by adsorption onto an inert material, such as diatomaceous earth, the other using bonded silica sorbents (Figure 7).

Columns packed with diatomaceous earth were first used in the mid-1970's for the extraction of drugs. Basically any existing liquid-liquid extraction procedure can be applied to this type of column extraction. The sample is simply applied to the column and eluted off with a suitable solvent.

Diatomaceous earth gives a similar extraction to the liquid-liquid method on which it is based. These columns tend to be less specific than bonded silica cartridges for the extraction of a range of drugs and have been used for drug screening purposes (Delbeke and Debackere, 1978). Recoveries from diatomaceous earth are, however, generally poorer than from the silica columns (Stewart et al., 1984). In addition, the procedures are fairly time consuming, requiring a

FIGURE 7.

Sorbent Extraction Cartridge



number of steps, and the lack of selectivity means that any interfering compounds co-extracted by liquid-liquid extraction may also be extracted by the diatomaceous earth (Breiter et al., 1976; Daenens et al., 1980).

In some cases, the isolated drugs from diatomaceous earth extractions required further clean-up procedures prior to GC analysis, resulting in a 30% loss of extraction efficiency (Kaempe, 1983).

In the case of bonded silica sorbents, there are a number of different types of column packing materials commercially available for drug extraction. These sorbents can be divided into three classes (non-polar, polar and ion-exchange) according to the nature of the interactions between isolates and solvents. This study addresses the interactions of all three classes.

The majority of solid-phase methods for the extraction of drugs from body fluids, which have been reported, involve the use of columns packed with non-polar adsorbent materials.

These consist of silica onto which is bonded a long chain hydrocarbon, principally C8 or C18, producing an essentially non-polar adsorbent phase similar to that present in a reversed phase HPLC column.

Since most drugs have the potential for some non-polar interaction, a number of methods have been described for the extraction of individual drugs (Kabra et al., 1983; Gault, 1985; Kabra and Nzekwe, 1985; Harrison et al., 1986; Stubbs et al., 1986; Fami et al., 1987;

Carlucci, 1988) or groups of structurally related drugs (Frethold and Sunshine, 1980; Ford et al., 1983; McDonald, 1985; Sample et al., 1986; Moore and Tebbett, 1987;), using C8 or C18 bonded silica columns.

Polar sorbents are used much less frequently than non-polar sorbents. This is unusual, since their selectivity is much greater than the non-polar cartridges which extract anything with some degree of non-polar character from biological matrices. (Stewart et al., 1984; Lensmeyer et al., 1986).

Ion-exchange cartridges are potentially the most useful from a drug screening point of view. Their use, so far, is not as extensive as that of non-polar cartridges, although they have been used to extract various drugs including catecholamines (Wu and Gornet, 1985) steroids (Axelson et al., 1981), and also xanthurenic acid (Ubbink et al., 1988).

Extraction efficiency comparisons between the various types of sorbents available have been carried out by several researchers.

The use of a granular support material as a column packing is compared with XAD-2 resin and conventional solvent extraction methods for various drugs, the diatomaceous earth material giving superior results in terms of drug recovery and solvent volumes required (Breiter et al., 1976).

Sep-Pak® C18 cartridges have been compared with Clin-Elut® (diatomaceous earth), XAD-2 resins and

Bond-Elut® C18, silica and cyanopropyl (CN) columns for a cross-section of drugs of abuse. Overall, the C18 packing materials gave the best results regarding drug recovery and reproducibility of extraction. However, good extraction of the basic drugs was obtained from the XAD-2 resins, diatomaceous earth and cyanopropyl columns. XAD-2 gave good recoveries for amphoteric drugs and Clin-Elut was good for hydrophobic drugs (Stewart et al., 1984).

Conventional solvent extractions at pH 9.3 or pH 5.2 into dichloromethane, were compared with XAD-2 resins, diatomaceous earth (Celite 560) and Bond-Elut C18 cartridges. The Bond-Elut extractions proved to be far simpler and more efficient than the other extractions (Hyde, 1985).

Therefore, a solid-phase extraction approach to sample preparation would give new data for those drugs not yet extracted using bonded sorbents (for example, mazindol and xylazine) as well as providing more selective screening methods for groups of drugs liable to abuse (for example, benzodiazepines). The extractions are rapid, simple, cheap, allow simultaneous extraction of multiple samples and are easily automated.

For this reason the use of bonded silica sorbents was studied.

2.3.1. Sorbent Extraction.

Theory.

Sorbent extraction is a physical process that involves a liquid and a solid phase. The solid phase has a greater affinity for the drug to be isolated than for the matrix in which the isolate is present. In theory, very selective extractions resulting in highly purified and concentrated isolates can then be achieved by choosing sorbents with an attraction for the isolate but not for the sample components. The specific properties of a bonded cartridge are a result of the functional group covalently bonded to the silica substrate through reaction of the activated silica with organosilanes.

Synthesis.

Bonded silicas are formed by the reaction of organosilanes with activated silica. The product is a sorbent with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage. The intention is to create a surface whose principal properties are due only to the functional group with minimum interactions from the silica substrate.

Physical Properties.

Bonded silicas are rigid materials that do not shrink or swell in different solvents. This is a distinct advantage over polystyrene based resins since a wide range of solvents can be employed for washing and

eluting the isolate. The bonded sorbents equilibrate rapidly to new solvent conditions. The particles of the sorbent are usually irregularly shaped to allow rapid solvent flow through the sorbent bed. This does have certain disadvantages in terms of extraction reproducibility, particularly from batch to batch of the same cartridges and certainly when cartridges are supplied by different manufacturers.

Chemical Stability.

The sorbents are stable within a pH range of 2-7.5. Above pH 7.5, the silica substrate is susceptible to dissolution in aqueous solutions and below pH 2, the silyl-ether linkage is labile. As a result, the functional groups bonded to the surface will begin to cleave, so altering the sorptive properties of the bed. In practical terms, though, it is possible to use the beds over the whole pH range since degradation is a finite process and sorbents are usually only exposed to solvents for a short length of time.

Solvation/Conditioning.

For a sorbent to react reproducibly with an isolate, the sorbent must be solvated or "conditioned". This means, essentially, wetting the sorbent in order to create an environment suitable for isolate retention. Any solvent which will wet both the polar surface and the functional group can be used, most commonly methanol or acetonitrile is employed.

The conditioning solvent must be miscible with the

matrix of the isolate and the sorbent bed must not be allowed to dry out.

Retention and Elution.

Retention of the isolate is a function of the degree of attraction of a chemical species for the sorbent.

Elution is brought about by introducing a solvent to which the isolate is more strongly attracted than it is to the sorbent. The eluent should elute the isolate from the bed in the smallest volume possible.

Capacity and Selectivity.

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. Capacities of silicas vary so it is necessary to consider capacity requirements of the isolate as well as any impurities likely to be present. Larger columns may retain all the isolate but will then require larger volumes of eluent to remove it.

Selectivity is the ability of the sorbent to discriminate between the isolate and all other sample matrix components. The selectivity, is then a function of the chemical structure if 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 functional groups common only to the isolate and not the other components of the matrix.

Sorbent choice, then, is based on interaction between

isolate and sorbent. In this study, the non-polar character, the polar character and the basic nature of the isolates are all considered in the development of solid-phase extraction methods.

a) Non-polar Interactions.

Compounds containing alkyl, aromatic, alicyclic or other functional groups with significant hydrocarbon structure will exhibit non-polar interactions. Most isolates, including most drugs, have some degree of non-polar character and so will be adsorbed onto non-polar sorbents, for example, C8 or C18 cartridges. Non-polar extraction is useful when the isolates vary widely in chemical structure. In general, non-polar sorbents are not very selective since most matrix components tend to have some degree of non-polar character. Non-polar interactions can be very strong with non-polar isolates of high molecular weight, in which case, isolates are often difficult to remove. Buffers, or aqueous matrices of high ionic strength may promote isolate elution by reducing the sorbent functional groups' interactions with the isolate. The effect of this can be reduced by lowering the ionic strength of the sample through dilution and equilibrating the sorbents with organic buffers only.

C18 (Octadecyl) Sorbents.

C18 is the most non-polar sorbent available. It is the most retentive of all sorbents for isolates being

retained by non-polar interactions. Very non-polar compounds are difficult to remove from this sorbent. It is generally regarded as the least selective sorbent since it retains almost everything from aqueous matrices, but final extracts are often of poor quality. The potential for polar interactions between this sorbent and isolates is less significant with C18 than with any other sorbent because of the predominant effect of the hydrocarbon chain.

C8 (Octyl) Sorbents.

C8 is very similar in properties to C18, but not quite as retentive for non-polar isolates, due to it's shorter hydrocarbon chain. It is therefore useful for isolates which are too strongly retained on C18 columns. The potential for polar interactions with this sorbent are somewhat higher than for C18, but it's polar interactions are not a significant feature of this sorbent.

b) Polar Interactions.

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 exhibiting polar interactions (dipole/dipole, induced dipole, pi-bonding etc.) exist. Polar interactions are very

useful for highly selective separation of molecules having similar structures. They are the least widely used set of sorbents.

CN (Cyanopropyl) Sorbents.

A medium polarity sorbent with many uses, CN is ideal for applications in which extremely non-polar isolates would be irreversibly retained on non-polar sorbents such as C18 or C8. Conversely, CN can be used as a polar sorbent that is less retentive for very polar isolates that might be retained irreversibly on the more polar sorbents, such as silica or diol. The cyano group gives CN a unique selectivity, which can be moderated by intelligent use of solvents.

20H (Diol) Sorbents.

Since diol cartridges are fairly polar, they are usually used for polar extractions from non-polar solvents. The diols resemble unbonded silica in their tendency for strong hydrogen bonding with isolates. They also have the ability to discriminate between compounds of close similarity, for example, structural isomers. In addition to their polar interactions, diols can also be used as non-polar sorbents because the hydrocarbon spacers on their functional groups provide enough non-polar character for retention of non-polar isolates.

NH2 (Aminopropyl) Sorbents.

In common with many of the ion-exchange sorbents, NH2 is capable of exhibiting all possible interactions to

some degree. NH2 is a very polar sorbent and a strong hydrogen bonder, so it can function as an anion-exchanger. The pH of the NH2 sorbent is 9.8, so at any pH below 9.8, NH2 is positively charged. Although NH2 can be used for non-polar interactions, it's extreme polarity makes its non-polar character less significant than its other properties.

c) Ion-exchange Interactions.

Cation exchange cartridges are mainly used to extract molecules containing functional groups capable of exhibiting a positive charge, that is, basic groups. Isolates can be removed from cation-exchangers by neutralising the charge on the sorbent or neutralising the charge on the isolate. Also, the presence of a high ionic strength buffer will cause the high concentration of cations in the buffer to compete with the cationic isolate for the sorbent, so promoting elution of the isolate.

PRS (Sulfonylpropyl) Sorbents.

A strong-cation exchange sorbent which is also very polar, PRS does not exhibit any appreciable degree of non-polar interactions. In non-polar solvents, PRS is capable of polar and hydrogen bonding interactions. The pKa of PRS is very low, and usually cationic isolates must be eluted by high ionic strength or by neutralising the charge on the cationic isolate.

SCX (propylbenzenesulfonyl) Sorbents.

SCX is a very strong cation exchanger with a low pKa. The main difference between this and PRS is the much higher potential for non-polar interactions of the SCX sorbent, due to the presence of the benzene ring on it's surface.

This non-polar character should be considered when the cartridge is used for ion-exchange from aqueous solvent systems.

This dual nature is very useful for the isolation of molecules exhibiting cationic and non-polar character. After retention on the column, the sorbent can be washed with non-polar and high ionic strength solvents without displacing the isolate. The isolate can then be removed with a solvent which disrupts both ionic and non-polar interactions simultaneously, such as methanolic hydrochloric acid.

2.3.2 Benzodiazepines.

Solvent Extraction

Typical solvent extraction procedures for 1,4 benzodiazepines have been described. These are often extremely long methods: sometimes requiring several solvent extractions and combining the various extracts (Steyn and Hundt, 1975; Shaw et al., 1983; Stanworth, 1984; Zilli and Nisi, 1986); often incorporating back extractions (Baselt et al., 1977; Pape and Ribick, 1977; Dhar and Kutt, 1979; Horton-McCurdy et al., 1979; Klotz, 1981; Vasiliades and Sahawneh, 1982); invariably including sample evaporation (Baktir et al., 1985; Locniskar et al., 1985); and occasionally derivatisation (Brooks et al., 1977; Langas, 1977; Vessman et al., 1977; Kaithsa and Tadrus, 1978).

Drug recoveries from solvent extractions are dependant on the number of extractions performed. The more extractions and back extractions that are carried out, the greater percentage of drug is extracted; conversely, each extra procedure allows an opportunity for drug loss. Often, the release of metabolites from their conjugated forms is necessary prior to extraction, particularly for benzodiazepine metabolites. Conjugated glucuronides in urine have been isolated and determined by reverse-phase HPLC (Mascher et al., 1984), but more commonly, drugs are released from their conjugated forms by some form of enzyme hydrolysis or digestion (Osselton, et al., 1977; Osselton, 1979).

Solid-Phase Extraction

The advantages of solid-phase extraction using diatomaceous earth, over solvent extraction, for the extraction of benzodiazepines, are numerous. A cheap, reliable extraction requiring no evaporation or concentration of the extract and the possibility of using the same sample aliquot for screening and quantitation are some of the positive aspects of solid-phase extraction (Samuels, 1977; Horton-McCurdy et al., 1981). Reviews of solid-phase techniques outlining the best use of bonded sorbents have been published (Harkey and Stolowitz, 1984; Tippens, 1987). For the use of non-selective sorbents (for example, diatomaceous earth) no recovery data is usually given. This is because much of the literature concerning non-selective solid-phase extraction is used for qualitative assays only (drug screening procedures) (Samuels, 1977). The use of diatomaceous earth (Extrelut®) as a quantitative procedure as well as a qualitative one requires considerable optimisation of the procedure for the drugs being extracted (Christensen, 1984).

The use of more selective octadecylsilane bonded sorbents has been reported. Recovery of benzodiazepines and their metabolites from human serum (Good and Andrews, 1981) was compared with recovery from liquid-liquid extraction (Strojny et al., 1978). In all cases, the bonded phase gave superior extraction

recovery, for example, chlordiazepoxide - bonded phase: 91±5.4%; solvent: 80±4%.

A rapid method for the isolation of twenty-two benzodiazepines from human samples using Sep-Pak C18 cartridges has recently been reported (Suzuki et al., 1988). This paper, unfortuneately, gives no recovery data, except to say that all drug recoveries were excellent from urine samples but somewhat lower for plasma samples. The recovery of medazepam, however, is given as 10 to 20% in plasma! Some deproteinization of the samples improved recoveries dramatically, but this involves an extra stage in an extraction intended to save time and money. No standard working concentrations are given or detection levels for drug analysis.

No literature involving the polar or ion-exchange extraction of these drugs was available.

Diazepam

Solvent Extraction

The recovery of diazepam and its metabolites from various biological samples using solvent extraction has generally been good. Solvent extractions are usually carried out using ether as the extracting solvent. Recoveries of 87.8 to 95.5% for diazepam from brain tissue (Komiskey et al., 1985); 96±3% for diazepam, 94±4% for desmethyldiazepam from plasma (Brodie et al., 1978); 82.6 to 107% from human serum (Lau et al., 1987) and 89 to 96% from human and dog urine and plasma

for diazepam and its metabolites (Vree et al., 1979), have been reported using ether extraction.

A recovery of 65 to 70% for drug and metabolites from post mortem blood (Wong, 1983) was attained using toluene as the extracting solvent, and slightly improved recoveries were obtained using benzene-methylene chloride to extract the drugs from plasma and urine samples of humans and cats (human plasma: 60 to 89%; human urine: 81 to 93%; cat plasma: 74 to 85%; cat urine: 79 to 88%)(Cotler et al., 1981). Using chloroform only as an extracting solvent gave 95.2 to 102% recoveries for diazepam and its metabolites from human serum (MacKichen et al., 1979).

Solid-Phase Extraction

Specific solid-phase extraction methods, all using non-polar cartridges, for diazepam and it's metabolites from biological fluids have also been extensively reported (Good and Andrews, 1981; Rao et al., 1982; Sample et al., 1986). These procedures are often preferred to other extraction systems because of their reproducibility and simplicity. However, problems with the cleanliness of extracts which prevents direct injection onto HPLC or GC have been encountered. This was overcome using a combined bonded-phase liquid-liquid extraction (de Groot and Grotenhuis-Mullenders, 1983).

From an efficiency point of view, drug loss is minimal

since extraction involves a single step. The simultaneous detection and measurement of the parent compound and metabolites is essential for many benzodiazepines because of their extensive pharmacological biotransformation into several metabolites. Recoveries of more than 88% for diazepam and its metabolites (Rao et al., 1982) and over 90% for diazepam (Hyde, 1985) are common.

Triazolam.

Solvent Extraction

Various solvents have been used to extract parent triazolam and its metabolites from biological fluids. Using ether, low recoveries were obtained from human plasma: 59 to 76% for triazolam; 54 to 66% for 1-hydroxytriazolam (Coassolo et al., 1983).

Better results were achieved using methylene chloride as an extracting solvent: 91.4% and 92.6% respectively for triazolam, from dog blood and plasma (Eberts, 1977).

Solid-Phase Extraction

Triazolam is included in solid-phase benzodiazepine screening methods (Suzuki et al., 1988). An automated screening method for benzodiazepines in human urine and plasma using C2 bonded cartridges (therefore having a much shorter hydrocarbon chain than C18 or C8 cartridges and so having a smaller capacity for non-polar interactions) gave drug recoveries of 97.9±2.2% for triazolam. The longer chain cartridges

were tried and found to give tailing peaks in analysis (Mura et al., 1987).

Sep Pak® C18 cartridges were used to extract triazolam and its metabolites from human urine (Inoue and Suzuki, 1987). Their recoveries were pH dependant, but satisfactory results (over 90%) were obtained right across the pH range 5 to 11.

For post mortem blood samples, Amberlite resin was used to extract parent triazolam, giving a recovery of 96-98% (Koves and Wells, 1986).

Flunitrazepam

Solvent Extraction

Ether, again, is the preferred extracting solvent for flunitrazepam. Recoveries of 70% for 7-aminoflunitrazepam and 50% for 7-acetamidoflunitrazepam in human plasma have been reported (Sumirtapura et al., 1982).

A slightly better recovery for flunitrazepam (75.3% from body fluids) had been reported using hexane (Vree et al., 1977), but the best recoveries were those obtained using extraction into benzene (99.7±4.9% for flunitrazepam; 98.6±7.8% for desmethylflunitrazepam) from human serum.

Solid-Phase Extraction

Flunitrazepam is included in solid-phase benzodiazepine screening methods (Mura et al., 1987; Suzuki et al., 1988), the former quoting 102±2.8% extraction recovery for flunitrazepam.

2.3.3 Xylazine.

Solvent Extraction

Xylazine has been extracted from biological fluids and tissues mainly by solvent extraction. A typical procedure would be as follows (Rogstad and Yndestad, 1981):

Serum (2g) was alkalinized by concentrated ammonia and internal standard (diphenhydramine hydrochloride) was added. The aqueous phase was extracted with diethyl ether (2 x 4ml) and the combined ether phases were washed with water which was discarded. After extraction with 0.05 M sulphuric acid (2 x 2ml), the combined aqueous solutions were alkalinised and extracted twice with 3-ml portions of chloroform. The chloroform was dried off under a gentle stream of nitrogen, and the residue was dissolved in methanol (50ul), of which 2ul were injected into the gas chromatograph.

Recovery using this method was reported as 100% from cattle serum samples and 93% from cattle meat samples. Extremely high recoveries were also obtained from white blood cells (97.7±3.5%), red blood cells (98.7±3%) and plasma (93.8±3.7%) of horses, using ether as an extracting solvent (Akbari, 1988). Chloroform alone was not as efficient, with recoveries of only 76.4±3.4% reported from human plasma (Alvinerie and Toutain, 1981).

Solid-Phase Extraction

Xylazine has also been extracted using XAD-2 resin (Poklis et al., 1985), but no recovery data is given. There is no record of bonded sorbent extraction for this drug.

2.3.4. Mazindol.

Solvent Extraction

The current extraction procedure for mazindol hydrolysis product from the urine of racehorses is extremely long, involving overnight enzyme hydrolysis followed by heating the sample for four hours at 110^{o}C and solvent extraction into a trisolvent of cyclohexane-dichloromethane-ethyl acetate (65:20:15) in 10% isopropyl alcohol.

A somewhat simpler extraction procedure involving the extraction of urine (to pH14 using 6M sodium hydroxide), into dichloromethane-isopropanol (90:10) has been described (Timmings et al., 1985). However, this method is also time-consuming and extract quality was reported as being extremely poor.

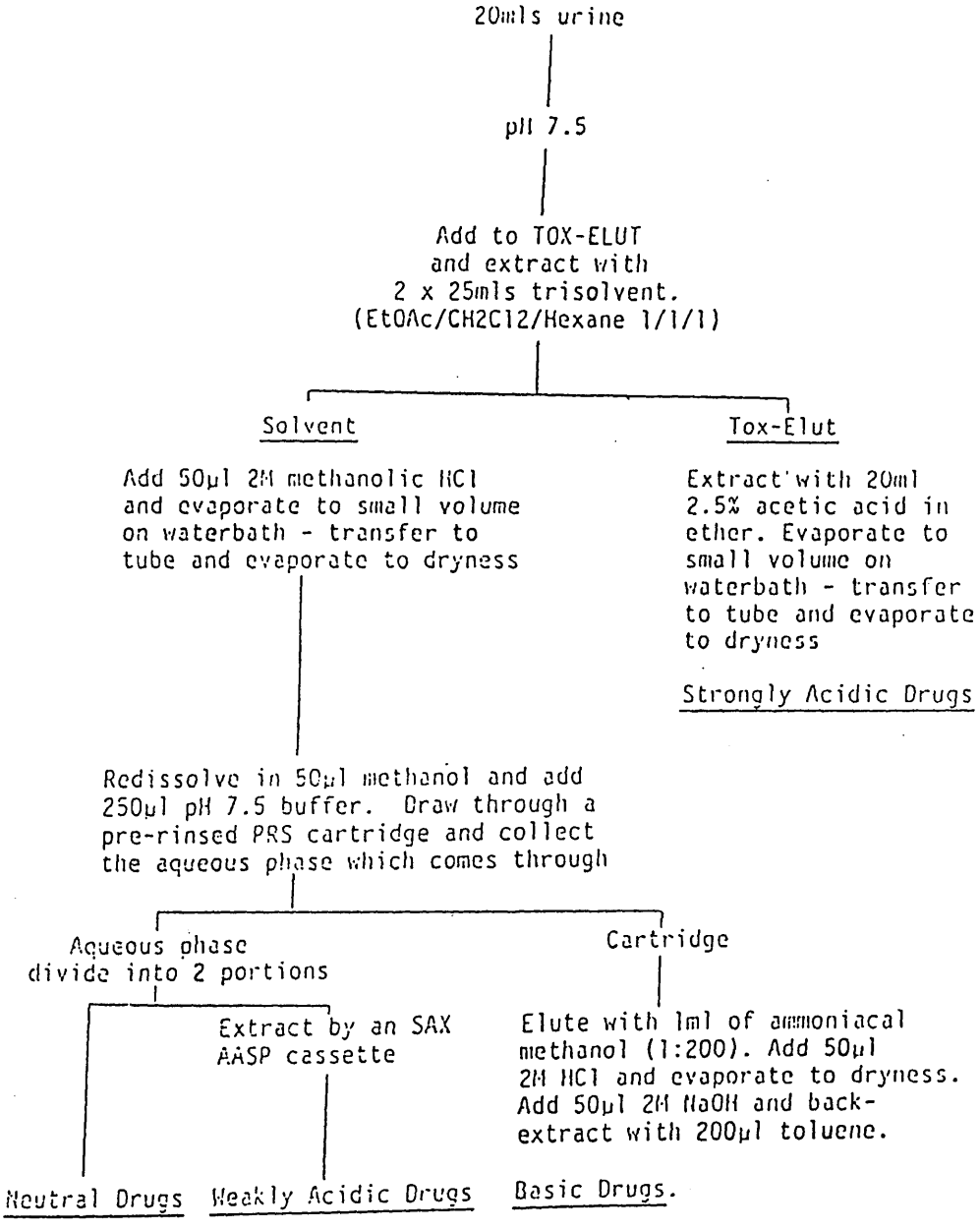
Solid-Phase extraction

To date, there are no solid-phase extraction procedures published for this particular drug.

However, a routine screening procedure involving solid-phases for the extraction of the urine of racehorses is in current use at the Horseracing Forensic Laboratory, Newmarket, England (Figure 8).

FIGURE 8.

Routine screening procedure for drugs from racehorse urine.



2.3.5. Drug Screening in the Urine of Racing Greyhounds.

Solvent Extraction

From a greyhound drug screening point of view, the development of a faster, more efficient extraction process is economically important. A single extraction for the recovery of acidic, basic and neutral drugs from greyhound dog urine was described by Hill et al., (1982), which is similar to that currently used as the procedure at British greyhound racing stadiums.

For confirmatory testing, the acidic drugs are extracted using a diatomaceous earth sorbent, while the basic drugs are extracted using a liquid-liquid extraction method:

Greyhound urine (20ml) was made basic with approximately 3.5M ammonia solution (10ml). This was extracted with ethyl acetate (50ml) in a 100ml capacity separating funnel. After shaking, the aqueous layer was discarded. The organic layer was extracted with 2M sulphuric acid (10ml). The acidic layer was transferred to another separating funnel, made alkaline with approximately 3.5M ammonia solution and extracted into ethyl acetate (50ml). The aqueous layer was discarded and the organic layer was dried over anhydrous sodium sulphate.

The extract was then evaporated to dryness on a water bath and reconstituted in papaverine solution (0.5mg/ml, 25ul), as an external standard.

2.3.6. Basic Drug Screening in Post-Mortem Urine Samples.

Solvent Extraction

Basic drug screening methods for post-mortem are well documented and widely used. Almost all routine toxicology laboratories employ solvent extraction methods, because these tend to give sufficiently clean extracts for chromatographic analysis. Recently, though, solid-phase extractions are being seriously considered as an alternative, mainly because of their automation potential which is a massive bonus for clinical testing laboratories whose workload is ever increasing.

3. EXPERIMENTAL

3.1. Methods of Analysis

3.1.1. Benzodiazepines - HPLC analysis

Equipment and column

The chromatographic system was based on a Gilson 302 pump incorporating a 5S piston head which was used to deliver solvent at a given flowrate. The eluent was monitored at 254 and 230 nm using a Pye Unicam PU4025 variable wavelength ultra-violet detector incorporating an Analytical flowcell assembly, volume 8ul, pathlength 10mm. Spectra were recorded on a Servoscribe chart recorder, operated at 1cm/minute and 10mV full scale deflection.

The column was 25cm x 4.6mm internal diameter, pre-packed with Hypersil 5um ODS C18 (HPLC Technology) and fitted with a 20ul Rheodyne 7125 injection valve.

a) Diazepam and metabolites

Preparation of standard solutions

Standard stock solutions of diazepam, oxazepam, desmethyldiazepam and temazepam were made up in methanol (1mg/ml). These solutions were diluted with mobile phase in order to obtain standard solutions of 10, 100 and 1000 ng/ml.

Separation of diazepam from its metabolites

To separate diazepam from its metabolites, the following mobile phase was prepared: methanol - deionised water - phosphate buffer(pH8) - acetonitrile (200:125:100:75) (Gill et al, 1986).

Retention times for diazepam and its metabolites using this HPLC system were determined.

b) Triazolam and 1-hydroxy triazolam

Preparation of standard solutions

Triazolam and 1-hydroxy triazolam were dissolved in methanol to give standard stock solutions (1mg/ml). The solutions were diluted with mobile phase in order to obtain standard solutions of 10, 100 and 1000 ng/ml.

Separation of triazolam from its metabolite

Two HPLC methods were developed for the separation of triazolam from its 1-hydroxy metabolite:

i) Triazolam was separated from its main metabolite using the HPLC system described above.

ii) Triazolam was also separated from its metabolites using the following HPLC system:

Equipment and column

The chromatographic system consisted of a continuous flow Gilson 302 pump incorporating a 5S piston head which was used to deliver solvent at a rate of 1.5ml/min.

The eluent was monitored at 230 nm with a Pye Unicam PU4025 variable wavelength ultra-violet detector.

The column was 25cm x 4.6mm internal diameter pre-packed with Hypersil 5um octadecylsilane C18 universal cartridge column (Capital HPLC Specialists) and fitted with a Rheodyne 7125 injection system incorporating a 20ul loop.

Elution solvent

The mobile phase used was that described by Gill et al., 1986 with the phosphate buffer replaced by acetate buffer: ammonium acetate buffer (pH7) - deionised water - acetonitrile - methanol (100:125:75:200 v/v).

Using the above systems and stock solutions described, the retention times for triazolam and 1-hydroxy triazolam were determined.

c) Flunitrazepam and metabolites

Preparation of standard solutions

Flunitrazepam and its metabolites were dissolved in methanol to give standard stock solutions (1mg/ml). The solutions were diluted with mobile phase in order to obtain standard solutions of 10, 100 and 1000 ng/ml.

Separation of flunitrazepam from its metabolites

Flunitrazepam was separated from its metabolites using the HPLC method described above (b(ii)) and retention times determined.

d) The separation of nineteen common benzodiazepines

Preparation of standard solutions

Nineteen benzodiazepines were dissolved in methanol at a concentration of 500 ng/ml.

Separation of nineteen benzodiazepines

The HPLC system described in (b(ii)) was then used for the separation of nineteen common benzodiazepines and retention times determined.

3.1.2. Xylazine - HPLC analysis

Preparation of standard solutions

A xylazine standard stock solution was prepared containing 1mg/ml in methanol. The solution was diluted with mobile phase in order to obtain standard solutions of 10, 100 and 1000 ng/ml.

Three HPLC systems were tried:

i) The HPLC system described above (b(ii)) was used to determine xylazine using a detection wavelength of 220 nm.

ii) Equipment and column

The HPLC system consisted of a continuous flow Kratos Spectroflow 400 pump, which was used to deliver solvent at a rate of 2ml/min. The eluent was monitored at 225 nm with a Pye Unicam PU4025 variable wavelength ultra-violet detector. The system was operated at room temperature ($20^{o}C$).

The column was 30cm x 4.0mm internal diameter pre-packed with Hypersil 10um octadecylsilane C18 and fitted with a Rheodyne 7125 injection system incorporating a 20ul loop.

Elution solvent

The composition of the mobile phase was : 2% glacial acetic acid in water-methanol-heptanesulphonic acid (55:45:0.2,v/v) (Alvinerie and Toutain, 1981).

iii) This system was a variation on a published method for bromazepam determination (Hirayama and Kasuya, 1983).

Equipment and column

The HPLC system consisted of a continuous flow Kratos Spectroflow 400 pump, which was used to deliver solvent at a rate of 2ml/min.

The eluent was monitored at 225 nm with a Pye Unicam PU4025 variable wavelength ultra-violet detector. The system was operated at room temperature ($20^{o}C$).

The column was 25cm x 4.6mm internal diameter pre-packed with Hypersil 5um octadecylsilane C18 and fitted with a Rheodyne 7125 injection system incorporating a 20ul loop.

Elution solvent

To determine xylazine, the following mobile phase was prepared: 1g of tetramethyl ammonium hydroxide dissolved in deionised water (250ml)-acetonitrile-methanol (250:150:10 v/v).

Using this system, the retention time for xylazine was determined.

3.1.3. Basic Drug Screening System

a) HPLC analysis

Preparation of drug standards

One hundred basic drugs were made up in methanol at concentrations of 1 and 5ug/ml.

Separation of basic drugs

Equipment and column

HPLC analysis of 100 basic drugs commonly encountered in urinary drug screening was performed using a gradient pumping system (Varian) operated at 1.5 ml/minute, incorporating a 10ul Rheodyne injection valve.

The detector was a UV/visible diode array spectrophotometer (Hewlett Packard) with a 9000/300 series data system. The eluent was monitored at 200nm and full spectra were recorded from 190 to 400nm for each peak. A spectral library was acquired under laboratory conditions for each compound examined.

The column was a Hibar Lichrospher 100 CH-8/II (25cm x 4.6mm) (Merck).

Elution solvent

The initial mobile phase composition was 10% acetonitrile in 0.05M pH3.2 potassium phosphate buffer. This was increased to 50% over 15 minutes, and the final composition was maintained for 5 minutes. A re-equilibration time of 5 minutes was required between injections (total cycle time 25 minutes).

Using this system, the retention times for all 100 drugs were determined.

b) GC analysis

Equipment and column

Gas chromatography of basic drugs was performed using a Chrompack-Packard, Model 427 equipped with a nitrogen-phosphorus detector (NPD) and a flame-ionisation detector (FID).

The capillary column was a WCOT fused silica, 25m x 0.32mm inside diameter, 0.45mm outside diameter, 0.41um CP Sil 5 CB (Chrompack).

Temperature programme

The injection port was held at 290^{o}C, detector at 290^{o}C and the oven was programmed from 120^{o}C to 290^{o}C at 8^{o}C per minute. The initial time was 2 minutes and final time 5 minutes.

The instrument was equipped with a split/splitless injector.

Gas flowrates

Helium was used as the carrier gas. The flowrate was approximately 1.5 ml/minute through the column, achieved by maintaining a column head pressure of 100 kPa. The column effluent was split 1:1 and helium make up gas was introduced to give a flow of 10ml/minute of helium to each detector.

The other gas flowrates were:

NPD:- hydrogen and air:- 5 and 15ml/minute respectively.

FID:- hydrogen and air:- 30 and 250ml/minute respectively.

The sample volume used was 1ul.

These analysis methods were currently in use in the laboratories where this work was carried out, and gas chromatography was used only as a qualitative technique.

3.1.4. Mazindol

a) HPLC analysis

Preparation of standard solutions

A mazindol standard stock solution was prepared containing 1mg/ml in methanol. The solution was diluted with mobile phase in order to obtain standard solutions of 10, 100 and 1000 ng/ml.

Equipment and column

The HPLC system consisted of a Perkin Elmer series 2 pump which was used to deliver solvent at a rate of 1.5 ml/minute. The eluent was monitored at 254 nm with a

Perkin Elmer LC-15B ultraviolet fixed wavelength detector.

The column was a 30cm x 4.5mm internal diameter 5um Bondapak C18 (Waters), fitted with a Rheodyne injection system incorporating a 20ul loop.

Elution solvent

The determination of mazindol was carried out using various compositions of 0.005M pentane sulphonic acid-acetonitrile- 85% phosphoric acid.

Mobile phases in the ratio 90:10:5, 50:50:5, 60:40:5 and 75:25;5 were prepared and the optimum eluent evaluated.

Using the chosen system, the retention time of mazindol was determined.

b) GC-MS analysis

Equipment and column

The GC-MS system used was a Hewlett Packard 5890 GC with a 5970 mass selective detector (MSD) and Chem Station Data System.

The column was a DB-1 methyl silicone fused silica capillary (J + W), 5m x 0.25mm internal diameter and 0.25 um film thickness.

The GC was operated in splitless injection mode.

Temperature programme

The oven was programmed from 50 to 280^{o}C at 30^{o}C/minute after 1 minute initial delay, and was held at final temperature for 5 minutes.

Gas flowrate

Helium carrier gas was used at a flowrate of 1ml/minute.

This mass spectrometry method was only used as a qualitative technique.

3.2 Methods of Extraction

All initial work involved the extraction of drugs from the urine of racing greyhounds.

3.2.1. Extraction Methods exploiting Non-Polar Sorbent Interactions.

1. Benzodiazepines

DIAZEPAM AND OXAZEPAM

Preparation of aqueous standard solutions

Diazepam was dissolved in deionised water to give a solution of concentration 300ng/ml.

Oxazepam, the main urinary metabolite of diazepam, was dissolved in deionised water to give a standard solution (100ng/ml).

Initial extraction procedure for oxazepam

Conditioning of the columns

Bond Elut® C8 columns of 3ml capacity and 1ml capacity were conditioned with two column volumes of methanol followed by one column volume of 1% ammonium hydroxide (v/v) to ensure a basic environment.

Addition of sample

The sample solution (1ml of 100ng/ml standard) was added to the column and drawn through under vacuum. The columns were then washed with deionised water (2 x 0.1ml) to displace any remaining methanol.

Elution of the drugs

The drugs were eluted from the column with:

a) methanol - 1%ammonium hydroxide - acetonitrile (2:2:1),

b) methanol-acetonitrile (1:1) or,

c) methanol-acetonitrile (3:1) (3 x 0.5ml) (K.C. Van Horne et al., 1985).

Analysis of the sample

The extract was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in mobile phase (0.1ml) prior to HPLC analysis.

Extraction efficiency of the columns

a) The effect of pH of the sample

Preparation of the working solution

A solution of labelled diazepam (approximately 10000 counts per minute) was made up by diluting 1ml of the supplied tritiated standard with 1 litre of deionised water.

Tritiated diazepam solution (0.1ml) was added to standard diazepam solution (300ng/ml)(1ml) to give a working solution (WS).

Buffering of the working solution

Aliquots of this solution (0.5ml) were buffered with 0.05M sodium bicarbonate buffer (pH9) (0.5ml) to pH 4.15, 7.63 with sulphuric acid and 10.25 with sodium hydroxide.

Conditioning of the extraction columns

The C8 solid-phase cartridges (3ml capacity) were conditioned with methanol (2 x 3ml).

Addition of sample

The standard solution was passed through the column under vacuum and collected.

Liquid scintillation counting

The liquid scintillation counter used was a Tricarb Packard. The counter was preset to count for one minute. For region A, the lower limit was set at 0.0 keV and the upper limit at 18.6 keV. For region B, the lower limit was 2.0 keV and the upper limit, 18.6 keV. Scintillant (Ecoscint®, suitable for use with aqueous solutions) (4ml), was added to the extract and the concentration of radiolabelled drug determined in each sample by liquid scintillation counting.

Elution of the sample

The drugs on the column were eluted with methanol-acetonitrile (3:1), scintillant was added (4ml) and the percentage recoveries determined.

b) The effect of elution solvents

Oxazepam standards on HPLC

Prazepam (2.4ug/ml in methanol) was added to the oxazepam standards prepared in 3.1.1.(a) (10, 100 and 1000 ng/ml concentrations) and the samples were injected onto the HPLC. From the series of ratios produced, at chart ranges 0.01, 0.02 and 0.04 absorbance units full scale, the linearity of response

of oxazepam to HPLC was determined so that percentage recoveries of oxazepam after extraction could be calculated.

Extraction of standard solutions

Conditioning of the extraction columns

The C8 solid-phase cartridges (3ml capacity) were conditioned with methanol (2 x 3ml).

Addition of sample

The standard solution was passed through the column under vacuum.

Elution solvents

For the elution step, the following combinations of solvents were tried:

1. Methanol-10% ammonium hydroxide-acetonitrile (3:1:1)

At this point, the sample was buffered to pH 10.25 with 0.05M sodium dihydrogen phosphate / sodium hydroxide (0.5ml) prior to extraction.

2. Methanol-acetonitrile-chloroform (2:1:1)

3. Methanol-acetonitrile-chloroform (2:1:3)

4. Chloroform

Analysis of drug

Analysis of the extracts was carried out using the HPLC system previously described and the percentage of oxazepam recovered was determined.

Oxazepam in greyhound urine samples

Blank greyhound urine was obtained by placing the greyhound in a metabolic cage and collecting the urine produced over a period of 24 hours.

Spiked urine samples in the range 10 to 1000 ng/ml were prepared by adding aliquots of a standard solution of oxazepam (1mg/ml) in methanol to blank urine (20 ml). The samples were then mixed on a vortex mixer.

Recovery of oxazepam from greyhound urine samples

Extraction procedure

Conditioning of the extraction columns

Bond Elut® columns, of 3 ml capacity, containing C8 packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned by washing with methanol (2 x 3ml), followed by phosphate buffer (pH 10.25) (1 x 0.5ml).

Addition of sample

Before the column could dry completely, the spiked urine sample (2.5 ml) + buffer (pH 10.25) (0.5ml), was applied to the column and drawn through.

Elution of the drug

The adsorbed drug was eluted from the column with chloroform (3 x 0.5ml).

Analysis of the drug

The combined eluent was evaporated to dryness under a stream of nitrogen at 50^{o}C and the residue was re-dissolved in the mobile phase (0.25ml) and prazepam standard (0.25ml) (2.4 ug/ml) as an external standard. Twenty microlitre (ul) samples were then injected onto HPLC.

The percentage of oxazepam recovered from greyhound urine was then determined.

Collection of greyhound samples

Four racing greyhounds were each administered diazepam (a single oral dose, 5mg). Urine samples were freely collected at timed intervals from the greyhounds. The samples were collected after 1, 2, 3, 4, 5, 6, 7, 24, 25, 26, 28, 30, 48 and 72 hours.

The samples were stored at -20^{o}C before use.

Each greyhound was dosed three times, allowing sufficient time between dosings for all the drug to have been excreted from the system.

The release of oxazepam from its conjugated state.

Oxazepam was released from its conjugated environment in the urine using enzyme hydrolysis.

Enzyme hydrolysis

Helix Pomatia Juice-B-glucuronidase releases both glucuronide and sulphates from their conjugated states. The optimum pH environment for this enzyme is pH5 (Axelson et al., 1981).

B-glucuronidase (25 ul) and 0.1M sodium acetate buffer (pH5; 0.5ml) were added to the urine (2.5 ml). The sample was then incubated at 60^{o}C for two hours.

Extraction of greyhound samples

The extraction procedure outlined above was performed using untreated urine samples and enzyme treated samples.

TRIAZOLAM AND 1-HYDROXY TRIAZOLAM

Triazolam and 1-hydroxy triazolam standards on HPLC

Prazepam (2.4ug/ml in methanol) was added to the triazolam and metabolite standards prepared in 3.1.1.(b) (10, 100 and 1000 ng/ml concentrations) and the samples were injected onto the HPLC analysis system (i). From the series of ratios produced, at chart ranges 0.01, 0.02 and 0.04 absorbance units full scale, the linearity of response to HPLC was determined so that percentage recoveries after extraction could be calculated.

Triazolam in greyhound urine samples

Triazolam and 1-hydroxytriazolam standards were made up in greyhound urine at 50, 100 and 500 ng/ml concentrations. These were extracted using a modification of the procedure developed for oxazepam.
A wash step was incorporated prior to elution (1 x 0.5ml deionised water). The percentage recovery of the drugs from greyhound urine was determined.

Collection of greyhound samples (0.25 mg administration)

Four greyhounds were each administered 1 x 0.25mg triazolam tablet. Urine samples were freely collected at timed intervals from the greyhounds. The samples were collected after 1, 2, 3, 4, 5, 6, 7, 24, 25, 26, 28, 30, 48 and 72 hours.

The samples were stored at -20^{o}C before use.

Each greyhound was dosed three times, allowing sufficient time between dosings for all the drug to have been excreted from the system.

Extraction of greyhound samples

Extraction was carried out as previously outlined and analysis was using HPLC.

The effect of increased capacity cartridges

Conditioning of the extraction columns

Bond Elut® columns, of 6 ml capacity, containing C18 packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned with two column volumes of methanol and one of water.

Addition of sample

Urine (3ml) was diluted with buffer pH10 (0.5ml) and applied to the column. The column was washed with deionised water (2 x 0.5ml).

Elution of the drug

The adsorbed drug was then eluted with chloroform (3 x 0.5ml).

Analysis of the drug

The eluent was evaporated to dryness and reconstituted in mobile phase (0.1ml) and prazepam standard (0.1ml) prior to HPLC injection and the percentage recovery of the drug was determined.

Collection of greyhound samples (0.5 mg administration)

The greyhounds were each administered 1 x 0.5mg triazolam tablet. Urine samples were freely collected at timed intervals from the greyhounds. The samples were collected after 1, 2, 3, 4, 5, 6, 7, 24, 25, 26, 28, 30, 48 and 72 hours.

The samples were stored at -20^{o}C before use.

Each greyhound was dosed three times, allowing sufficient time between dosings for all the drug to have been excreted from the system.

Extraction of greyhound samples

Greyhound urine samples taken one and two hours after oral administration of 1 x 0.5mg triazolam tablet only were extracted using this procedure. Some of the samples were enzyme hydrolysed (see oxazepam procedure) prior to extraction.

2. Xylazine

Xylazine standards on HPLC

The xylazine standards prepared in 3.1.2. (10, 100 and 1000 ng/ml concentrations) were injected onto the HPLC. From the peak areas produced, at chart ranges 0.01, 0.02 and 0.04 absorbance units full scale, the linearity of response to HPLC was determined so that percentage recoveries after extraction could be calculated.

Xylazine in greyhound urine samples

Spiked urine samples were made up at 100, 500 and 1000 ng/ml concentrations.

Recovery of xylazine from greyhound urine samples

Extraction procedure

Conditioning of the extraction columns

Columns were conditioned with two column volumes of methanol, one column volume of deionised water followed by one column volume of phosphate buffer (pH 10.25).

Addition of sample

Spiked urine (3ml), to pH 10.25 with buffer (0.5ml), was applied to the column and drawn through under vacuum.

The column was washed with deionised water (2 x 0.5ml) and the column was allowed to dry for 3 or 4 minutes.

Elution of the drug

The drug was eluted with chloroform (3 x 0.5ml).

Analysis of the drug

The extract was blown to dryness and reconstituted in methanol (0.5ml) before analysis by HPLC.

Twenty microlitre (ul) samples were then injected onto HPLC and the percentage recovery of xylazine determined.

The effect of increased capacity cartridges

Bond Elut® columns, of 6 ml capacity, containing C18 packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned with two column volumes of methanol and one of water.

Addition of sample

Urine (3ml) was diluted with buffer pH10 (0.5ml) and applied to the column. The column was washed with deionised water (2 x 0.5ml).

Elution of the drug

The adsorbed drug was then eluted with chloroform (3 x 0.5ml).

Analysis of the drug

The extract was blown to dryness and reconstituted in methanol (0.5ml) before analysis by HPLC.

Twenty microlitre (ul) samples were then injected onto HPLC and the percentage recovery of xylazine determined.

Changes in extraction procedure

A methanol wash was incorporated into the system.

Acetone was used as an alternative eluent.

Direct injection of the extract in chloroform onto HPLC was attempted.

Collection of greyhound samples

Four greyhounds were injected with 2% xylazine solution (Rompun) (0.05ml/kg). The average greyhound weighs 36 kg.

Urine samples were collected after 1, 2, 3, 4, 5, 6, 7, 24, 26, 28 and 30 hours.

Extraction of greyhound samples

Conditioning of the extraction columns

Bond Elut® columns, of 3 ml capacity, containing C8 packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned by washing with methanol (2 x 3ml), followed by phosphate buffer (pH 10.25) (1 x 0.5ml).

Addition of sample

Before the column could dry completely, the urine sample (2.5 ml), buffer (pH 10.25) (0.5ml), and 100ul of a 15ug/ml methanolic solution of diazepam was applied to the column and drawn through.

Elution of the drug

The adsorbed drug was eluted from the column with chloroform (3 x 0.5ml).

Analysis of the drug

The combined eluent was evaporated to dryness under a stream of nitrogen at 50°C and the residue was re-dissolved in the mobile phase (0.5ml).

Twenty microlitre (ul) samples were then injected onto HPLC.

3.2.2. Extraction Methods exploiting Polar Sorbent Interactions.

1. Benzodiazepines

TRIAZOLAM AND METABOLITES

Triazolam and 1-hydroxy triazolam standards on HPLC

Prazepam (2.4ug/ml in methanol) was added to the triazolam and metabolite standards prepared in 3.1.1.(b) (10, 100 and 1000 ng/ml concentrations) and the samples were injected onto the HPLC analysis system (ii). From the series of ratios produced, at chart ranges 0.01, 0.02 and 0.04 absorbance units full scale, the linearity of response to HPLC was determined so that percentage recoveries after extraction could be calculated.

Evaluation of polar sorbents

Conditioning of the extraction columns

Bond-Elut® columns containing cyanopropyl (CN), aminopropyl (NH2) or diol (2OH) packing material (100mg) with a capacity of 1ml were positioned in a Vac-Elut® system. Vacuum pressure was adjusted to 15 mm Hg and each column was conditioned with methanol (2 x 1ml) followed by deionised water (pH7, 1 x 1ml).

Addition of sample

Without allowing the column to dry out, the urine sample spiked with triazolam (500 ng/ml)(1ml) + deionised water (pH7, 1ml), was added to the column.

The urine was drawn through and the column was dried under vacuum for 3 minutes.

The cartridge was washed with deionised water (pH7, 2 x 0.25ml).

Elution of the drug

The drug was then eluted with methanol, to pH4 with hydrochloric acid or to pH10 with ammonium hydroxide (2 x 0.25ml) (See Appendix A2.6.).

Analysis of the drug

The eluent was then directly analysed by HPLC and the percentage recovery of the drug determined.

Triazolam and its metabolites in greyhound urine samples

The 4-hydroxy metabolite of triazolam had been received for use as a drug standard.

Spiked urine samples in the range 10-1000 ng/ml were prepared by adding aliquots of a standard solution of the triazolam and its metabolites in methanol (1mg/ml) to blank greyhound and human urine, and stored at -20^{o}C until required for extraction.

Extraction of greyhound samples

Spiked urine samples and authentic greyhound urine samples (taken following the administration of 1 x 0.5mg triazolam tablet) were extracted according to the following procedure. Some of the samples were enzyme hydrolysed (see oxazepam procedure) and some were not:

Conditioning of extraction columns

Bond Elut® columns, of 1ml capacity, containing CN packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned by washing with methanol (2 x 1ml), followed by deionised water (pH 7) (1 x 1ml).

Addition of sample

Before the column could dry completely, the urine sample (1ml), buffer (pH 7) (1ml), and 100ul of a 15ug/ml methanolic solution of diazepam was applied to the column and drawn through.

The urine was drawn through and the column was dried under vacuum for 3 minutes.

The cartridge was washed with deionised water (pH7, 2 x 0.25ml).

Elution of the drug

The drug was then eluted with methanol, to pH4 with hydrochloric acid (2 x 0.25ml).

Analysis of the drug

Twenty microlitre (ul) samples were then injected onto the HPLC and the percentage recoveries of triazolam and its metabolites were determined.

Triazolam and its metabolites in human urine samples

Four healthy female volunteers (average age: 26; average weight: 67 kg) were each administered a single tablet (1 x 0.25mg) of triazolam. The subjects

abstained from alcohol and nicotine twenty-four hours before and after administration. Urine samples were collected 2, 3, 4, 5 and 12 hours after administration and were stored at -20°C until required for extraction.

The samples were extracted according the extraction procedure above.

FLUNITRAZEPAM

Flunitrazepam and its metabolites on HPLC

The standard solutions prepared in 3.1.1.(c) (10, 100 and 1000 ng/ml concentrations) were injected onto the HPLC analysis system (3.1.1.b(ii)). From the peak areas produced, at chart ranges 0.01, 0.02 and 0.04 absorbance units full scale, the linearity of response to HPLC was determined so that percentage recoveries after extraction could be calculated.

Flunitrazepam and its metabolites in greyhound urine samples

Spiked greyhound urine samples were made up by adding aliquots of flunitrazepam (1mg/ml) and its metabolites desmethylflunitrazepam (1mg/ml) and 7-aminoflunitrazepam (1mg/ml) in methanol, to blank greyhound urine. Extractions were carried out at 0.5 ug/ml level and the percentage recovery determined as for triazolam and its metabolites.

Collection of greyhound samples

Four racing greyhounds were dosed with 1 x 2mg tablet of flunitrazepam and urine samples were collected 1, 2, 3, 4, 5, 6, 7, 24, 26, 28, and 30 hours after administration and stored at -20^{o}C until required for extraction.

Extraction of greyhound samples

The urine samples were extracted according to the procedure described above.

BENZODIAZEPINE SCREENING PROCEDURE

HPLC analysis of benzodiazepines

All nineteen benzodiazepines were injected onto the HPLC system described in 3.1.1.b(ii) so that percentage recoveries of the drugs could be determined after extraction.

Extraction of standard benzodiazepines

Nineteen benzodiazepines (0.5 ug/ml standards) were subsequently extracted using the procedure described above and the percentage recovery of each was determined.

2. Xylazine

The HPLC system previously described for the determination of xylazine was used.

Xylazine in greyhound urine samples

Urine samples in the range 10-1000 ng/ml were prepared by adding aliquots of a standard solution of xylazine in methanol (1mg/ml) to blank greyhound urine.

Collection of actual samples

Four greyhounds were injected with 2% xylazine solution (Rompun) (0.05ml/kg). The average greyhound weighs 36 kg. Urine samples were collected after 1, 2, 3, 4, 5, 6, 7, 24, 26, 28 and 30 hours.

The samples were stored at -20 ^{0}C before extraction. Each greyhound was dosed three times allowing sufficient time between dosings for total excretion to have occurred.

Recovery of xylazine from greyhound urine samples

Extraction procedure

Conditioning of the extraction columns

Bond Elut® columns, of 1ml capacity, containing CN packing material were positioned in a 10 column capacity Vac Elut® system. Pressure was adjusted to 10→15 mm Hg and then each column was conditioned by washing with methanol (2 x 1ml), followed by deionised water (pH 7) (1 x 1ml).

Addition of sample

Before the column could dry completely, the urine sample (1ml), buffer (pH 7) (1ml), and 100ul of a

15ug/ml methanolic solution of diazepam was applied to the column and drawn through.

The urine was drawn through and the column was dried under vacuum for 3 minutes.

The cartridge was washed with deionised water (pH7, 2 x 0.25ml).

Elution of the drug

Methanol (50ml) was added to 36% w/w hydrochloric acid (3ml) to give methanolic hydrochloric acid. The drug was then eluted with methanolic hydrochloric acid - acetonitrile (50 : 50 v/v, 2 x 0.25ml).

Analysis of the drug

Twenty microlitre (ul) samples were then injected onto the HPLC and the percentage recovery of xylazine was determined.

3.2.3. Extraction Methods exploiting Ion-exchange Sorbent Interactions.

1. Basic Drugs

HPLC analysis

The drug standards were analysed by HPLC (3.1.3.(a)) and from the peak areas obtained, the percentage recoveries of the drugs after extraction were calculated.

GC analysis

The GC analysis method was used for quantitative analysis only.

Cation-exchange extraction

Thirty basic drugs at 1 and 5ug/ml concentrations were made up in methanol and extracted using the following cation exchange extraction procedure:

Conditioning of extraction columns

Bond Elut® strong cation exchange (SCX) columns (1ml capacity). These contained 40um silica particles with an alkyl bonded benzenesulphonylpropyl moiety. The cartridges were conditioned under vacuum on a Vac-Elut® manifold with methanol (2ml), water (1ml) and 7mM phosphoric acid (pH3.4)(0.5ml).

Addition of sample

Urine (1ml) and 7mM phosphoric acid (0.5ml) were mixed thoroughly and applied to the column. The column was air dried for approximately 30 seconds, and then washed with 7mM phosphoric acid (1ml), 0.1N acetic

acid (0.5ml) and methanol (1ml).

Elution of the drug

The column was again air dried and ammoniacal methanol (1%)(1ml) was passed through the column and collected. An aliquot of the residue (20ul) was removed for analysis.

Analysis of the drug

The remainder was evaporated to dryness under nitrogen at 45^{o}C, and reconstituted in 50ul of solvent, 10ul of which were analysed by HPLC so that the percentage recoveries of each drug could be determined.

Method Validation

Human post-mortem urine samples

Twenty-four post-mortem urine samples were extracted by conventional solvent extraction methods (Foerster and Mason, 1974; Anderson and Stafford, 1983) in parallel with the solid-phase extraction procedure described and the following non-selective method:

Liquid/Solid extraction on diatomaceous earth

Absorption extraction columns were prepared in 10ml pipettes by closing the bottom with a plug of glass wool, and filling the pipette with a diatomaceous earth material (Chem Elut®, Analytichem International, Harbor City, California) to a level 1-2cm below the top.

Urine samples (5ml) were diluted with 0.2M borate buffer (pH9;3ml), then applied to the columns and

allowed to absorb for 2-3 minutes. Solvent (n-butyl chloride) was applied and allowed to flow through until the required volume had been collected (12ml). The eluting solvent was evaporated to dryness, reconstituted in mobile phase and analysed.

Basic drugs in greyhound urine samples

The cation-exchange procedure was carried out on spiked greyhound urine samples containing twenty-five basic drugs, in order to determine percentage recoveries.

Method Validation

Greyhound urine samples

Thirty urine samples from greyhound racing tracks in the UK, received between December 1988 and January 1989 were extracted using solid-phase extraction in parallel with the existing liquid/liquid method.

a) Solid-phase extraction

Strong cation exchange (SCX) cartridges of 100mg/1ml capacity (Analytichem, Harbor City. California).

Conditioning of the extraction columns

The columns were conditioned under vacuum on a Vac-Elut® manifold with methanol (2 x 1ml), deionised water (1 x 1ml) and 7mM phosphoric acid (pH3.4, 1 x 0.5ml).

Addition of sample

Greyhound urine (1ml), adjusted to pH3.4 with phosphoric acid, was applied to the cartridge and drawn through. The column was air dried for 3 minutes, then washed with 7mM phosphoric acid (1 x 1ml), 0.1N acetic acid (0.5ml) and methanol (1 x 1ml).

Elution of the drug

The column was again air dried, then basic methanol (pH10 with ammonium hydroxide, 2 x 0.5ml) was passed through the column and collected.

Analysis of the drug

The eluent was evaporated to dryness and reconstituted in papaverine solution (0.5mg/ml, 25ul) for GC analysis.

b) Liquid/liquid extraction

Greyhound urine (20ml) was made basic with approximately 3.5M ammonia solution (10ml). This was extracted with ethyl acetate (50ml) in a 100ml capacity separating funnel. After shaking, the aqueous layer was discarded. The organic layer was extracted with 2M sulphuric acid (10ml). The acidic layer was transferred to another separating funnel, made alkaline with approximately 3.5M ammonia solution and extracted into ethyl acetate (50ml). The aqueous layer was discarded and the organic layer was dried over anhydrous sodium sulphate. The extract was then evaporated to dryness on a water bath and reconstituted in papaverine solution (0.5mg/ml, 25ul), as an external standard, for GC analysis.

2. Mazindol

Mazindol on HPLC

The standard solutions prepared in 3.1.4. (10, 100 and 1000 ng/ml concentrations) were injected onto the HPLC analysis system (3.1.4.a). From the peak areas produced, at chart range 0.02 absorbance units full scale, the linearity of response to HPLC was determined so that percentage recoveries after extraction could be calculated.

GC-MS analysis

GC-MS analysis was used as a qualitative procedure only.

Mazindol in racehorse urine samples

Horse urine samples were prepared by adding microlitre aliquots of a standard solution of the drug (1mg/ml) in methanol to urine (1ml). Solutions containing 100 ng to 10 ug/ml of drug were prepared in this manner, mixed by vortexing and kept at $4^{o}C$ until required for analysis.

Extraction of racehorse samples

Three racehorses were each given an oral dose of 50mg of mazindol, and urine samples were taken at timed intervals of 0-1, 1-2, 2-4, 4-6, 6-8, 28 and 52 hours after administration.

This was repeated following an oral dose of 5mg of mazindol.

The spiked and authentic samples were extracted as

previously described, with slight modifications. The larger capacity SCX columns were used (3ml) and 10ml of sample, buffered to pH3.5 with 7mM phosphoric acid (5ml) was applied to the column.

For HPLC analysis, the sample was directly injected into the system, and the percentage recovery determined from the spiked samples, but for GC-MSD analysis, the extract was evaporated to dryness and reconstituted in chloroform (25ul) prior to injection.

4. RESULTS AND DISCUSSION

4.1. Methods of Analysis

4.1.1. Benzodiazepines - HPLC analysis

a) Diazepam and metabolites

Benzodiazepines are a widely available group of sedatives and so are potential drugs of abuse in greyhound racing. Their extraction from urine and subsequent quantitative analysis by HPLC is desirable.

Diazepam and its metabolites were separated using the chromatographic system described (3.1.1.(a)). The eluent was first monitored at 254nm using a Pye Unicam PU4025 variable wavelength ultra-violet detector, but 230nm was found to give much greater sensitivity.

The column used was a reverse-phase C18, and the eluent consisted of methanol-deionised water-sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75).

The optimum flowrate for separation was 1.5 ml/minute. Diazepam and its metabolites were separated within 13 minutes (Table 1) and generally peak shapes were good (Figure 9).

Because oxazepam is the major metabolite of diazepam in greyhound urine, it was more intensely examined than the other metabolites.

Prazepam (2.4ug/ml in methanol) was used as an external standard for all diazepam related HPLC work.

TABLE 1.

RETENTION TIMES FOR DIAZEPAM AND ITS METABOLITES USING HPLC

Drug	Retention Time
Oxazepam:	7.5 minutes
Temazepam:	9.3 minutes
N-desmethyldiazepam:	10.4 minutes.
Diazepam:	12.2 minutes

Column: Hypersil 5um C18 25cm x 4.6mm i.d.
Mobile phase: Methanol-deionised water- sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75)
Detection wavelength: 230nm

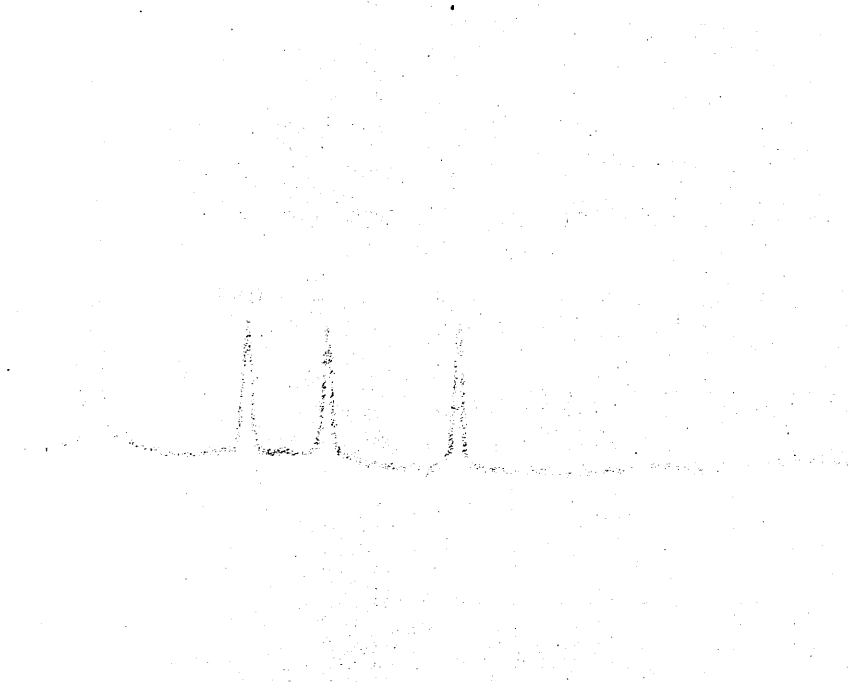


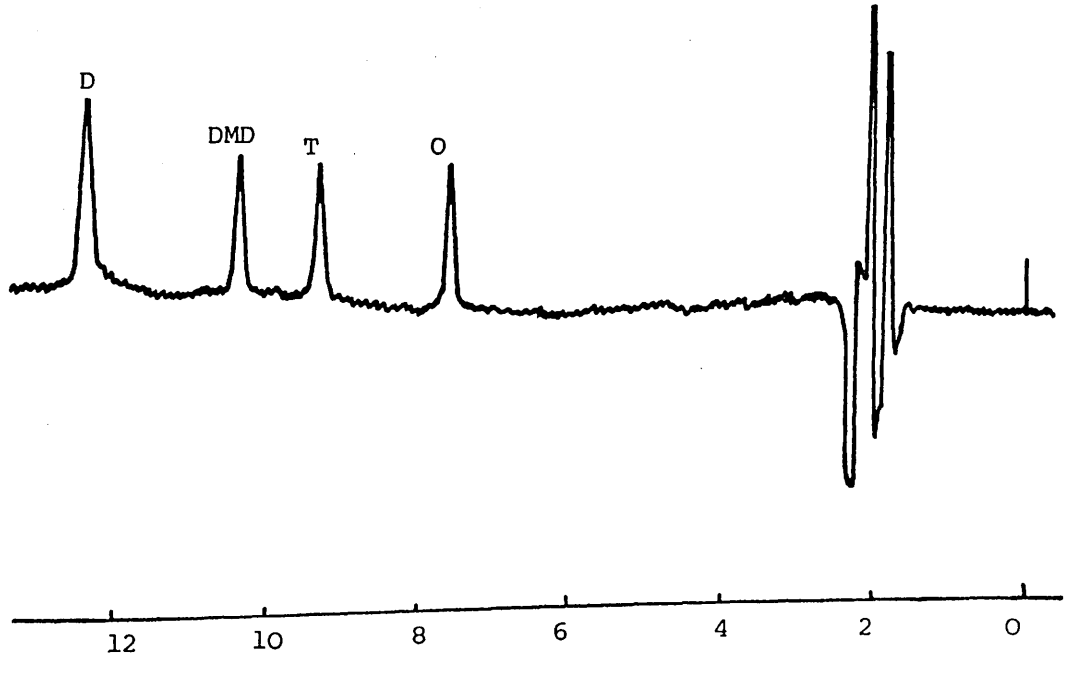
FIGURE 9.

Separation of diazepam from its major metabolites using HPLC.

Column: Hypersil 5um ODS C18 (25cm x 4.6mm i.d.)
Mobile phase: Methanol-deionised water-sodium phosphate buffer (pH8)-acetonitrile (200:125:100;75 v/v)
Detection wavelength: 230 nm
Range: x0.02

Concentration of standard solutions: 100 ng/ml

D= diazepam
O= oxazepam
T= temazepam
DMD= desmethyldiazepam



Oxazepam standards on HPLC

A series of ratios, oxazepam standard peak area divided by prazepam standard peak area, were calculated, varying both the oxazepam concentration and the range of the chart recorder.

The response of aqueous oxazepam standards to the HPLC conditions previously described was linear for three different ranges over the concentration range 0-1000ng/ml oxazepam (Table 2).

Straight line graphs were produced relating the ratio to oxazepam concentration from which oxazepam concentrations were calculated, with a coefficient of variation of 4.60% at 0.02 absorbance units full scale (AUFS).

Standard solutions of 50ng/ml were easily detected which was adequate for the purposes of this work.

The range x0.02 was the optimum setting for signal to noise ratio.

TABLE 2.

RELATIONSHIP BETWEEN OXAZEPAM CONCENTRATION AND PEAK AREA USING HPLC ANALYSIS

Range (AUFS)	Peak Area Relationship
x0.01	y=800x
x0.02	y=769.2x ± 4.6% over three days
x0.04	y=1111.1x

y = concentration of oxazepam standard (ng/ml)

$$x = \frac{\text{area of oxazepam peak}}{\text{area of prazepam peak}}$$

Column: Hypersil 5um C18 25cm x 4.6mm i.d.
Mobile phase: Methanol-deionised water- sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75)
Detection wavelength: 230nm

b) Triazolam and 1-hydroxy triazolam

Two HPLC methods were developed for the separation of triazolam from its 1-hydroxy metabolite:

i) Triazolam was separated from its main metabolite using the HPLC system described. The column used was a reverse-phase C18, and the eluent consisted of methanol-deionised water-sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75).

The optimum flowrate for separation was 1.5 ml/minute. Using this system, triazolam was separated from its main metabolite and peak shapes were good for both drugs (Figure 10a).

Triazolam standards on HPLC

The retention times were determined (Table 3) and the linearity of response was determined over the concentration range 0-500 ng/ml and over three chart ranges for both triazolam and its metabolite (Table 4). Standard solutions of 50ng/ml were easily detected. Prazepam was used as the external standard in benzodiazepine determinations.

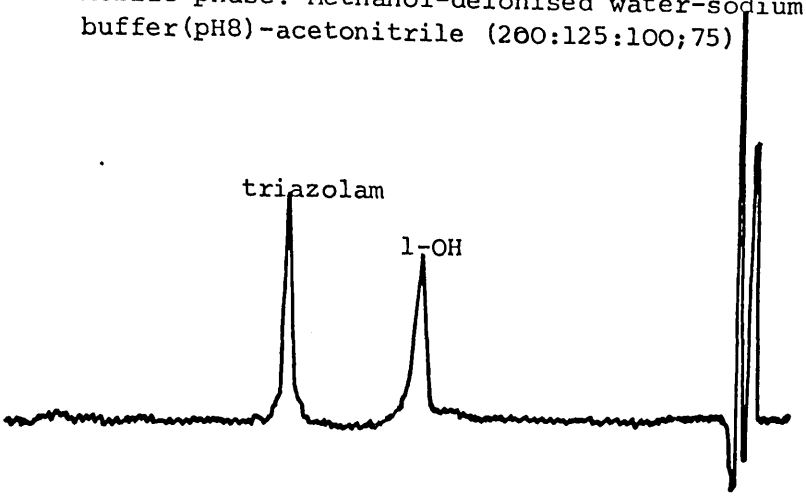
Modification of mobile phase for use with LC-MS

The presence of a phosphate buffer in the HPLC system described above caused problems with column blocking. Also, the detection limit of 50ng/ml for triazolam and its main metabolite was inadequate for the purposes of this work. A more sensitive analysis method was required.

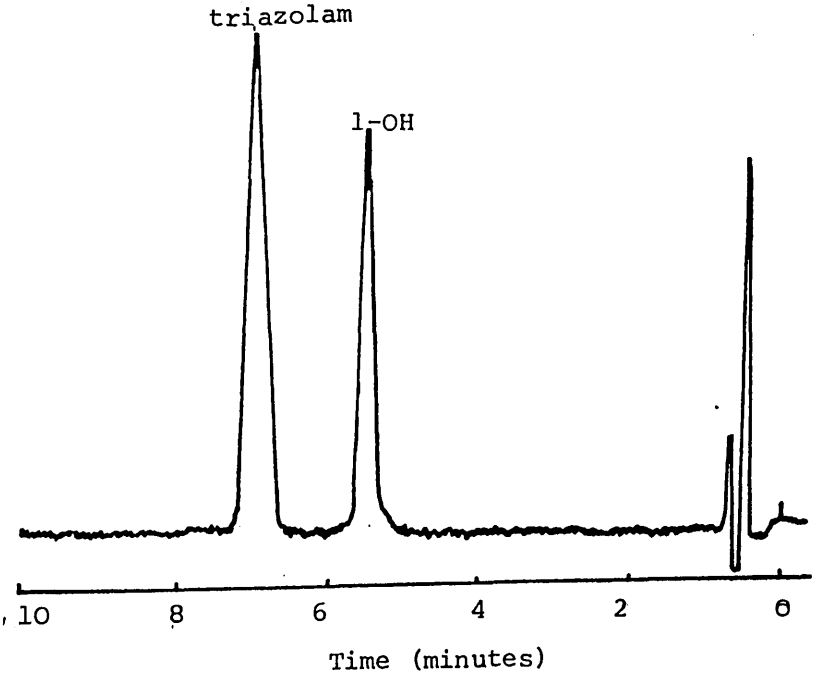
FIGURE 10.

Separation of triazolam from 1-hydroxy triazolam using HPLC.

a) Column: C18
Mobile phase: Methanol-deionised water-sodium phosphate buffer(pH8)-acetonitrile (200:125:100;75)



b) Mobile phase: Methanol-deionised water-ammonium acetate buffer(pH7)-acetonitrile (200:125:100:75)



Concentration of standard solutions: 500 ng/ml
Detection wavelength: 230nm
Range: x0.02

TABLE 3.

RETENTION TIMES FOR TRIAZOLAM AND ITS METABOLITE USING HPLC

Drug	Retention Time
Triazolam	6.6 minutes
1-hydroxy triazolam	4.8 minutes

Column: Hypersil 5um C18 25cm x 4.6mm i.d.
Mobile phase: Methanol-deionised water- sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75)
Detection wavelength: 230nm

TABLE 4

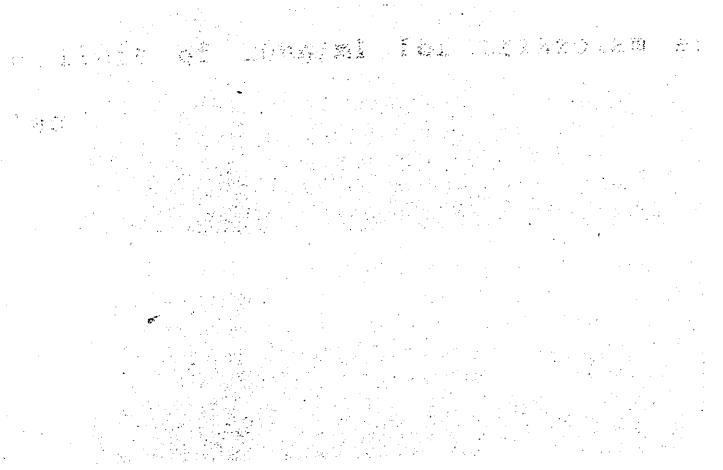
RELATIONSHIP BETWEEN TRIAZOLAM AND 1-HYDROXY TRIAZOLAM CONCENTRATION AND PEAK AREA USING HPLC ANALYSIS

Range (AUFS)	Triazolam	1-hydroxy triazolam
	Peak Area Relationship	
x0.01	y = 937x	y = 734.2x
x0.02	y = 1803x ± 5.4%	y = 1303 ± 0.5%
x0.04	y = 1703x	y = 1392x

y = concentration of drug standard (ng/ml)

$x = \frac{\text{area of drug peak}}{\text{area of prazepam peak}}$

Column: Hypersil 5um C18 25cm x 4.6mm i.d.
Mobile phase: Methanol-deionised water- sodium phosphate buffer (pH8)-acetonitrile (200:125:100:75)
Detection wavelength: 230nm



The possibility of LC-MS was considered. The HPLC system described above was not compatible with LC-MS because of the presence of phosphate buffer, so an alternative mobile phase was developed (ii).

In the modified mobile phase the phosphate buffer was replaced with ammonium acetate buffer. The composition of the modified mobile phase was: ammonium acetate buffer (pH7) - deionised water - acetonitrile - methanol (100:125:75:200 v/v).

Using the above system and stock solutions described, the retention times for triazolam and 1-hydroxy triazolam were determined and found to be similar to those obtained using the original mobile phase (Table 5).

Further, the acetate buffer system produced peak shapes which were as good as those produced by the phosphate system (Figure 10b) and it also provided greater sensitivity. The developed HPLC method gave a detection limit of 10ng/ml for triazolam and its two metabolites.

TABLE 5.

RETENTION TIMES FOR TRIAZOLAM AND ITS METABOLITE USING HPLC

Drug	Retention Time
Triazolam	7.2 minutes
1-hydroxy triazolam	5.4 minutes

Column: Hypersil 5um C18 Universal cartridge (25cm x 4.6mm i.d.)
Mobile Phase: Ammonium acetate buffer (pH7)-deionised water-acetonitrile-methanol (100:125:75:200 v/v)
Detection wavelength: 230nm

c) Flunitrazepam and metabolites

The system described above is LC-MS compatible and because of its greater sensitivity, it was subsequently investigated for the separation of flunitrazepam from its metabolites.

Flunitrazepam was separated from its metabolites using this system. Peak shapes were good particularly for the metabolites (Figure 11).

d) Separation of nineteen common benzodiazepines

The possibility of interference in these assays from other related compounds was considered. Nineteen benzodiazepines were separated using the HPLC system described above to see whether any other common benzodiazepines were co-eluting with diazepam and its metabolites, triazolam and its metabolites or flunitrazepam and its metabolites.

Day to day reproducibility of the analysis method was evaluated by constructing calibration curves for all drugs on three different days (Table 6).

Overall reproducibility was good, with the coefficient of variation ranging between 1.2% for lormetazepam and 13% for nitrazepam.

All the compounds were separated within 30 minutes although not all were chromatographically resolved from one another. The retention times are given (Table 7).

FIGURE 11.

Separation of flunitrazepam from its metabolites using HPLC.

Column: C18
Mobile phase: Methanol-deionised water-ammonium acetate buffer (pH7)-acetonitrile (200:125:100:75)
Detection wavelength: 230nm
Range: x0.02

Concentration of standard solutions: 500 ng/ml

F= flunitrazepam
DMF= desmethylflunitrazepam
7AF= 7-aminoflunitrazepam

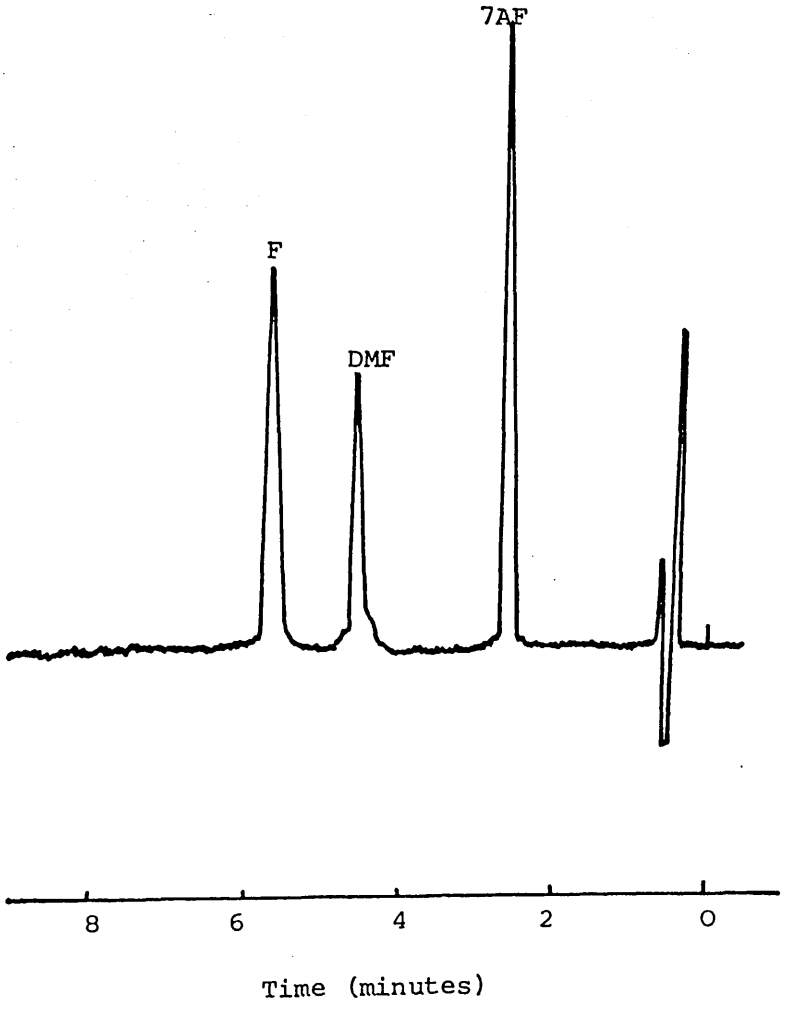


TABLE 6.

REPRODUCIBILITY OF HPLC ANALYSIS PROCEDURE FOR BENZODIAZEPINES

Drug	Average Peak Area (mm^2)	COV (%)
Alprazolam	113.2	4.7
Clobazam	196	7.1
Clonazepam	60	4.2
Desmethyldiazepam	154	2.3
Diazepam	125.8	7.1
Flunitrazepam	94.5	4.2
Desmethylflunitrazepam	72	7.7
7-aminoflunitrazepam	172	6.5
Flurazepam	20	3.2
Lorazepam	242.7	4.8
Lormetazepam	50	1.2
Loprazolam	36	2.4
Midazolam	60	3.1
Nitrazepam	39.3	12.8
Oxazepam	190	3.0
Temazepam	220	4.4
Triazolam	289.4	8.9
1-hydroxytriazolam	159	1.9
4-hydroxytriazolam	165	9.0

Drug concentrations: 500ng/ml
Column: Hypersil 5um C18 Universal cartridge (25cm x 4.6mm i.d.)
Mobile Phase: Ammonium acetate buffer (pH7)-deionised water-acetonitrile-methanol (100:125:75:200 v/v)
Detection wavelength: 230nm

TABLE 7.

RETENTION TIMES OF BENZODIAZEPINES ANALYSED BY HPLC

Drug	Retention Time (minutes)
Alprazolam	7.7
Clobazam	7.2
Clonazepam	5.2
Desmethyldiazepam	9.9
Diazepam	12.8
Flunitrazepam	5.6
Desmethylflunitrazepam	4.5
7-aminoflunitrazepam	2.5
Flurazepam	27.3
Lorazepam	6.3
Lormetazepam	9.2
Loprazolam	8.1
Midazolam	12.5
Nitrazepam	5.2
Oxazepam	6.7
Temazepam	8.5
Triazolam	7.2
1-hydroxytriazolam	5.4
4-hydroxytriazolam	5.8

Column: Hypersil 5um C18 Universal cartridge (25cm x 4.6mm i.d.)
Mobile Phase: Ammonium acetate buffer (pH7)-deionised water-acetonitrile-methanol (100:125:75:200 v/v)
Detection wavelength: 230nm

Clobazam had the same retention time as triazolam. Flunitrazepam was eluted between the metabolites of triazolam. The developed system adequately separates triazolam and its metabolites from all commonly occurring benzodiazepines except clobazam and flunitrazepam. It also separates all the metabolites of diazepam and flunitrazepam from possible interfering benzodiazepines.

The system was designed to be LC-MS compatible (no phosphate buffers are involved) so that, if necessary interfering peaks could be positively identified using this technique. LC-MS analysis was not carried out in this work.

The detection limit for most of the benzodiazepines was 50 ng/ml at 0.02 AUFS although for 7-aminoflunitrazepam, clobazam, desmethyldiazepam, lorazepam, oxazepam, temazepam, triazolam, 1-OH triazolam and 4-OH triazolam, the limit was lower (10 ng/ml). Loprazolam and flurazepam were not detected below 100ng/ml.

4.1.2. Xylazine

Xylazine, a veterinary sedative, is potentially a drug of abuse in racing greyhound situations due to its high potency. A reproducible and sensitive analysis system was required for its determination and quantitation in greyhound urine.

The HPLC system described above (C18 column; methanol-deionised water-ammonium acetate buffer (pH8)-acetonitrile (200:125:100:75) did not allow good chromatography of xylazine. The peak shapes were too broad and the retention time was long.

The published method for xylazine HPLC analysis (C18 column; 2% glacial acetic acid in water - methanol - heptanesulphonic acid (55:45:0.2,v/v); detection wavelength 225nm) (Alvinerie and Toutain, 1981) gave very broad peak shapes and so an alternative method was developed.

Xylazine is similar in structure to bromazepam so a published HPLC method for bromazepam was modified to separate xylazine. The system (C18 column; mobile phase: 1g tetramethyl ammonium hydroxide dissolved in deionised water (250ml) - acetonitrile - methanol (250:150:10,v/v); flowrate 2ml/minute; detection wavelength 225nm) was a modification of an existing method used for bromazepam (Hirayama and Kasuya, 1983). The peak shapes were excellent and the detection limit for xylazine was 10ng/ml.

Xylazine standards on HPLC

The linear relationship was calculated between peak area ratio and the concentration of xylazine in urine between 10 and 1000 ng/ml. Standard curves constructed on three different days showed good reproducibility over the concentration range used, with a coefficient of variation of 5.95% at 0.02 absorbance units full scale (AUFS) over three days (Table 8) from which xylazine concentrations in extracted samples could be calculated.

The detection limit was 10 ng/ml and the retention time for xylazine was determined (Table 8).

TABLE 8.

RELATIONSHIP BETWEEN XYLAZINE CONCENTRATION AND PEAK AREA USING HPLC ANALYSIS

Drug concentration (ug/ml)	Average Peak Area (mm^2)
0.05	4.9
0.1	10.2
0.5	45.7
1.0	89.9

Range	Peak Area Relationship
x0.01	y=151.5x
x0.02	y=95.3x ± 5.95%
x0.04	y=31.25x

x = concentration of drug (ug/ml)
y = area of drug peak

RETENTION TIME FOR XYLAZINE USING HPLC : 6.5 minutes

Column: Hypersil 5um C18, 25cm x 4.6mm i.d.
Mobile phase: 1g tetramethyl ammonium hydroxide dissolved in 250 ml deionised water-acetonitrile-methanol (250:150:10,v/v)
Detection wavelength: 225nm

4.1.3. Basic Drug Screening System

HPLC analysis

The screening of urine for the presence of basic drugs is commonly encountered in pre-employment screening, post-mortem analysis and drug testing in sport. The development of an HPLC system for a wide range of basic drugs to aid specific drug identification was desirable.

HPLC analysis of 100 basic drugs commonly encountered in urinary drug screening was performed using a C8 column, and a gradient pumping system operated at 1.5 ml/minute. The eluent was monitored at 200nm and full spectra were recorded from 190 to 400nm for each peak. The initial mobile phase composition was 10% acetonitrile in 0.05M pH3.2 potassium phosphate buffer increasing to 50% over 15 minutes, and the final composition was maintained for 5 minutes. A re-equilibration time of 5 minutes was required between injections (total cycle time 25 minutes) (Logan, 1988).

One hundred basic compounds were analysed using this system and the retention times are given (Table 9). The absorbance maxima or points of inflexion were also noted to allow comparison of these values with published data (Clarke, 1986).

TABLE 9.

RETENTION TIMES OF COMPOUNDS ANALYSED BY HPLC/DAD SPECTROSCOPY

Drug	Retention Time (minutes)	Drug	Retention Time (minutes)
Alprazolam	16.1	Metaclopramide	9.9
Amitriptyline	17.2	Methadone	18.2
Amoxapine	14.6	Methamphetamine	7.5
Amphetamine	6.75	Methaqualone	16.6
Atropine	8.5	Methylecgonine	10.6
Benzocaine	14.1	Methylphenidate	10.2
Benzoylecgonine	7.9	Methyprylon	6.0
Benztropine	15.3	Metoprolol	9.8
Bupivacaine	13.3	Morphine	3.2
Butorphanol	12.1	Nalorphine	15.0
Caffeine	6.7	Naloxone	5.9
Chlordiazepoxide	13.5	Nordiazepam	16.4
Chloroprocaine	8.9	Normeperidine	11.0
Chlorpheniramine	12.3	Norpropoxyphene1	8.5
Chlorpromazine	22.0	Nortriptyline	16.6
Clonazepam	16.1	Oxazepam	14.9
Cocaine	11.6	Oxycodone	6.9
Codeine	6.2	Oxymorphone	3.7
Cyproheptadine	16.7	Papaverine	10.0
Desipramine	15.9	Pentazocine	13.1
Dextromethorphan	13.9	Phencyclidine	14.2
Diazepam	18.6	Phenelzine	5.2
Dihydrocodeine	5.7	Phenmetrazine	7.1
Diphenhydramine	14.9	Phentermine	7.7
Doxepin	15.4	PPA	5.0
Ephedrine	5.6	Procaine	6.6
Ethchlorvynol	13.9	Promazine	15.9
Homocaine	14.5	Promethazine	15.8
Ethylmorphine	7.9	Propoxyphene	17.2
Fentanyl	15.0	Propanolol	13.4
Flurazepam	14.2	Protryptiline	16.2
Glutethimide	11.5	Quinine	9.6
Haloperidol	16.3	Strychnine	8.6
Hexobarbital	14.1	Temazepam	18.4
Hydrocodone	7.4	Tetracaine	14.5
Hydromorphone	4.3	Theobromine	5.1
Imipramine	16.3	Theophylline	4.5
Ketamine	8.5	Thioridazine	19.6
Lidocaine	8.6	Triazolam	18.5
Lorazepam	15.1	Trifluroperazine	19.0
Loxapine	15.4	Trimipramine	17.5
LSD-25	12.1	Tripelennamine	12.5
Mazindol	12.5		
Meperidine	11.5		
Mesoridazine	14.5		

PPA = phenylpropanolamine

Column: Hibar Lichrospher 100 CH-8/II (25cm x 4.6mm)

Mobile Phase: 10% Acetonitrile in 0.05M pH3.2 potassium phosphate buffer increasing to 50% over 15 minutes. Final composition maintained for 5 minutes.

This comparison was not always possible as some compounds display bathochromic or hypochromic shift depending on the pH and solution conditions under which measurement is made. While not all compounds listed were completely resolved, in most cases peak identities were initially assigned on the basis of retention time and confirmed by an examination of the UV spectra.

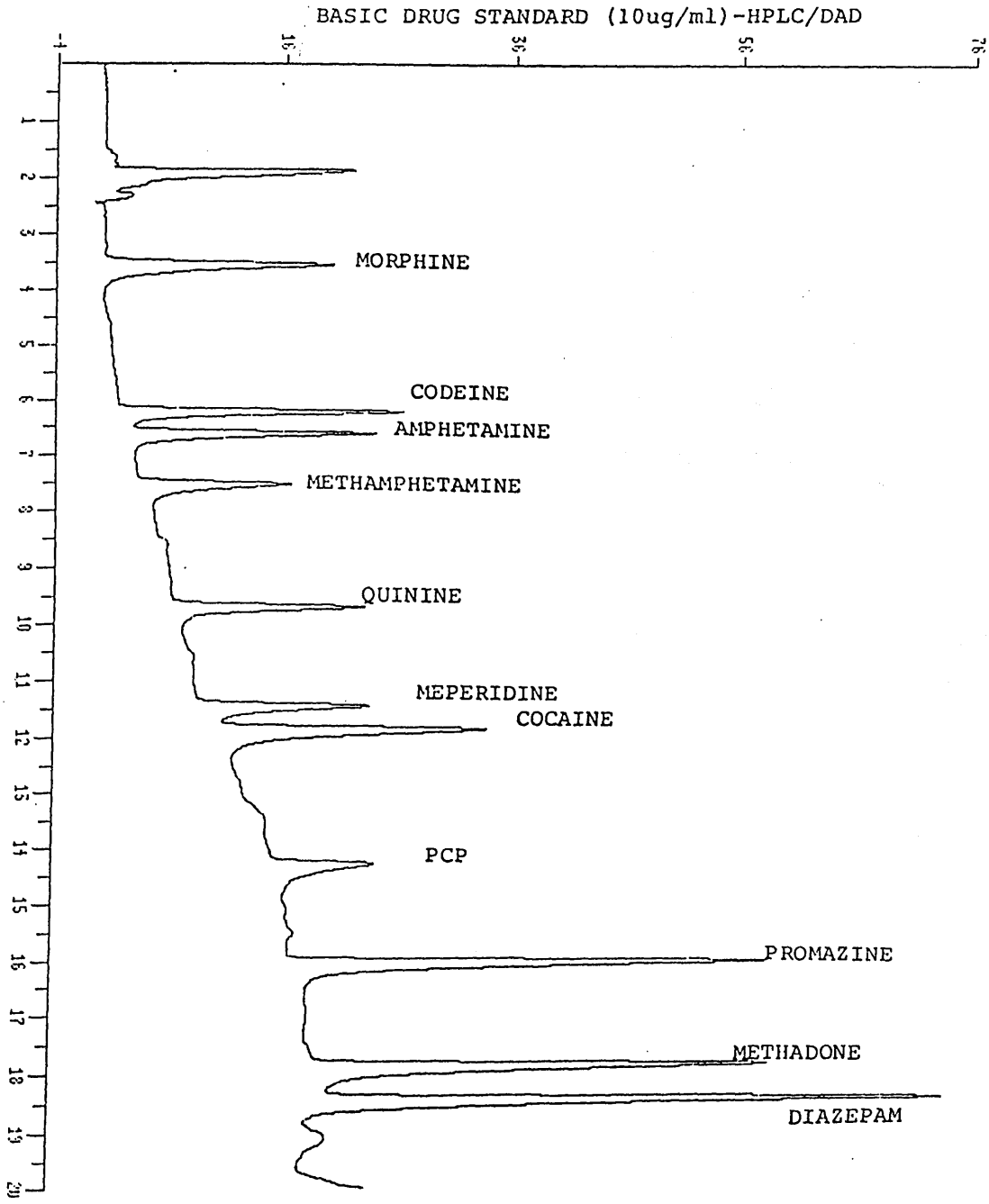
The reproducibility of retention times was measured for 10 repeat runs over a period of three weeks.

This was found to be good with a mean variation of ± 6 seconds. The reproducibility of retention times in HPLC is recognised as being poorer than in GC (Smith et al., 1987) and reproducibility of methods between laboratories is even less reliable.

The use of alkyl aryl ketones and other markers to calculate retention indices for HPLC has been shown to have some merit (Smith et al., 1988), but generally individual laboratories prepare their own data bases of retention times and UV spectral library under local conditions.

Peak shapes were generally good due to the use of gradient elution. A chromatogram of 11 drugs extracted from an aqueous drug standard is shown (Figure 12).

FIGURE 12.


BASIC DRUG STANDARD (10ug/ml)-HPLC/DAD
MORPHINE
CODEINE
AMPHETAMINE
METHAMPHETAMINE
QUININE
MEPERIDINE
COCAINE
PCP
PROMAZINE
METHADONE
DIAZEPAM
End of plot. Time = 0.01 to 19.99 minutes Chart speed = 1.00 cm/min

UV spectroscopy does not give as specific structural information for individual compound identification as does nuclear magnetic resonance (NMR), infra-red (IR) or mass spectroscopy (MS). This lack of specificity does, however, have the advantage that compounds belonging to the same class very often display similar UV absorbance patterns. For example, figure 13 shows the UV spectra of three opiates; dihydrocodeine, hydrocodone amd hydromorphone, three phenothiazines; promazine, promethazine and trifluroperazine, and three benzodiazepines; alprazolam, triazolam and diazepam.

Each group of compounds is well resolved chromatographically (Table 9) and is sufficiently different spectroscopically to allow discrimination on that basis alone, however each group displays absorbances in characteristic regions, so compounds which have not been characterised on the HPLC system can be tentatively assigned to a compound class in order to assist with further analytical determinations. The sensitivity of this HPLC method is comparable to those quoted for other HPLC/UV detection methods. The sensitivity of the diode array detector is also similar to that of other UV detectors. The advantage of diode array detection is to allow the operator to examine, post-run, a spectrochromatogram and select the wavelength providing optimum signal to noise ratio for any peak of interest.

UV Spectra of three opiates, three phenothiazines, three benzodiazepines.

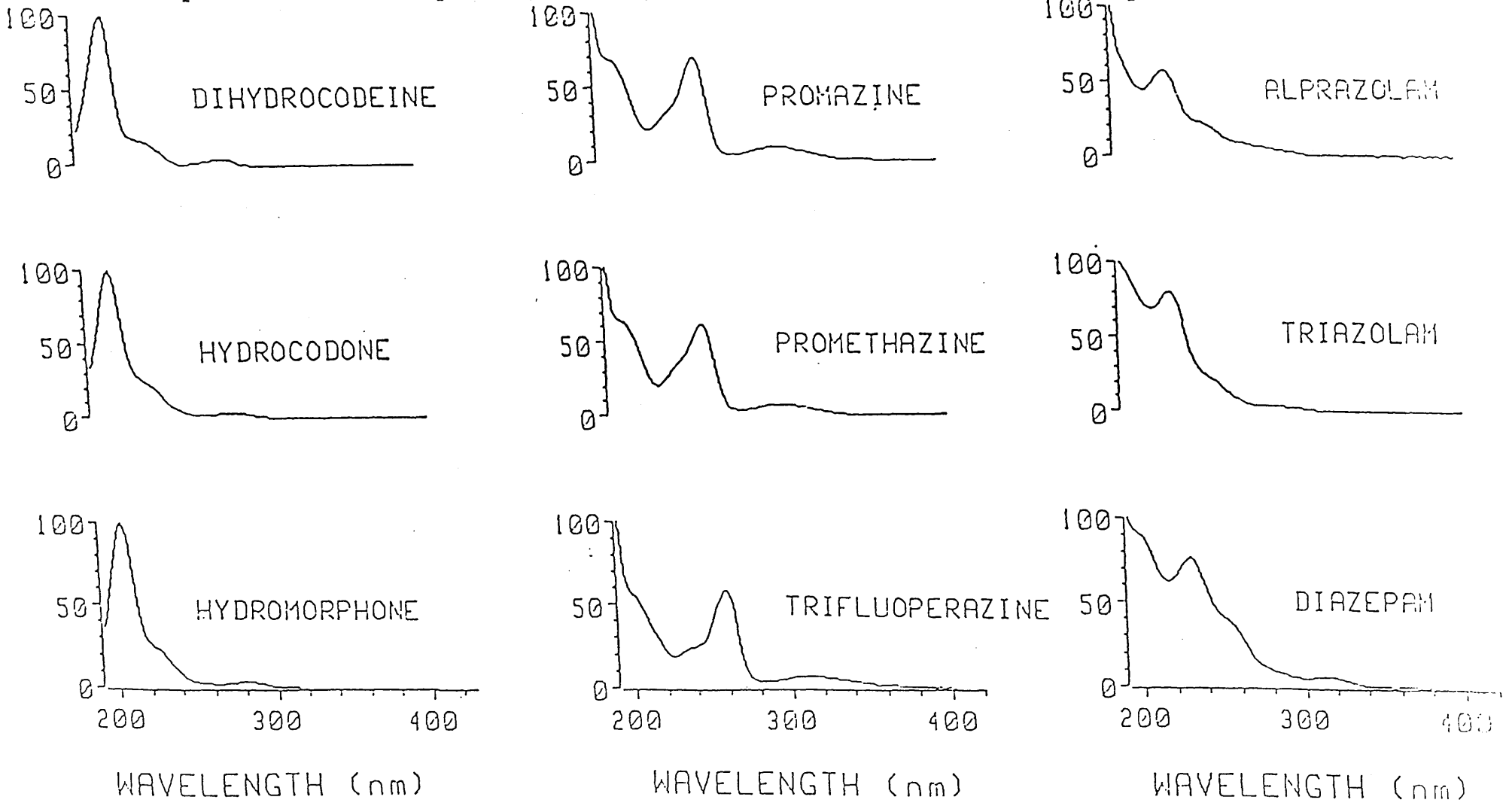


FIGURE 13.

4.1.4. Mazindol

HPLC analysis

Mazindol, a stimulant, is potentially a widely abused drug in horse racing. Since no reversed-phase HPLC methods for the analysis of mazindol have been previously reported, a quantitative analysis procedure was required to determine mazindol in racehorse urine. Since mazindol has a pKa value of 8.6 (Clarke, 1986), it would be poorly retained in a revese-phase system at pH 7 or 8 since there would be some degree of ionisation at such a pH. The possibility of ion-pair HPLC was then considered.

For basic drugs, an acidic eluent is usually chosen and the most widely used ion-pairing agents are the sodium salts of alkylsulfonic acids. The mobile phase permits ion-pair chromatography by adjusting the pH so that the sample is present in its ionic form (pH4).

The first system described (3.1.4.(a) C18 column; 0.005M pentane sulphonic acid-acetonitrile-85% phosphoric acid 90:10:5,v/v); detection wavelength 254nm) gave a very long retention time for mazindol. The next two systems, which used an increased amount of acetonitrile relative to the ion-pairing agent, gave a very short retention time for the drug,- mazindol was eluted too closely to the solvent front.

The optimum mobile phase suitable for the separation of mazindol was 0.005M pentane sulphonic acid - acetonitrile - 85% phosphoric acid (75:25:5,v/v) on a reversed phase HPLC system (C18 column) and the detection wavelength was 254nm (Table 10a). Peak shapes were generally good.

Mazindol standards on HPLC

The linear relationship was found between peak area ratio and the concentration of mazindol in urine between 10 and 1000 ng/ml. Standard curves constructed on three different days showed good reproducibility over the concentration range used, with a coefficient of variation of 5.40% at 0.02 absorbance units full scale (AUFS) over three days (Table 10b) from which mazindol concentrations in extracted samples could be calculated.

The detection limit was 25 ng/ml.

TABLE 10.

10a).

RETENTION TIME OF MAZINDOL USING VARIOUS ELUENT COMPOSITIONS

Mobile phase composition 0.005M PSA: ACN: HPO_4	Retention time of mazindol
90 : 10 : 5	30.2 minutes
50 : 50 : 5	1.8 minutes
60 : 40 : 5	3.7 minutes
75 : 25 : 5	12 minutes

10b).

RELATIONSHIP BETWEEN MAZINDOL CONCENTRATION AND PEAK AREA USING HPLC ANALYSIS

Range x0.02 AUFS

10-1000ng/ml $y = 68.97x \pm 5.4\%$ over three days

Column: 5um Bondapak C18, 30cm x 4.5mm i.d.
Detection wavelength: 254nm

4.2. Methods of Extraction

4.2.1. Extraction Methods exploiting Non-polar Sorbent Interactions

1. Benzodiazepines

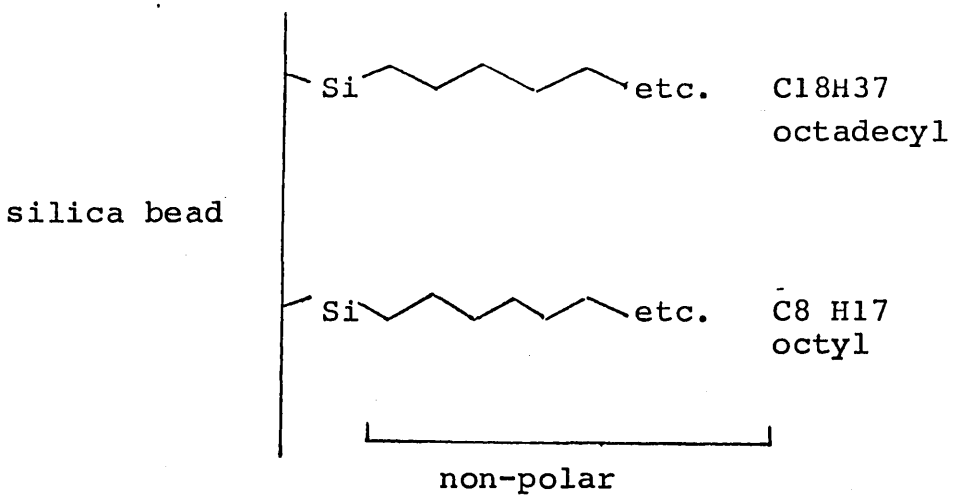
Non-polar interactions are those that occur between the carbon-hydrogen bonds of the sorbent functional groups and the carbon-hydrogen bonds of the isolate. These forces are commonly known as "Van der Waals" or "dispersion" forces. Since most organic molecules show some degree of non-polar character (including benzodiazepines), non-polar interactions can be used to retain the drug on the sorbent.

Unbonded silica does not exhibit non-polar interactions.

Benzodiazepines have a substantial degree of non-polar character, so non-polar interactions between them and a non-polar sorbent was an appropriate starting point for their extraction from biological fluids. The non-polar sections of the drug (C-H bonds in particular) are attracted to the non-polar side chain of the bonded sorbent and this interaction is broken by a non-polar solvent for which the isolate has a stronger affinity (Van Horne et al., 1985) (Figure 14).

FIGURE 14.

Primary interactions of a non-polar sorbent.



DIAZEPAM AND OXAZEPAM

Initial extraction procedure

The initial extraction procedure for aqueous standards of oxazepam made use of non-polar C8 cartridges (3ml capacity). Retention of non-polar isolates is facilitated by polar solvents, so methanol was used to condition the sorbents. A basic buffer was then passed through the sorbent to ensure that any basic drugs subsequently passed through the column would remain in unionised form during adsorption onto the sorbent bed. The inclusion of a wash step gave very clean extracts. A maximum recovery of 62.4% was achieved.

Even a solvent as polar as methanol, then, has sufficient non-polar character to disrupt the non-polar interactions between the isolate and the sorbent. In an attempt to improve on this recovery, the column eluent was altered to methanol-acetonitrile (1:1), a step which reduced the efficiency of extraction to 46.3%.

The use of more methanol than acetonitrile (3:1) gave a similar extraction efficiency (Table 11) showing that addition of acetonitrile results in a loss of efficiency.

The question of whether retention or elution of the drug was at fault was addressed using labelled diazepam.

TABLE 11.

MAXIMUM EXTRACTION EFFICIENCY OF OXAZEPAM FROM AQUEOUS SOLUTIONS USING C8 CARTRIDGES

Eluent	Percentage Recovery
Methanol-1% ammonium hydroxide-acetonitrile (2:2:1)	62.4%
Methanol-acetonitrile (1:1)	46.3%
Methanol-acetonitrile (3:1)	46.1%

An aqueous standard solution of tritiated diazepam was passed through the sorbent and the effluent was collected.

The retention capacity of the cartridge was assessed by measuring the amount of radioactivity remaining in the aqueous sample of tritiated diazepam after being passed through the bonded sorbent. From the results in Table 12, the amount of diazepam not retained on the column was on average 6%. The retention capacity of the column was 94% (Table 12a).

a) The effect of pH of the sample

The standard solution was buffered to various pH values prior to extraction and the results in table 12a show that the retention capacity was not affected at all by pH.

Table 12b shows that with the use of methanol - acetonitrile (3:1) as the eluent, the extraction efficiency was greatest when the sample was buffered, prior to extraction, to a basic pH.

This is not an unexpected conclusion since benzodiazepines are basic drugs and would be in unionised form in basic conditions, so making them more soluble in organic solvents and less soluble in ionic or aqueous solutions.

Also, unionised forms of drugs are retained longer than ionised drugs on reversed-phase HPLC systems, suggesting that an unionised form would aid retention.

TABLE 12.

DETERMINATION OF DIAZEPAM RETENTION USING LIQUID SCINTILLATION COUNTING

12.a)

pH	Counts per minute (CPM)	Percentage drug unretained
Reference standard (0.5ml ws + 0.5ml water)	12781	
4.15	911	7.1%
4.15	256	2.0%
7.63	674	5.2%
7.63	905	7.1%
10.25	786	6.1%
10.25	657	5.1%
Unbuffered	632	4.9%
Unbuffered	956	7.5%

12.b)

pH	Counts per minute (CPM)	Percentage drug eluted
4.15	3752	29.4%
7.63	6491	50.8%
10.25	8934	69.9%

WS = working solution = Diazepam aqueous standard solution (300ng/ml) (1ml) + 0.1ml of tritiated diazepam (1ml) made up to 1l with deionised water.

From these results, the problem lay in the inadequate elution of the drug from the sorbent, and not in inadequate retention. Various elution solvents were therefore evaluated.

b) The effect of elution solvents

Four elution solvents of different polarities were prepared and used to elute oxazepam from the columns. The recoveries obtained in order of decreasing polarity of the eluents are given (Table 13).

With the exception of the first eluent which was alkaline, the samples were buffered to pH 10.25 with 0.05M sodium dihydrogen phosphate/sodium hydroxide (0.5ml) prior to extraction, because the results in table 12b show that buffering of the sample to an alkaline pH prior to extraction potentially increases the efficiency.

1. Methanol-10% ammonium hydroxide-acetonitrile (3:1:1).

This elution solvent gave an extremely low recovery of approximately 14%. Since basic conditions aid retention, this eluent composition was probably too basic to allow elution.

2. Methanol-acetonitrile-chloroform (2:1:1)

The introduction of a more non-polar elution solvent (chloroform), and ensuring that the drug was in a basic environment prior to extraction, improved the extraction efficiency (66%).

TABLE 13.

RECOVERIES OF OXAZEPAM USING C8 CARTRIDGES WITH ELUTION SOLVENTS OF DECREASING POLARITY

Methanol-10% ammonium hydroxide-acetonitrile (3:1:1)	Recovery (%)
Reference (100 ng/ml oxazepam)	100
Average of two determinations:	
C8 cartridges: 1:	19.9
2:	16.3
3:	12.1
4:	12.5
5:	9.0
	14.0±4.2%
Methanol-acetonitrile-chloroform (2:1:1)	
Reference (100 ng/ml)	100
C8 cartridges: 1:	60.55
2:	84.60
3:	57.51
4:	66.45
5:	63.35
	66.5±10.7%
Methanol-acetonitrile-chloroform (2:1:3)	
Reference (100 ng/ml)	100
C8 cartridges: 1:	63.7
2:	76.9
3:	60.3
4:	68.8
5:	90.4
	72.1±12%
Chloroform	
Reference (100ng/ml)	100
C8 cartridges: 1:	81.7
2:	94.1
3:	87.7
4:	84.6
5:	87.6
6:	72.2
	84.7±7.4%

3. Methanol-acetonitrile-chloroform (2:1:3)

A further increase in chloroform concentration improved the extraction efficiency, to 72%

4. Finally, since the introduction of a very non-polar solvent increased the extraction efficiency, 100% chloroform was used as the eluting solvent, consequently increasing the extraction efficiency to 84%.

These results suggest that oxazepam is held onto the sorbent very strongly by non-polar interactions requiring a very non-polar eluent for successful elution.

Oxazepam, the main metabolite of diazepam in greyhound urine, was quantified using this extraction procedure rather than the parent diazepam, because diazepam is extensively metabolised in animal species (Schwartz et al., 1965).

Oxazepam in greyhound urine samples

Recovery of oxazepam from greyhound urine samples

The percentage oxazepam recovery from spiked urine samples, using the outlined procedure, was determined by comparison of peak areas obtained after injection of an extract of a urine sample spiked with a known concentration of drug, with that produced by the same concentration of the drug in methanol.

Each measurement was taken as the average of two determinations.

The positive identification of oxazepam was based on elution time, absorbance maxima (230 nm) and comparison with standards.

The extraction of oxazepam from spiked samples using C8 columns was good (Table 14).

An acceptable efficiency of 84% was achieved and the chromatograms produced were fairly clean, so the method was applied to actual samples received from administration of diazepam to racing greyhounds.

Temazepam and N-desmethyldiazepam were detected up to four hours after dosing, but only in very small quantities. Diazepam was not detected.

Chromatograms, in general, were very clean with no endogenous substances interfering with oxazepam identification (Figure 15).

The effect of enzyme hydrolysis

Oxazepam is considerably conjugated, mainly as oxazepam glucuronide when excreted in the urine (Tjaden et al., 1980).

Enzyme hydrolysis of the samples was carried out using the enzyme B-glucuronidase at pH5 (Axelson et al., 1981) in order to release oxazepam from its conjugated state and so allow the measurement of an increased amount of free oxazepam in the urine.

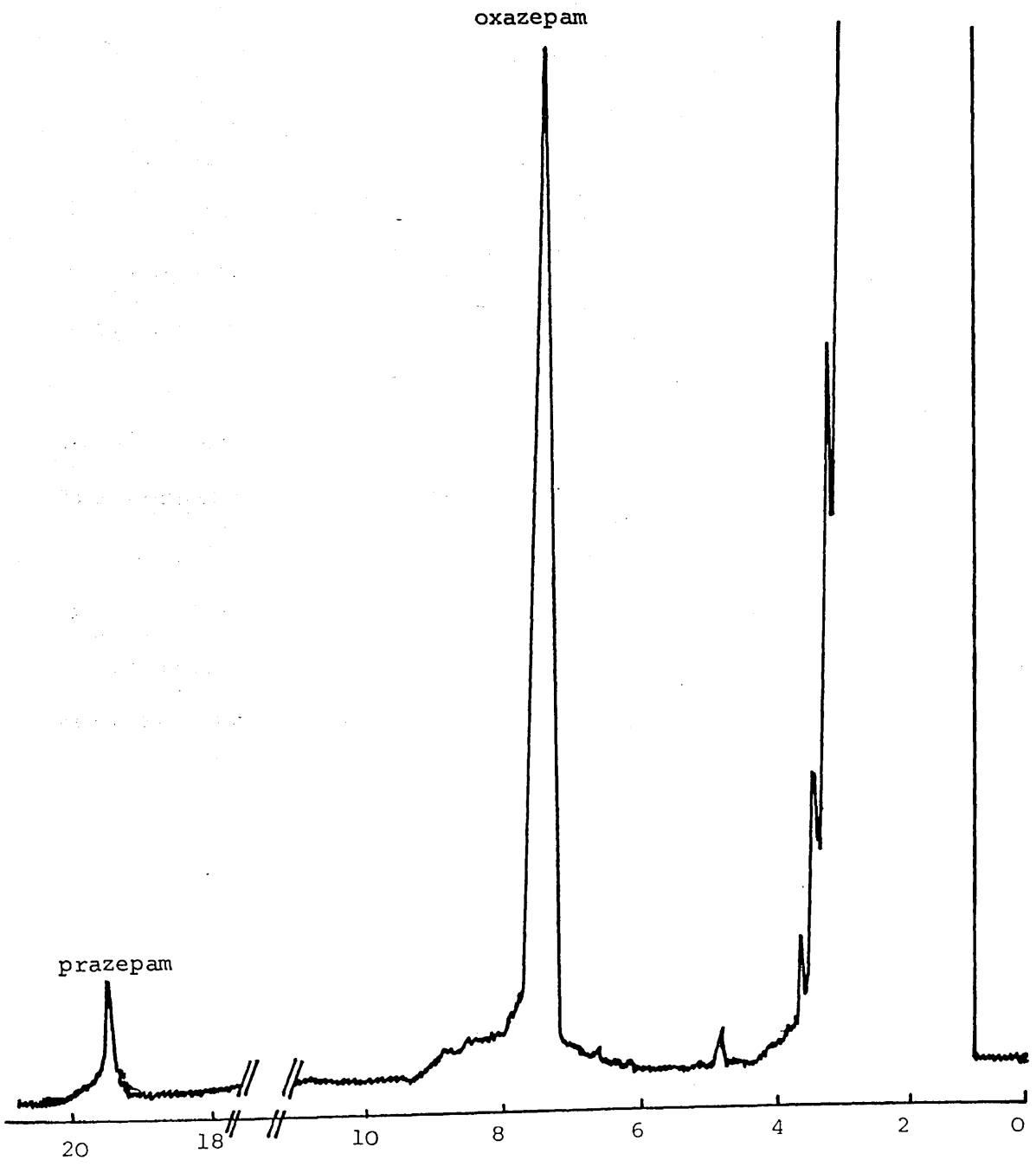
TABLE 14.

EXTRACTION EFFICIENCY FROM C8 CARTRIDGES FOR URINE SAMPLES SPIKED WITH OXAZEPAM

Spiked sample (50 ng/ml)		Peak Area (mm^2)	Percentage Recovery
Reference		85	100
Reference		100	100
Reference		90	100
C8 columns:	1:	77	83.9
	2:	76	82.9
	3:	80	87.2
	4:	77	83.9
	5:	80	87.2
	6:	78	85.1
	7:	75	81.8
	8:	70	76.3
			83.5±4.2%

FIGURE 15.

Oxazepam extracted from greyhound urine -
3 hours after a single oral dose of diazepam -
using C8 columns and HPLC analysis.



The results in table 15 show that enzyme hydrolysis of urine markedly increases the amount of free oxazepam available for extraction.

Extraction of greyhound samples

Quantitation of oxazepam

Four greyhounds were each given a single oral dose of 5mg of diazepam and urine samples were collected at timed intervals following administration.

The quantitation of oxazepam from greyhound urine samples was carried out using the ratio of oxazepam peak areas to prazepam standard peak areas. (Prazepam concentration was constant at 2.4 ug/ml). Total oxazepam concentrations were calculated by taking into account the 84% column extraction efficiency and the total urine volume collected at given time intervals.

The results are given in table 16.

Not all the greyhounds produced urine at the required time intervals, although hourly collections were attempted.

For all four greyhounds, the peak excretion time of oxazepam was between two and three hours after administration. Two of the greyhounds showed peak excretion values after 3 hours, one peaked after 2 hours and one greyhound did not give a sample after 3 hours at all. This greyhound's peak excretion time was taken as 2 hours.

TABLE 15.

COMPARISON OF ENZYME HYDROLYSED AND UNTREATED URINE SAMPLES CONTAINING OXAZEPAM AND EXTRACTED USING C8 CARTRIDGES

Sample		Retention Time (minutes)	Peak Area (mm^2)
Untreated Extracts			
Blank		---	---
02 hours:	1:	---	---
	2:	---	---
04 hours:	1:	7.5	112
	2:	7.6	100
06 hours:	1:	---	---
	2:	---	---
Treated Extracts			
Blank		---	---
02 hours:	1:	7.50; 9.20; 10.2	994; 54; 85
	2:	7.65; 9.40; 10.5	1008; 78; 58
04 hours:	1:	7.40; --- ; 10.2	1539; --; 24
	2:	7.50; 9.20; 10.3	1520; 63; 70
06 hours:	1:	7.80	1193
	2:	7.90	611
24 hours:	1:	7.80	800
	2:	7.60	532

Retention times:		
	Oxazepam	7.5 minutes
	Temazepam	9.3 minutes
	N-desmethyldiazepam	10.4 minutes

TABLE 16.

TOTAL OXAZEPAM CONCENTRATIONS DETECTED IN URINE OF RACING GREYHOUNDS

Time after Dosing (hours)	Greyhound Number	Total Urine Collected (mls)	Total Oxazepam * Present (ug)
01	1	31	NONE
	2	42	NONE
	3	50	NONE
02	1	34	54.58 → 64.97
	2	34	54.38 → 64.74
	3	14	58.63 → 69.79
	4	25	56.50 → 67.20
03	1	68	80.36 → 95.69
	2	68	78.58 → 93.55
	3	20	43.90 → 51.33
04	1	43	23.91 → 28.46
	2	45	24.89 → 29.63
	3	158	31.00 → 36.91
	4	123	27.9 → 29.70
05.25	1	36	11.88 → 14.14
	2	36	11.71 → 13.94
	4	109	9.80 → 10.12
06	1	28	3.64 → 4.34
	2	28	3.84 → 4.57
	3	25	3.87 → 4.50
	4	20	3.21 → 4.31
25	1	36	3.34 → 3.98
	2	36	3.95 → 4.71
	3	14	2.23 → 2.65
	4	50	1.90 → 2.01
26	1	20	1.03 → 1.23
	2	20	1.23 → 1.47
	3	25	0.09 → 1.23
28	1	20	0.44 → 0.52
	2	22	0.51 → 0.61
	3	35	0.43 → 0.51
30	1	25	0.34 → 0.40
	2	25	0.37 → 0.44

* Range covering four greyhounds

Overall, the results between greyhounds were very consistent (Table 16) and trends were similar, for example, no oxazepam was detected later than 30 hours in any of the greyhounds or in the one hour collection.

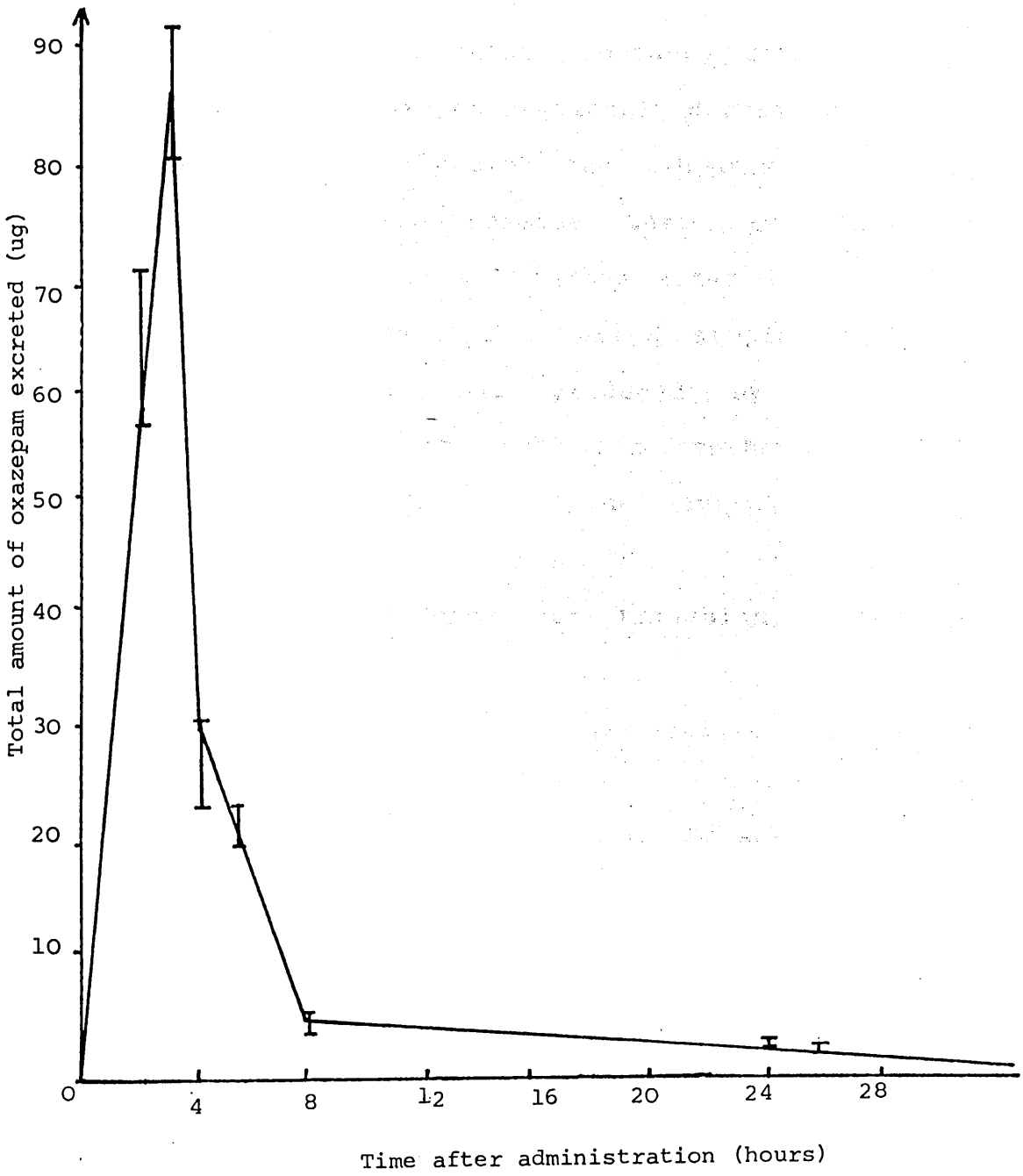
Peak oxazepam concentrations (mean values 1.38-1.4 ug/ml) were attained, three hours after dosing (Figure 15), and average oxazepam levels of 0.02 ± 12.8% ug/ml were still detected 30 hours after dosing (Figure 16).

Conclusion

The detection of oxazepam up to 30 hours after the administration of a single dose of diazepam demonstrates that the use of this analytical procedure is feasible because most samples from racing greyhounds are taken before and after racing. Generally, pre-race samples are taken approximately 1.5 hours before the race is due to start. The greyhounds are then allowed no contact with their owners. Any greyhound seen to improve or deteriorate markedly on previous performances is then sampled as soon as possible after the race, and the following morning. Therefore, a greyhound which has been given sufficient diazepam to produce an effect on performance, would give a positive sample post-race even if the pre-race sample (taken within one hour of doping) was negative.

FIGURE 16.

Average excretion of oxazepam from greyhound urine.



TRIAZOLAM AND 1-HYDROXY TRIAZOLAM

Recovery of triazolam and 1-hydroxy triazolam from greyhound urine samples

Aliquots of standard solutions of triazolam and its main metabolite (1mg/ml in methanol) were added to blank greyhound urine to produce spiked samples of 100 and 500ng/ml concentrations. These were extracted according to the extraction procedure developed for the extraction of oxazepam previously described.

The recoveries of triazolam and 1-hydroxy triazolam from greyhound urine samples were determined by comparing the peak areas obtained after injection of an extract from the spiked urine sample of known concentration, with that produced by the same concentration of each drug in methanol. Each measurement was taken as the average of two determinations.

The extraction efficiency for triazolam, using C8 (3ml) cartridges, was reasonable (80%) at low concentrations. However, the metabolite was poorly extracted (Table 17).

Reproducible results were difficult to obtain due to the wide variation in solvent flowrate through the cartridges. Further investigation showed that the actual packing level in the cartridges was inconsistent throughout the batch of columns being used.

TABLE 17.

EXTRACTION EFFICIENCY FOR URINE SAMPLES SPIKED WITH TRIAZOLAM AND ITS METABOLITE USING C8 CARTRIDGES

Prazepam concentration was constant at 2.4ug/ml

Triazolam Standard Injection (0.01 AUFS) y = 937x

1-hydroxytriazolam Injection y = 734.2x

Triazolam Spiked Samples

Triazolam
0.01 AUFS

Triazolam Concentration (ng/ml)	Amount on Column (ng)	Triazolam/Prazepam Ratio
550	1375	3.07
550	1375	3.12
110	275	0.47
110	275	0.51

1-hydroxytriazolam Concentration (ng/ml)		
500	1250	1.33
500	1250	1.37
100	250	0.29
100	250	0.31

Sample Calculation
Triazolam concentration = 550 ng/ml
Therefore, in 2.5ml added to column, = 1375 ng.

Extracts are reconstituted to 0.5 ml, so 100% extraction is equivalent to 1375 x 2 = 2750 ng/ml concentration.

Area ratio x 937 (see above) = 2900,
so, % recovery = 2900/2750 x 100
= 105.4%

Similarly, triazolam, 110 ng/ml, 80.1% recovery,
1-hydroxytriazolam, 500 ng/ml, 39.6% recovery,
1-hydroxytriazolam, 100 ng.ml, 44.1% recovery.

This variation was more marked between different suppliers of the columns.

Reproducible results could only be obtained by ensuring that all the columns used for extraction were from the same supplier, and where possible from the same batch of manufacture (Table 18).

At higher concentration levels (1 and 5ug/ml), the extractions were much less efficient, probably due to overloading of the column. However, this problem would not be encountered, due to the low levels of drug employed.

Extraction of greyhound samples (0.25 mg administration)

The greyhounds were each given a single oral dose of 0.25mg of triazolam and urine samples were collected at timed intervals following administration.

Triazolam is extensively biotransformed and its metabolites are conjugated in urine (Kitigawa et al., 1979). Enzyme hydrolysis of the samples was carried out to release the metabolites from their conjugated states according to the previous procedure used for oxazepam urine samples. Neither triazolam nor its main metabolite was detected from these greyhound samples.

TABLE 18.

IMPROVED EXTRACTION EFFICIENCY FOR URINE SAMPLES SPIKED WITH TRIAZOLAM AND ITS METABOLITE WITH C8 CARTRIDGES, USING THE SAME BATCH OF CARTRIDGES FROM THE SAME SUPPLIER

Triazolam Spiked Samples

Triazolam Concentration (ng/ml)	Amount on Column (ng)	Triazolam/Prazepam Ratio
x 0.04 y = 1703.6x		
550	1375	2.89
550	1375	3.00
x 0.02 y = 1803x		
110	275	0.77
110	275	0.78
x 0.01 y = 937x		
55	137.5	0.75
55	137.5	0.74
1-hydroxytriazolam Concentration (ng/ml)		
x 0.04 y = 1392.9x		
500	1250	4.38
500	1250	4.37
x 0.02 y = 1302.8x		
100	250	0.89
100	250	0.88
x 0.01 y = 734.2x		
50	125	0.85
50	125	0.86

Sample Calculation

Ratio (2.945) x 1703.2 (see above) = 5015.9
The extracts are reconstituted in 200 ul, so 5015.9/5 gives concentration /ml = 1003.2

1003.2/1375 x 100 = 72.9%

Mean Extraction Efficiencies

Triazolam : 550 ng/ml, recovery : 72.9%
110 ng/ml, recovery : 99.0%
55 ng/ml, recovery : 95.4%

1-hydroxytriazolam : 500 ng/ml, recovery : 97.6%
100 ng/ml, recovery : 92.7%
55 ng/ml, recovery : 99.8%

However, the analysis did reveal the presence of a substance in the extract which did not correspond to either triazolam or 1-hydroxy triazolam under the chromatographic conditions previously discussed (C18 column; methanol-deionised water-phosphate buffer (pH8)-acetonitrile (200:125:100:75,v/v); detection wavelength 230nm).

The peak had a retention time of six minutes and was observed in all samples except the blank urine, an observation suggesting that it was a possible metabolite of triazolam. However, six minutes corresponded neither to triazolam (retention time 6.6 minutes) nor to its major metabolite 1-hydroxy triazolam (retention time 4.8 minutes).

The elution of a substance prior to the parent drug suggests the substance is a metabolite (i.e. more water soluble, therefore a faster elution). However, reference to the literature for both humans and dogs shows 1-hydroxytriazolam to be the major metabolite. Indeed, up to 37% of a dose administered to dogs was excreted as the 1-hydroxy metabolite, and 13% as the 4-hydroxy metabolite (Eberts, 1977).

In order to increase the amount of this substance extracted, columns with a larger capacity for non-polar interactions were used.

The effect of increased capacity cartridges

C18 columns (6ml capacity) were conditioned with two column volumes of methanol and one of water.

Urine (3ml) was diluted with buffer pH10 (0.5ml) and applied to the column. The column was washed with deionised water (2 x 0.5ml).

The adsorbed drug was then eluted with chloroform (3 x 0.5ml) and the eluent was evaporated to dryness and reconstituted in mobile phase (0.1ml) and prazepam standard (0.1ml) prior to HPLC injection.

The extraction efficiency using this method was found to be excellent for both triazolam and its 1-hydroxy metabolite (Table 19). The extract quality seemed to be a function of initial sample quality, but in most cases, extracts were found to be fairly clean. The larger capacity columns were subsequently used for triazolam extraction because more urine could be extracted and hence, a larger amount of isolate obtained.

Extraction of greyhound samples (0.5 mg administration)

The greyhounds were each administered 1 x 0.5mg triazolam tablet. Urine samples were freely collected at timed intervals from the greyhounds.

Since the maximum amount of the unidentified substance was required, only the one and two hour urine samples were extracted.

TABLE 19.

EXTRACTION EFFICIENCY FOR TRIAZOLAM AND 1-HYDROXY TRIAZOLAM FROM GREYHOUND URINE USING C18 CARTRIDGES

Triazolam (100ng/ml):	92 ± 2.4%	(n=4)
(500ng/ml):	100 ± 3.1%	(n=4)
1-hydroxytriazolam (100ng/ml):	94 ± 6.2%	(n=4)
(500ng/ml):	96 ± 3.3%	(n-4)

These samples were chosen because triazolam is absorbed into the body very quickly, so the highest amounts of drug related substances are likely to be excreted within the first couple of hours.

Some of the samples were enzyme hydrolysed according to the procedure previously described for oxazepam hydrolysis. Using C18 cartridges, these samples were extracted along with the untreated samples (Table 20). The unknown peak was still present, and enzyme treatment increased the amount of isolate extracted.

Conclusion

The efficiency of non-polar sorbents for the extraction of triazolam and its metabolites from greyhound urine was good. When columns from the same supplier and same batch were used, reproducibility of extraction was excellent. Triazolam and its metabolites adsorbed well onto extremely non-polar sorbents and were eluted using an extremely non-polar solvent (chloroform).

However, a more selective extraction procedure which could exploit some of the polar nature of the drugs as well as the non-polar character may result in a more selective extraction of triazolam related compounds.

TABLE 20

COMPARISON OF ENZYME HYDROLYSED AND UNTREATED URINE SAMPLES CONTAINING TRIAZOLAM AND EXTRACTED USING C8 CARTRIDGES

Untreated Extracts

Sample	Retention Time (mins.)	Peak Area (mm^2)
Blank	---	---
01 hours	6.0	35
02 hours	6.0	45
03 hours	6.0	26

Treated Extracts

Blank	---	---
01 hours	5.8	370
	5.8	350
02 hours	6.0	988
	6.0	1002
03 hours	5.9	520
	5.9	524

Also, it was possible that the HPLC system described was not sufficiently sensitive to determine any triazolam or metabolites which had been simultaneously extracted from the urine as well as the unidentified substance. The possibility of LC-MS analysis to aid identification of the unknown peak was considered.

The development of an LC-MS compatible HPLC system would then have two advantages over the system incorporating a phosphate buffer:-

a) the identification of the unknown peak

b) the abolition of phosphate buffers, which were causing column blocking and resulting in inadequate sensitivity for triazolam and 1-hydroxy triazolam analysis.

The use of polar sorbents for triazolam extraction was studied and an HPLC system which provided a facility for interfacing with mass-spectrometry as well as increased sensitivity was developed.

2. Xylazine

Xylazine in greyhound urine samples

Recovery of xylazine from greyhound urine samples

The extraction procedure previously described (3.1.2.(2)), was used to extract xylazine from spiked greyhound urine samples:-

C8 columns (3ml capacity) were conditioned with methanol, deionised water and phosphate buffer (pH 10.25) prior to the addition of spiked urine buffered to pH 10.25. The column was washed, dried and the drug eluted with chloroform prior to analysis by HPLC. The recovery of xylazine was calculated from these samples by comparing the peak area obtained after analysis of an extract from a spiked urine sample of known concentration, with that produced by the same concentration of drug in methanol. Each measurement was taken as the average of two determinations.

The positive identification of xylazine was based on comparison with standards, absorbance maxima (225 nm) and retention time.

Initially, a low extraction recovery was obtained (Table 21), however, the efficiency increased with decreasing concentration, suggesting overloading of the column.

Larger C18 columns were then used to determine whether an increase in non-polar interaction capacity would increase extraction efficiency.

TABLE 21.

GREYHOUND URINE SAMPLES (SPIKED WITH XYLAZINE) EXTRACTED USING C8 CARTRIDGES

Concentration (ng/ml)	Peak Area (mm^2)	Percentage recovery
x 0.04 AUFS		
1000	37.5	
1000	32.5	28.4
1000	35.0	
500	40.0	
500	32.0	61.1
500	36.1	
x 0.02 AUFS		
100	35.2	
100	35.0	70.6
100	35.0	

Sample Calculation

1000ng/ml sample is equivalent to 3ug on column (3ml urine sample extracted). Reconstituted in 0.5 ml, then 100% efficiency should equal 3ug/0.5ml = 6ug/ml.

(37.5 + 32 + 35)/31.25/6 (at x 0.04, y = 31.25x), x 100 = 28.4%

Similarly for the other concentrations.

The effect of increased capacity cartridges

The mean xylazine recoveries were low using larger columns with a higher capacity for non-polar interactions (C18;6ml) (Table 22). This was probably due to strong retention of xylazine on the sorbent through non-polar interactions causing difficulties with elution of the drug.

Larger capacity columns require larger volumes of solvent to elute the drugs, consequently requiring an evaporation step. This means that one of the advantages of solid-phase extraction:- the increase in speed of extraction, is lost by the time-consuming need for evaporation.

Changes in extraction procedure

The xylazine extracts were reasonably clean using this procedure, and the water wash did not remove any of the drug suggesting that xylazine is strongly bonded to the sorbent by non-polar interactions.

To improve the cleanliness of the extract further, a methanol wash was incorporated into the extraction procedure.

This resulted in approximately 30% of the drug being eluted with the methanol, therefore this was not included in subsequent extractions.

TABLE 22.

MEAN EXTRACTIONS FOR XYLAZINE FROM GREYHOUND URINE USING C18 COLUMNS

Xylazine concentration (ng/ml)	% Recovery
5000	11.4
1000	21.7
500	17.9

Since xylazine is very soluble in acetone, this was evaluated as an alternative eluent to chloroform. However, acetone did not remove xylazine from the sorbent. This inability to break non-polar interactions was probably due to its very polar nature.

Elution, then, is not a function of isolate solubility in an eluent, but is dependant on interaction cleavage between sorbent and isolate.

Direct injection of the eluent (chloroform), onto the HPLC analysis system, was attempted in an effort to remove the evaporation step and shorten the time prior to analysis. The chloroform took approximately 5 minutes to be totally eluted from the HPLC column resulting in an extremely wide solvent front and hence poor chromatography. The evaporation of the extract followed by reconstitution in methanol were retained in the procedure.

The C8 columns gave better all round cleanliness of extracts and extraction efficiency, so the method was not modified.

Repeated extractions gave somewhat varied results, the assay giving only 80.3 ± 14% reproducibility at 100ng/ml concentration over a period of three days.

Assuming 80% efficiency, actual samples taken from the greyhounds were extracted and analysed.

Extraction of greyhound samples

Quantitation

The greyhounds were each given an intramuscular injection of xylazine (0.05ml/kg) and urine samples were collected at timed intervals following administration.

The quantitation of parent xylazine from greyhound urine samples was carried out using the previously calculated peak area ratios (Table 8). Parent xylazine concentrations were calculated (Table 23).

Each greyhound was dosed three times allowing sufficient time between dosings for all related compounds to have been excreted from the animal.

For all greyhounds, the peak excretion time was between 2 and 3 hours. No parent xylazine was detected in any of the dogs more than eight hours after administration.

Greyhound 1.

Between the second and third dosings (a period of four weeks), greyhound number one lost weight (37kg to 32.4kg). There was no apparent reason for this.

The first two administrations gave similar results regarding excretion pattern. The total values (taking into account the amount of urine produced) show a high xylazine level after two hours, a marked drop after three and a peak excretion level after four.

TABLE 23.

AMOUNT OF XYLAZINE EXCRETED DETERMINED BY C8 EXTRACTION PROCEDURES AND HPLC ANALYSIS

Greyhound Number / Time after dosing (hours)	1	2	3	4
	Xylazine excreted (ug/ml)			
2	0.05-0.07	0.19-0.24	0.10-0.12	0.41-0.51
	0.03-0.03	0.02-0.03	0.26-0.33	0.19-0.24
	0.41-0.51	0.20-0.25	0.21-0.26	0.13-0.17
3	0.05-0.06	0.12-0.14	0.15-0.18	0.19-0.24
	0.02-0.03	0.05-0.07	0.09-0.12	0.19-0.24
	0.42-0.53	0.15-0.18	0.18-0.22	0.18-0.22
4	0.12-0.15	0.12-0.15	0.06-0.08	0.14-0.18
	0.02-0.03	0.06-0.07	0.03-0.03	0.21-0.26
	0.16-0.20	0.13-0.17	0.12-0.14	0.30-0.38
5	0.13-0.16	0.05-0.06	0.02-0.03	0.04-0.05
	0.03-0.04	0.03-0.04	0.02-0.36	0.10-0.12
	0.14-0.18	0.08-0.10	0.12-0.15	0.27-0.34
6	0.03-0.04	0.03-0.04	0.03-0.03	0.02-0.03
	----------	0.01-0.01	0.01-0.02	0.08-0.10
	0.04-0.05	0.04-0.05	0.06-0.07	0.26-0.32
7	0.02-0.02	---------	---------	0.02-0.02
	----------	---------	---------	0.07-0.09
	----------	0.02-0.03	---------	0.1-0.13
	Total xylazine excreted (ug)			
2	16.5-20.8	5.85-7.3	14.3-17.8	6.0-7.5
	12.0-14.7	1.6-2.0	81.6-102	1.9-2.4
	104-130	5.6-7.0	25.4-31.7	10.6-13.3
3	3.8-4.8	11.0-13.7	33.5-42.2	2.0-2.5
	0.7-0.8	5.7-7.2	22.1-27.7	4.7-5.8
	50.8-63.5	12.3-15.3	9.6-12.0	9.1-11.4
4	34.6-43.2	9.8-12.3	5.2-6.5	30.7-38.5
	9.5-11.9	3.5-4.4	10.5-13.1	6.6-8.3
	23.7-29.6	6.4-7.9	6.35-7.9	15.7-19.6
5	25.4-31.7	6.0-7.5	2.9-3.7	19.0-23.7
	1.4-1.7	3.9-4.9	6.3-7.9	2.9-3.6
	5.5-6.9	2.5-3.2	6.1-7.6	30-37.5
6	3.3-4.1	1.0-1.3	1.9-2.3	10.8-13.5
	--------	0.4-0.4	2.1-2.7	2.5-3.1
	2.04-2.5	0.8-1.1	5.2-6.5	29.2-36.5
7	2 4-2.9	---------	---------	6.1-7.6
	---------	---------	---------	0.7-0.9
	---------	0.2-0.32	---------	1.0-1.3

However, the third administration, which involved a much lower dose of xylazine, gave higher excretion values, peaking after two hours.

The total amount of xylazine excreted within 7 hours of dosing for this dog was not consistent:

Administration 1: 0.39→0.49 ug/ml excreted in 7hrs.

Administration 2: 0.1→0.13 ug.ml excreted in 7 hrs.

Administration 3: 1.17→1.49 ug/ml excreted in 7 hrs.

This corresponded to 0.05%, 0.02% and 0.14% of the dose being excreted within 7 hours.

Greyhound 2.

In all dosings for this greyhound, the peak excretion time was 3 hours.

The total amount of xylazine excreted within 7 hours of administration was also consistent, although the second administration gave somewhat lower values:

Administration 1: 0.52→0.65 ug/ml excreted in 7 hrs.

Administration 2: 0.17→0.21 ug/ml excreted in 7 hrs.

Administration 3: 0.61→0.77 ug/ml excreted in 7 hrs.

This corresponded to 0.03%, 0.01% and 0.02% of the dose respectively being excreted in the first 7 hours.

Greyhound 3.

The peak excretion time for this greyhound (the only female) was three hours in the first administration, and two hours in the other two. The total excretion amounts are fairly consistent and compare well with results from other greyhounds:

Administration 1: 0.35→0.44 ug/ml excreted in 7hrs.

Administration 2: 0.41→0.51 ug/ml excreted in 7 hrs.

Administration 3: 0.61→0.77 ug/ml excreted in 7 hrs.

This corresponded to 0.04%, 0.092% and 0.04% of the dose respectively being excreted in the first 7 hours.

Greyhound 4.

The results for greyhound 4 were the least consistent, although one of the total excretion values within 7 hours (administration 1) compares well with results from other dogs.

Overall excretion values :

Administration 1: 0.67→0.86 ug/ml excreted in 7 hrs.

Administration 2: 0.83→1.04 ug/ml excreted in 7 hrs.

Administration 3: 1.24→1.55 ug/ml excreted in 7 hrs.

This corresponded to 0.05%, 0.01% and 0.06% of the dose respectively being excreted in the first 7 hours.

Greyhound 4 was the heaviest greyhound and so received the highest dose of xylazine. Its excretion values being higher than the other greyhounds is not entirely unexpected.

Overall, the percentage of the total dose exceted within 7 hours is reasonably consistent between dogs and between dosings, but there is a wide variation in the actual amount excreted.

The mean excretion values, within 7 hours is 0.67±60.4% ug/ml.

Inconsistency of results

a) Medium

Greyhound urine is a very difficult medium to work with because of the inconsistency of the nature of the sample and the high salt content. The large differences in the amount of urine excreted by the greyhounds at given time intervals affects the efficiency of extraction because of the salt content level. This can be compensated for somewhat by dilution of the sample with water or buffer (see next section).

To a certain extent, greyhound metabolism can be regulated, for example, by controlling the diet and exercise routine.

Consistency within a greyhound, if not between dogs would certainly be expected.

b) Extraction columns

While extracting two sets of samples, it was noted that the packing material in the columns was not even between cartridges. These were columns supplied by the same manufacturer and a comparison with the products of other companies showed there to be major differences in amount of packing material present.

Closer investigation showed that solvent flow through the columns under vacuum differed considerably within the same batch of columns, as well as between columns from different suppliers. This was also observed during the extractions of triazolam.

Furthermore, since it was not feasible to use a cartridge more than once (possibility of inadequate elution and hence carry-over) several batches had to be used in these extractions. This helps to explain some of the inconsistencies in both extraction recovery and greyhound administration.

As with triazolam, it was found that only columns from the same manufacturer should be used, preferably from the same batch, for related extractions.

Conclusion

The efficiency of non-polar sorbents for the extraction of xylazine from greyhound urine was good although not very reproducible. Xylazine was strongly adsorbed onto extremely non-polar sorbents and could not be eluted using an extremely non-polar solvent (chloroform).

The less polar C8 columns allowed good xylazine extractions at low concentrations (100ng/ml) but again extraction procedures were not very reproducible.

A more selective extraction procedure which could exploit some of the polar nature of xylazine as well as its non-polar character may result in a more selective extraction for xylazine.

The use of columns from the same batch and the same supplier may also result in more reproducible data.

A study of polar sorbents for xylazine extraction was then conducted.

4.2.2. Extraction Methods exploiting Polar Sorbent Interactions

1. Benzodiazepines

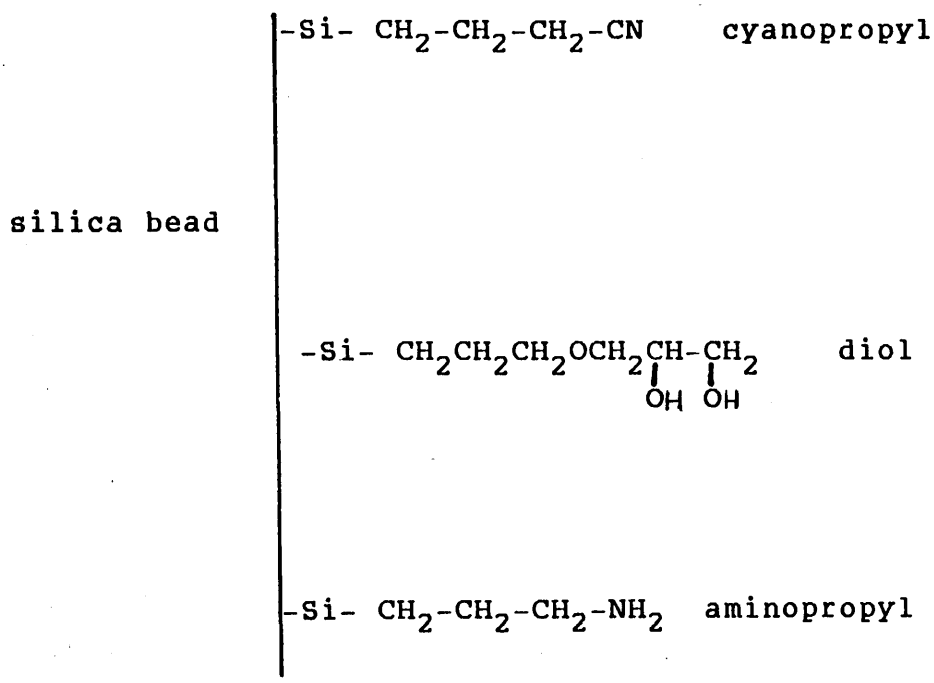
Polar interactions are exhibited by many different sorbents and functional groups on isolates. Polar interactions include hydrogen bonding, dipole/dipole, induced dipole/dipole, pi-pi and other interactions in which the distribution of electrons between individual atoms in the functional groups is unequal, causing positive and negative polarity. This property allows an isolate molecule bearing a polar functional group to interact with a polar group on the sorbent. Groups that exhibit this type of interaction include aromatic rings and groups containing hetero-atoms such as oxygen, nitrogen, sulphur and phosphorus.

Xylazine and triazolam both contain nitrogen atoms which could be used to interact selectively with the polar group on the sorbent (Figure 17). Retention of these drugs on the sorbent is also through the non-polar interaction of the side-chain of the sorbent with the non-polar character of the drug.

Because of the polar nature of the silica substrate (and especially of unbonded silanol groups), polar interactions are characteristic of all bonded silicas. (Van Horne et al., 1985).

FIGURE 17.

Primary interactions of a polar sorbent.



Evaluation of polar sorbents

All three common polar sorbents were studied.

Cyanopropyl (CN), aminopropyl (NH2) or diol (2OH) cartridges (1ml;100mg), were conditioned with methanol and deionised water. Blank greyhound and human urine samples, spiked with triazolam (500 ng/ml)(1ml) + deionised water (pH7, 1ml), were added to the columns and washed with deionised water.

The drug was eluted with methanol, to pH4 with hydrochloric acid or to pH10 with ammonium hydroxide (Appendix A1.6.).

The eluent was directly analysed by HPLC and the percentage recovery of the drug determined by comparison of peak areas obtained with peak areas produced by standard solutions of triazolam in methanol.

The efficiency of extraction of the three sorbents is shown in table 24. The diol cartridges were the least efficient regardless of eluent (maximum 25%), and both cyanopropyl and aminopropyl sorbents showed higher efficiencies of extraction using an acidic eluent (97-100%) rather than a basic eluent (55-72%). Cyanopropyl columns gave the highest extraction recoveries (100%), so these were chosen for subsequent extractions.

TABLE 24.

PERCENTAGE RECOVERIES OF TRIAZOLAM (1ug/ml) FROM SPIKED GREYHOUND AND HUMAN URINE USING POLAR SORBENTS.

Column		Greyhound Urine	Human Urine
Cyanopropyl:	Acidic eluent	100 ± 2.3%	100 ± 2.1%
	Basic eluent	65 ± 1.5%	72 ± 2.0%
Aminopropyl:	Acidic eluent	98 ± 0.5%	97 ± 2.0%
	Basic eluent	55 ± 5.2%	62 ± 6.3%
Diol:	Acidic eluent	16 ± 3.0%	22 ± 3.1%
	Basic eluent	18 ± 5.0%	25 ± 4.2%

Functional group interaction

These results can be explained by considering the functional group interactions of the drug with the sorbents (Tippens, 1987).

At pH7, the silanol groups on the surface of the solid phase cartridge which are not bonded to the side-chain are negatively charged.

At the same pH, basic drugs, will be positively charged.

The drug is retained on the column, due to non-polar interactions between the hydrocarbon side chain and the hydrocarbon character of the drug, the ionic interaction between the charged sections and the dipole moment present between the nitrogen groups in the drug and the polar groups on the surface of the sorbent.

The non-polar interactions are primary interactions and the polar interactions secondary. The ion-exchange interactions are the weakest form of interaction in these particular sorbents.

In order to disrupt the retention, that is, to elute the drug, it is necessary to neutralise either the charge on the drug by passing a strongly basic methanolic solution through the sorbent, or to neutralise the charge on the silanol groups by passing an acidic methanolic solution through the sorbent.

Methanol has sufficient non-polar character to disrupt the non-polar interactions present, since these are not as strong as on completely non-polar sorbents (previous section).

Interactions

In the case of the diol (Figure 17), the ionic interactions are so strong, occurring along the side-chain as well as at the silanol groups, that the pH4 eluent is not sufficiently strong to break the side-chain interaction. At a higher pH, for example pH14, the NH_4+ ionisation would be suppressed. However, the use of high pH values is not recommended with these cartridges.

The advantages that the aminopropyl and cyanopropyl cartridges have over the diol columns can be explained by noting that the functional group interaction does not occur directly. The main retention is caused by the non-polar interactions of the side-chain with the non-polar character of the drug. The dipole moment of the charged cyano group at the end of the side-chain attracts the unequally shared electrons in the drug, for example, lone pairs of electrons or dipole moments (C≡N; C=O etc.) so aiding specific retention.

Specific retention is also aided by ionic interaction between the silanol groups and the charged nitrogen atoms in the drug.

Deactivation of silanol groups

End-capping of the sorbents is a process which deactivates some of the silanol groups on the surface of the sorbent. Cyanopropyl cartridges are not subjected to this process during synthesis but aminopropyl columns are (Van Horne et al., 1985).

The CN cartridges are un-endcapped, and therefore have more silanol groups available for retention while the NH_2 cartridges are endcapped which explains the slightly higher recovery from the CN cartridges.

Elution solvents

Finally, the elution using the acidic methanol medium was found to be more efficient than basic methanol (Table 24).

This can be explained by considering the suppression of ionisation within the cartridge. The acidic elution which suppresses the ionisation of the silanol groups is more even, because of the similar pKa value of each silanol group. Therefore, this eluent is more efficient than the basic eluent which attempts to suppress ionisation of nitrogen molecules in the drug which have widely differing pKa values.

The extraction procedure employing polar sorbents is particularly pH dependant. The pH simply being 'basic' or 'acidic' is not sufficiently accurate.

The pH is critical because, in order for the maximum interaction between sorbent and isolate to occur, the pH must be sufficiently high so as to ionise the sorbent, and sufficiently low so as to ionise the weakly basic benzodiazepines. The pH of the eluting solvent is also critical since it must be sufficiently acidic to neutralise the charge on the silanol groups all over the sorbent.

TRIAZOLAM AND ITS METABOLITES

Triazolam and its metabolites in greyhound urine samples

Triazolam and its metabolites were determined from spiked greyhound urine samples using CN columns conditioned with methanol and deionised water. The urine sample, buffer (pH 7), and diazepam were applied to the column and drawn through, dried and washed with deionised water. (Diazepam was incorporated as an internal standard (1.5ug/ml). All previous use of a standard had been an external use to ensure HPLC reproducibility of injection).

The drug was eluted with methanol, to pH4 with hydrochloric acid.

The percentage recovery of triazolam and its metabolites were determined from spiked greyhound urine samples, using this procedure, by comparing the peak area obtained after injection of an extract from a spiked urine sample of known concentration, with that produced by the same concentration of drug in methanol. Each measurement was taken as the average of two determinations (Table 25(a)).

The recovery of 4-hydroxy triazolam was particularly poor. The wash solution was collected and analysed from the 4-hydroxy triazolam: 21.3% of the drug standard was found to elute with the deionised water during washing. The polar deionised water probably causes disruption of the polar interactions present, suggesting that the 4-hydroxy metabolite is more strongly held by polar interactions than either the parent triazolam or its 1-hydroxy metabolite.

The wash stage was discarded and the eluent was altered to methanolic hydrochloric acid (pH4)-acetonitrile (50:50 v/v). This being a less polar eluent, it would, theoretically, help to break any non-polar bonding present between the sorbent and the isolate.

The result of these changes was such that the extraction efficiency was greatly improved for the 4-hydroxytriazolam. However, no real change occurred in the extraction efficiencies for the other drugs (Table 25(b)).

TABLE 25.

25(a).

EXTRACTION EFFICIENCY FOR TRIAZOLAM AND 1-HYDROXY TRIAZOLAM FROM GREYHOUND URINE USING CN CARTRIDGES

Triazolam	(1ug/ml):	100%
	(0.5ug/ml):	101%
1-hydroxytriazolam	(1ug/ml):	74.4%
	(0.5ug/ml):	77.6%
4-hydroxytriazolam	(1ug/ml):	41.1%
	(0.5ug/ml):	32.1%

25(b).

MODIFIED EXTRACTION PROCEDURE FOR 4-HYDROXY TRIAZOLAM

Triazolam	(1ug/ml):	100%
	(0.5ug/ml):	100%
1-hydroxytriazolam	(1ug/ml):	73.2%
	(0.5ug/ml):	78.9%
4-hydroxytriazolam	(1ug/ml):	101.1%
	(0.5ug/ml):	91.4%

Polar sorbent extraction of greyhound samples followed by HPLC analysis incorporating an acetate buffer

A more sensitive, LC-MS compatible HPLC system incorporating an acetate buffer was developed for the analysis of triazolam and its metabolites.

The same urine samples collected from the greyhounds following the administration of 1 x 0.5mg tablet of triazolam were extracted using cyanopropyl columns and analysed by HPLC using this system.

The unidentified peak previously observed in the non-polar extracts was not present in these extracts. The peak was not a system peak, since it was not present in the blank urine from the same dogs.

Therefore, as the unidentified peak was not present in the HPLC system incorporating an acetate buffer, identification of the unknown by LC-MS was not possible.

The only difference in the extraction of the urine samples between non-polar and polar columns was that the non-polar extracts had been analysed using a mobile phase containing a phosphate buffer whereas the polar extracts were analysed using a mobile phase containing an ammonium acetate buffer.

Therefore, the substance causing the peak was being produced either during the extraction, hydrolysis or analysis of the urine sample.

Polar sorbent extraction of greyhound samples followed by HPLC analysis incorporating a phosphate buffer

To try to determine the origin of this peak, the urine samples were extracted with polar (CN) cartridges and analysed on HPLC using a phosphate buffer.

In the three and four hour samples, this unidentified peak was again observed, therefore both the extraction and the hydrolysis of the samples did not affect triazolam or its metabolites. The presence of the unknown peak occurred only when a phosphate buffer was incorporated into the HPLC system. A possible explanation for this, is that during the analysis, the triazolam metabolites were converted to a substance with a shorter retention time on the system incorporating a phosphate buffer, possibly a drug:phosphate complex.

To avoid this problem, greyhound samples were analysed using polar columns and an HPLC system incorporating acetate buffer as previously described.

Extraction of greyhound urine samples

Cyanopropyl columns were employed in the extraction of urine samples collected from the greyhounds following the administration of 1 x 0.5mg tablet of triazolam. Each dog was dosed twice, allowing sufficient time between dosings for all the drug to have been excreted.

The results obtained from greyhound dosing show a wide variation between dogs (Table 26).

1-hydroxy and 4-hydroxy metabolites were detected in some of the dogs (Figure 18), but not all.

Parent triazolam was detected in the urine of one of the greyhounds up to eight hours after dosing, but in the other two, triazolam was only detected up to two hours post-administration. Therefore, mean values, for the latter portion of the graph (Figure 19) are based on results from one greyhound only.

There was a wide variation between dogs, but each dog showed a consistent response to dosings. The linear plot of triazolam and its metabolite concentration (uM x 10-4) versus time post-administration (Figure 19), suggests a rapid conversion of triazolam into 1-OH and 4-OH triazolam on administration, and the continuous conversion throughout the excretion phase for 1-OH. 1-OH triazolam is excreted more quickly than triazolam. This is to be expected since it is more water soluble, and its concentration appears to be dependant on triazolam concentration.

However, this is not true of 4-OH triazolam. A rapid conversion into 4-OH triazolam on administration gives a maximum concentration time of 2 hours and its elimination is complete before that of triazolam and 1-hydroxy triazolam, suggesting that it is not continuously formed in the body.

TABLE 26.

AMOUNT OF TRIAZOLAM AND ITS METABOLITES DETERMINED IN GREYHOUND URINE USING CN CARTRIDGES

Hours after administration	Triazolam (ug/ml)						Mean (uM x 10-4)
	1	2	3	4	5	6	
1	0.023	0.023	0.073	0.05	0.057	0.071	1.4
2	0.027	0.231	0.047	0.041	0.045	0.42	1.1
3	0.026	0.025					0.74
4	0.026	0.023					0.68
5	0.027	0.021					0.67
6	0.016	0.018					0.47
7	0.011	0.013					0.34
8	0.01	0.01					0.28
	1-OH Triazolam (ug/ml)						
1	0.06		0.035	0.067			1.5
2	0.05	0.012	0.034	0.065			1.1
3	0.04	0.04	0.035	0.063	0.042		1.2
4	0.03	0.026	0.007	0.051	0.027		0.8
5		0.01		0.04	0.02		0.65
6				0.01	0.018		0.53
7					0.016		0.45

The female greyhound was on heat at the time of one dosing so only one set of urine samples was obtained from this greyhound.

	4-OH Triazolam (ug/ml)				
1	0.026		0.08	0.081	1.7
2	0.04	0.013	0.18	0.092	2.4
3	0.037	0.03	0.04	0.06	1.2
4	0.026	0.036	0.03	0.04	0.92
5	0.023	0.043	0.03	0.02	0.81
6	0.019	0.013	0.02	0.01	0.43
7	0.003	0.006	0.01	0.00	0.18

4-OH Triazolam was only detected in the urine of two of the greyhounds.

HPLC system: C18 column; mobile phase methanol-ammonium acetate buffer (pH8)-deionised water- acetonitrile (200:100:125:75, v/v); detection wavelength 230nm.

FIGURE 18.

Triazolam and metabolites extracted from greyhound urine - 3 hours after a single oral dose of triazolam - using CN columns and HPLC analysis.

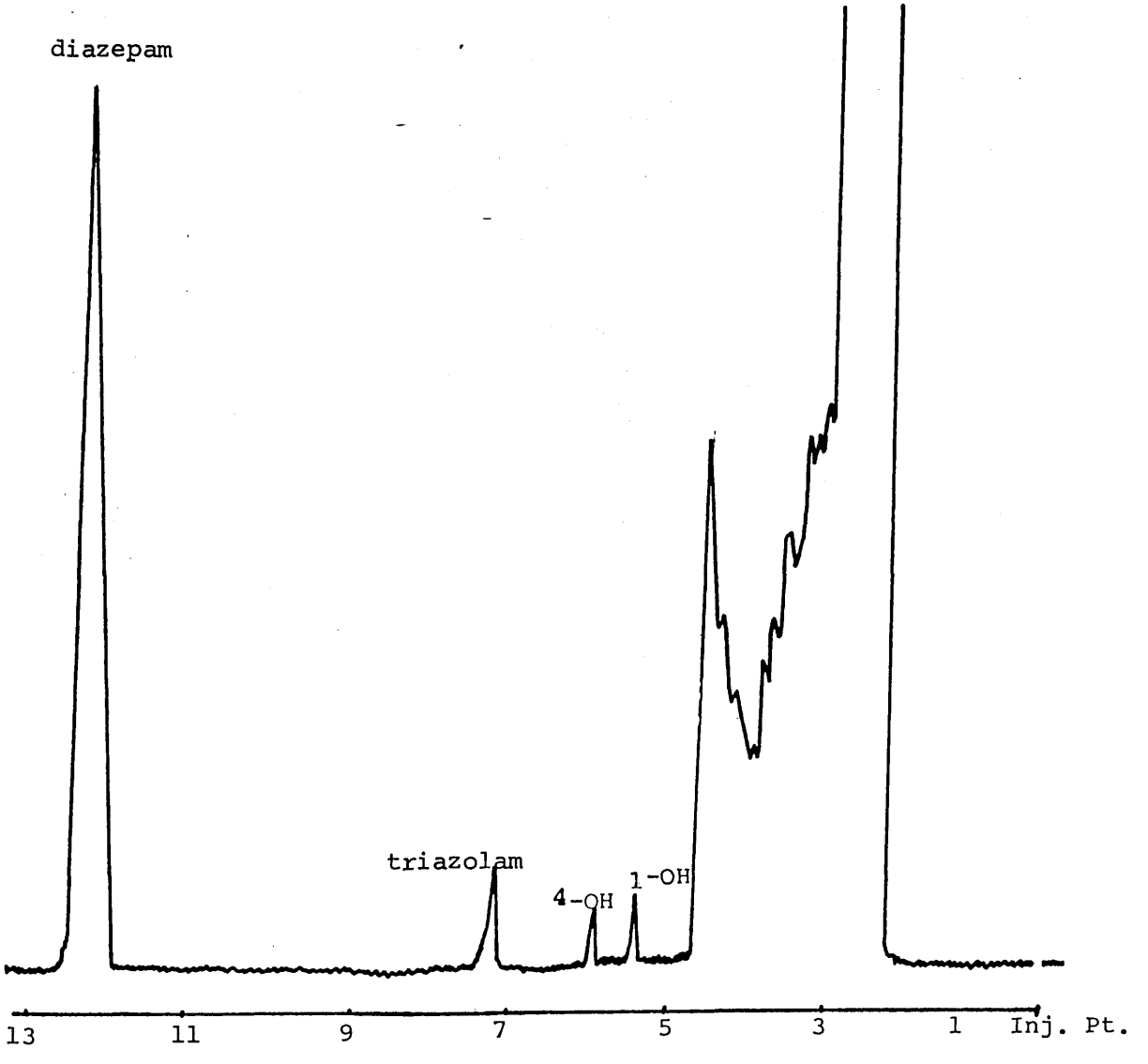
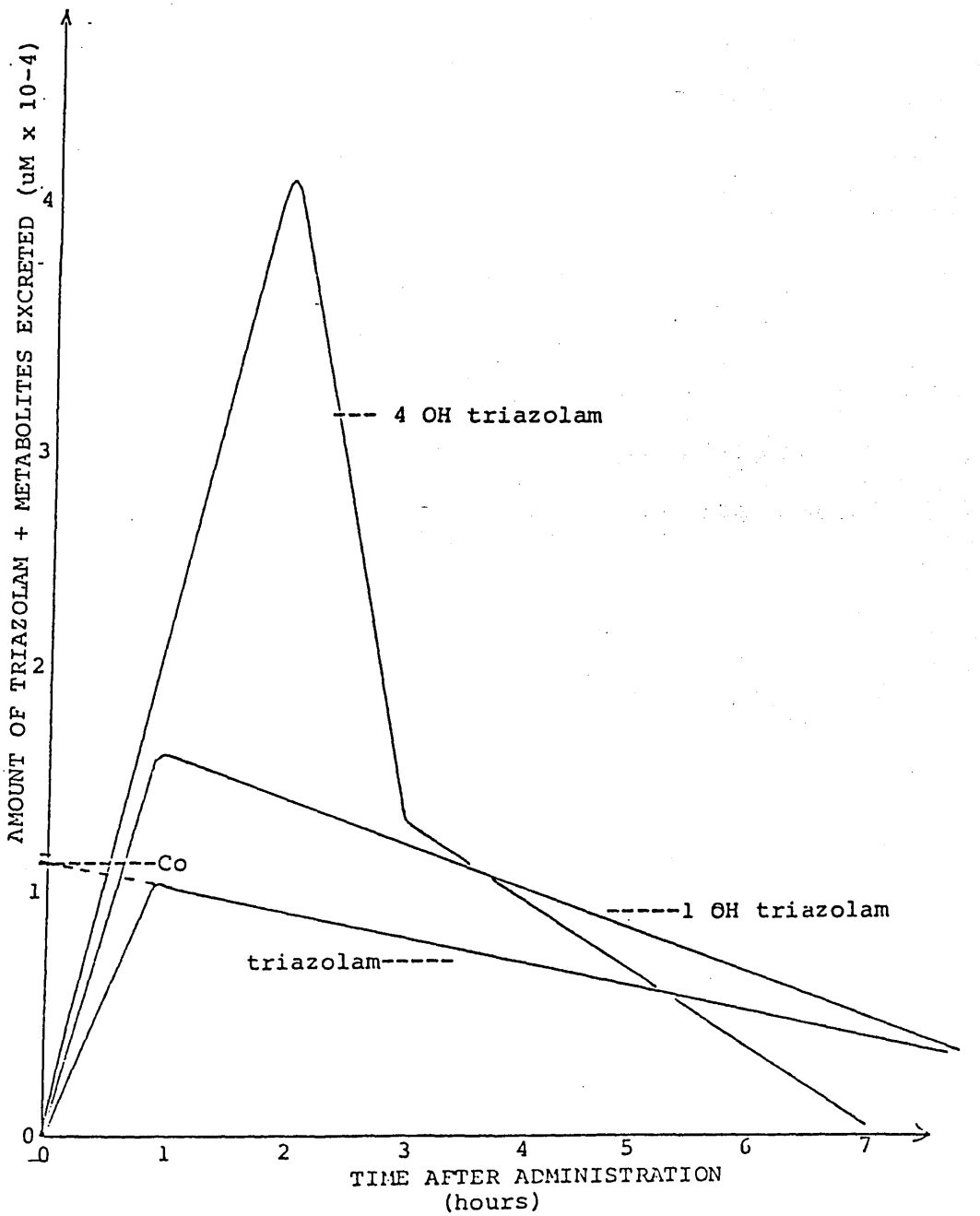


FIGURE 19.

Average triazolam and metabolite excretion from greyhound urine.



This also suggests that it is more water soluble and hence more polar than the other metabolite or parent triazolam since it is excreted quickly.

This agrees with the earlier observation that the 4-hydroxy metabolite was removed from the bonded sorbent by washing to a greater extent than either the 1-hydroxy metabolite or parent triazolam.

The fact that 4-OH triazolam was not detected at all in the urine of one of the greyhounds may suggest very rapid elimination of this metabolite.

Pharmacokinetics

Figure 20 shows a semi-logarithmic plot of the concentrations of triazolam and its metabolites (ug/ml) versus time after administration. Pharmacokinetic parameters are difficult to measure when a drug is as extensively metabolised as triazolam.

Parameters for the parent drug and its metabolites were calculated using mean values (Appendix 2) (Table 27), and were compared with literature values for beagle dogs (dose 0.5mg/kg) (Eberts, 1977).

In the greyhound, triazolam is absorbed at a faster rate than it is eliminated, as would be expected. The elimination rates of both metabolites are much faster than for the parent drug, and are similar to the elimination rates in beagles.

FIGURE 20.

Semi-logarithmic plot of triazolam and its metabolites excreted from greyhound urine.

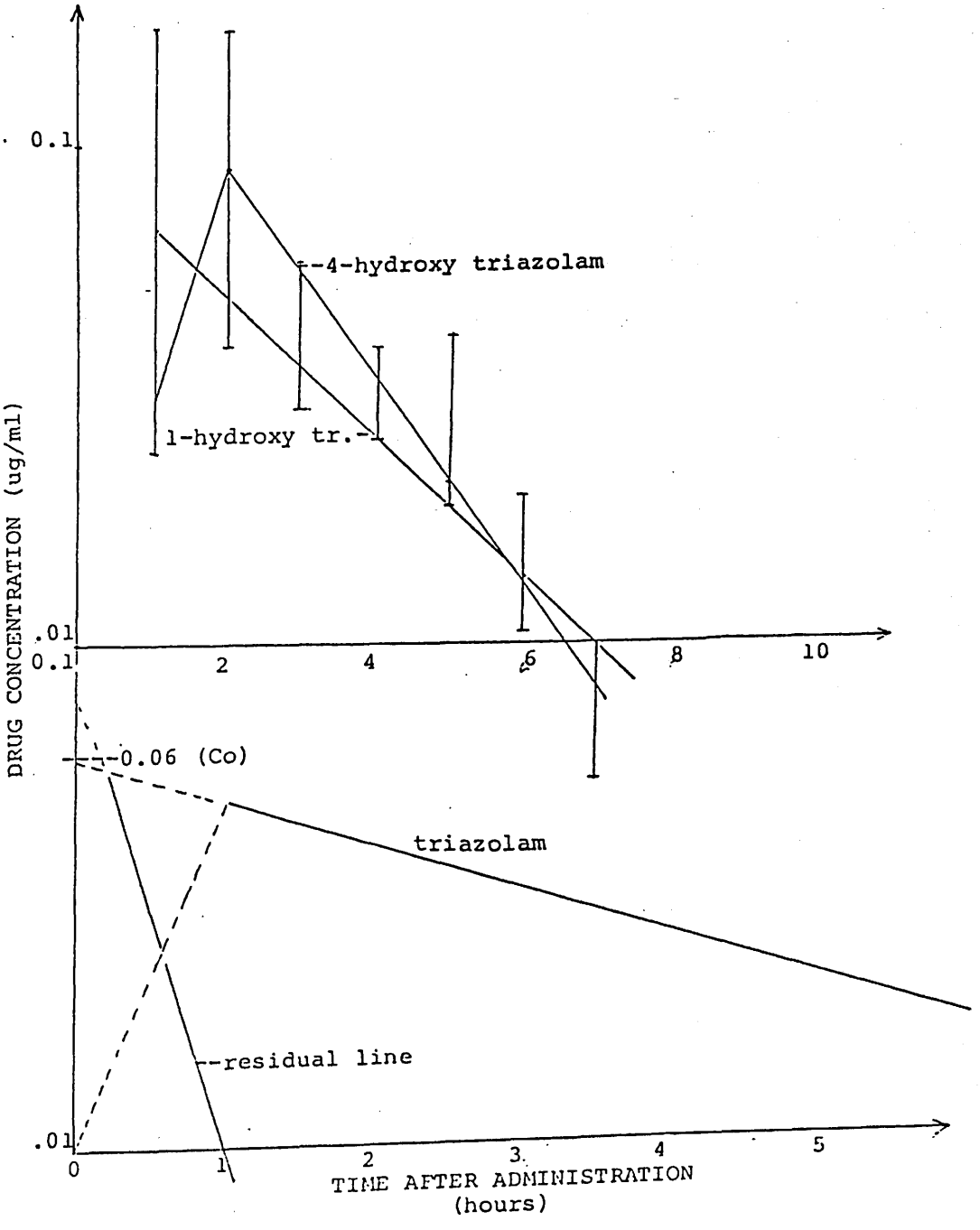


TABLE 27.

PHARMACOKINETIC PARAMETERS FOR TRIAZOLAM AND ITS METABOLITES IN THE URINE OF RACING GREYHOUNDS

Co = Apparent value for urine concentration at zero time.

$T_{0.5}$ = Time taken for the concentration of drug in urine to decline to half its original value.

Vd = Volume of distribution = dose/Co.

k_{el} = Rate of elimination = 0.693/ $T_{0.5}$.

Clu = Clearance rate = Vd x k_{el}.

AUC = Area under curve = Co/k_{el}.

k_{ab} = Rate of absorption = residual line slope x 2.303

$T_{0.5}ab$ = Half-life of absorption = $0.693/k_{ab}$.

Fraction of oral dose absorbed, F (bioavailability)
$F = ((Co/k_{el}) - (Co/k_{ab}))/AUC$.

Triazolam

	Greyhounds (n=3) (dose = 500 ug)	Domestic Dogs (dose = 5000 ug)
Co (ug/ml)	0.06	0.131±65
$T_{0.5}$(hrs)	3.3	0.85
Vd (litres)	8.3	not given
k_{el}(hrs-1)	0.21	0.82
Clu (l/hr)	1.7	not given
AUC	0.208	0.29
k_{ab}(hrs-1)	2.07	not given
$T_{0.5}ab$(hrs)	0.34	not given
F (%)	88	not given

	1-OH Triazolam	4-OH Triazolam	Metabolites (lit.)
k_{el}	0.350	0.462	0.398
$T_{0.5}el$	2.0	1.5	1.74

Overall, greyhounds excrete the parent drug much more slowly than beagles, although this may be accounted for to an extent by the dose difference.

This suggests that the metabolism of triazolam in racing greyhounds, is different to that in beagles. Comparison with the literature is difficult, since there are no available studies which use racing greyhounds as subjects, and metabolic pathways for triazolam excretion vary widely between animal species (Kitigawa et al., 1979). Greyhound metabolism is likely to differ from that of domestic dogs due to the very low fat content of greyhounds.

Triazolam and its metabolites were not detected in urine 12 hours after administration.

Triazolam and its metabolites in human urine samples

Four healthy female volunteers (average age: 26; average weight: 67 kg) were each administered a single tablet (1 x 0.25mg) of triazolam. The subjects abstained from alcohol and nicotine twenty-four hours before and after administration. Urine samples were collected at timed intervals after administration and were stored at -20^{o}C until required for extraction.

The samples were extracted using polar columns according the extraction procedure above.

Extraction of human urine samples

The extracts were sufficiently clean for direct injection onto the HPLC system and the peaks observed in the chromatograms corresponded to the retention times of 1 and 4-hydroxy triazolam standards, the main metabolites of triazolam (Figure 21).

The overall profiles (Table 28; Figure 22) between volunteers are in good agreement. Triazolam and 1-OH triazolam levels peaked after 2 hours then declined exponentially over the next 10 hours. 4-OH triazolam concentration increased up to five hours and none was detected after twelve hours, but there was insufficient data for any meaningful pharmacokinetic parameters to be calculated for this metabolite from this data.

Pharmacokinetics

Figure 23 shows a semi-logarithmic plot of triazolam and 1-OH triazolam concentrations (ug/ml) versus time after administration (mean values). Pharmacokinetic parameters for parent triazolam were calculated (Table 29) and compared to published data (Smith et al., 1986).

Smith's data was obtained from male subjects (average weight 77kg) following a dose of 500ug of triazolam. This study involved females (average weight 68kg) who were each administered 250ug triazolam.

FIGURE 21.

Triazolam and metabolites extracted from human urine - 3 hours after a single oral dose of triazolam - using CN columns and HPLC analysis.

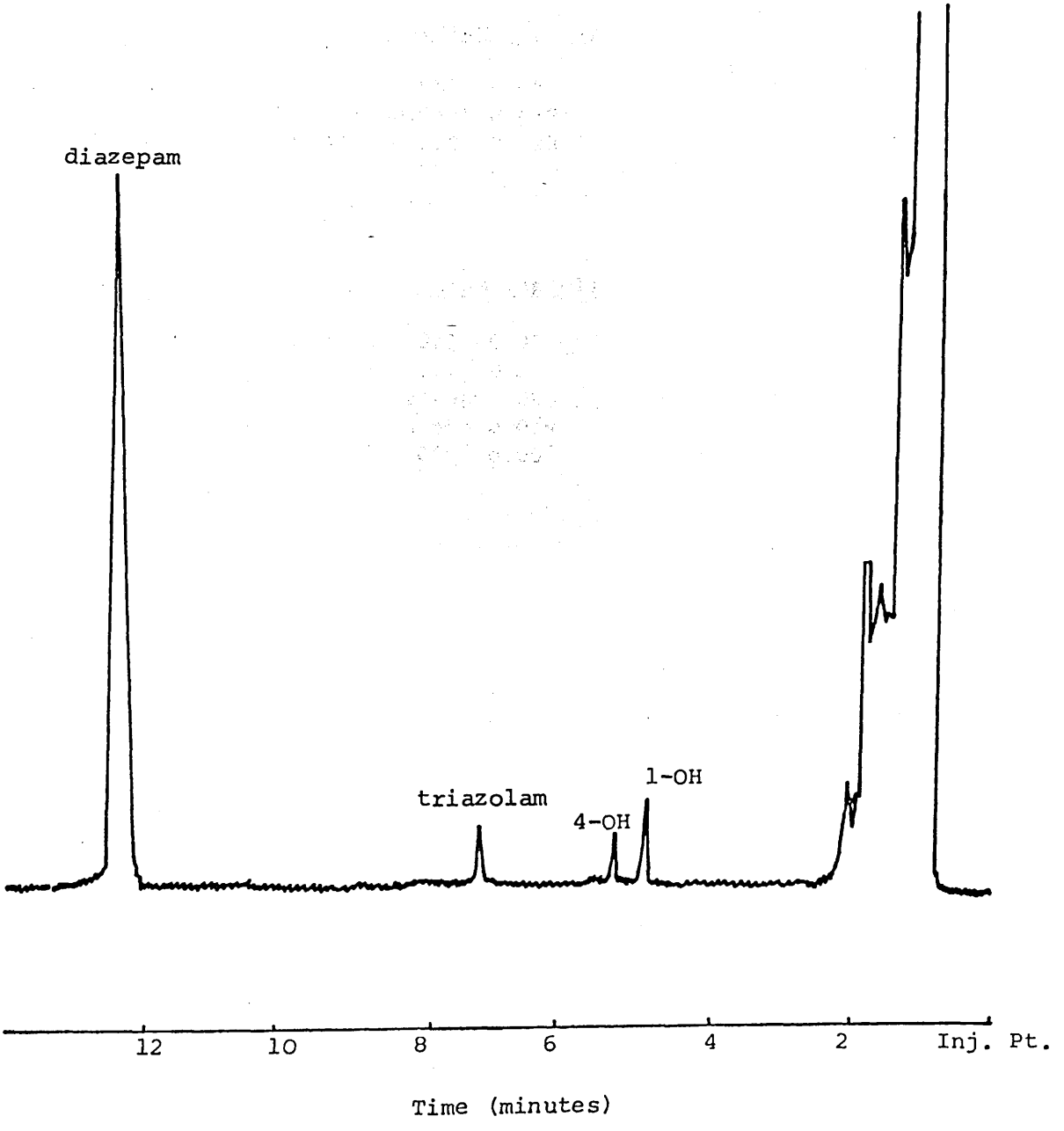


TABLE 28.

AMOUNT OF TRIAZOLAM AND ITS METABOLITES DETERMINED IN HUMAN URINE USING CN CARTRIDGES

Hours after administration	Triazolam (ug/ml)				Mean (uM x 10-4)
	1	2	3	4	
2	0.02	0.045	0.0272	0.035	0.98
3	0.013	0.025	0.0237	0.0272	0.65
4	0.013	0.012	0.01	0.02	0.34
5	0.013	0.008	0.0084	0.01	0.27
12	0.00	0.00	0.00	0.00	0.00
	1-OH Triazolam (ug/ml)				
2	0.062	0.082	0.051	0.047	1.7
3	0.041	0.034	0.032	0.08	1.3
4	0.041	0.032	0.023	0.07	1.2
5	0.022	0.031	0.022	0.025	0.698
12	0.012				0.34
	4-OH Triazolam (ug/ml)				
2	0.026	0.021	0.02		0.6
3	0.0325	0.032	0.03		0.9
4	0.04	asleep	0.040		1.2
5	0.06	asleep	0.039		1.5
12	0.00	0.00	0.00		0.00

FIGURE 22.

Average triazolam and metabolite excretion from human urine.

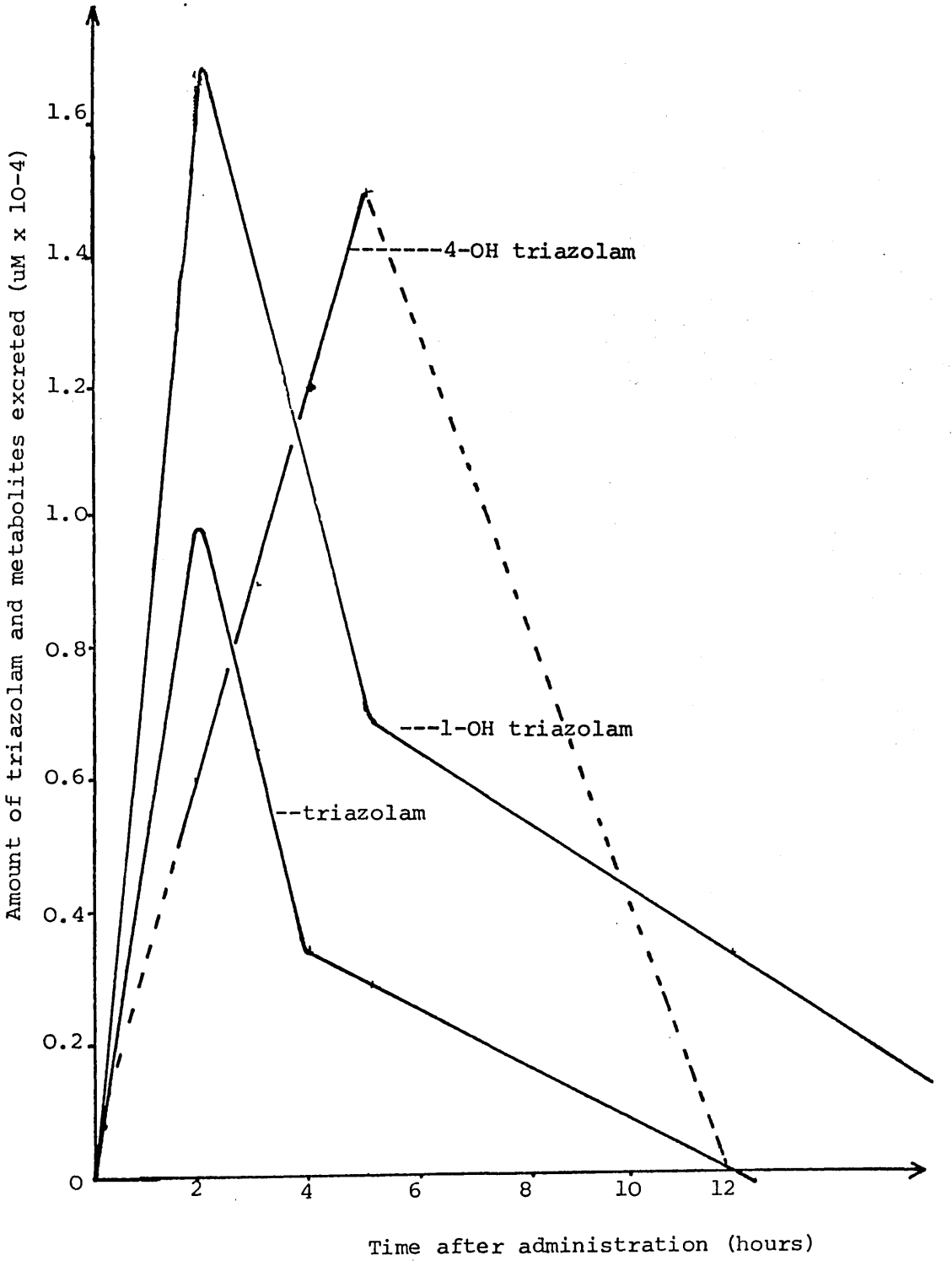


FIGURE 23.

Semi-logarithmic plot of triazolam and its metabolites excreted from human urine.

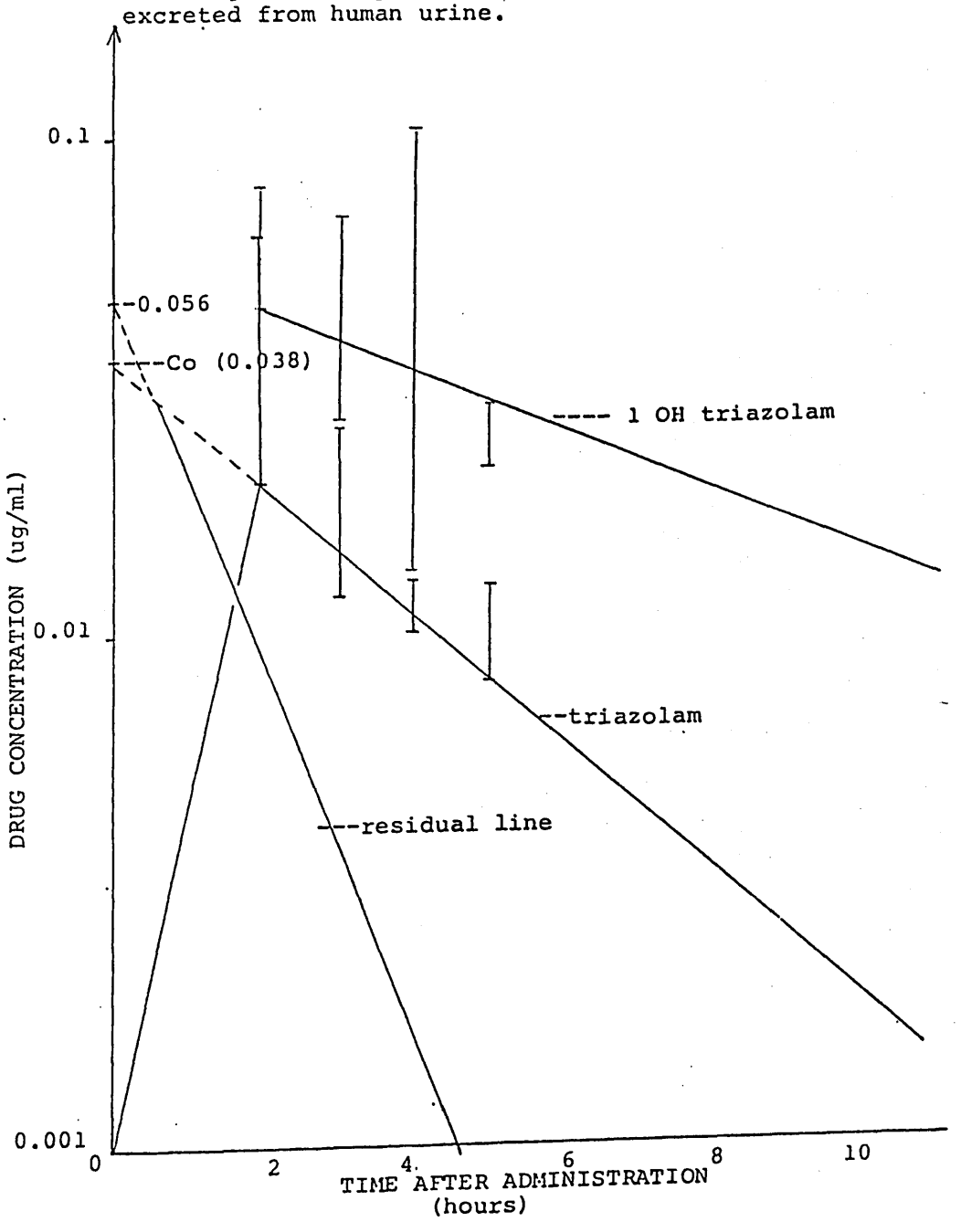


TABLE 29.

PHARMACOKINETIC PARAMETERS FOR TRIAZOLAM AND ITS METABOLITES IN HUMAN URINE

Triazolam

	Calculated data (dose=250 ug)	Published data (dose=500 ug) (Smith et al., 1986)
Co (ng/ml)	38	3.99±1.47
$T_{0.5}$(hrs)	2.3	2.94
Vd (litres/kg)	0.094	1.31±0.39
k_{el}(hrs-1)	0.30	0.24
Clu (ml/min/kg)	2.0	5.56±1.88
AUC	0.13	not given
k_{ab}(hrs-1)	0.693	not given
$T_{0.5}$ab(mins)	60	13.28±6.9
F (%)	62	not given

	1-OH Triazolam	Metabolites (Pakes et al., 1981)
k_{el}	0.124	0.18
$T_{0.5}$el	5.6	3.9

Taking this into consideration, the calculated values compare very well with the literature. The half-life and elimination rate constant give similar values to published data, but the clearance rate is somewhat higher in males.

A comparison of elimination half-life and rate constant for the metabolites of triazolam with published data (Pakes et al., 1981) showed very similar trends, although the dose administered in the work by Pakes was 0.88mg of triazolam.

Further corroboration of these results comes from comparing the results with other published data (Wendt, 1985). The bioavailability and half-life in the beta phase for human subjects reported by Wendt, were 60% and 1.7 to 3 hours respectively. The corresponding calculated values were 62% and 2.3 hours respectively.

4-OH triazolam was excreted much more quickly than triazolam or 1-hydroxy triazolam, suggesting that, as in the greyhound, 4-OH concentration is not dependant on triazolam concentration.

Again, 1-OH triazolam concentration seems to be dependant on the amount of parent drug present.

The results were reproducible and the extraction method efficient enough to determine extremely low drug levels.

FLUNITRAZEPAM

Flunitrazepam in greyhound urine samples

Spiked greyhound urine samples were made up by adding aliquots of flunitrazepam (1mg/ml) and its metabolites in methanol, to blank greyhound urine. Extractions were carried out according to the procedure outlined (CN columns; no wash stage; acidic methanol pH4 eluent) at 0.5 ug/ml level and the percentage recoveries for flunitrazepam and its metabolites were determined, by comparing the peak area obtained after injection of an extract from a spiked urine sample of known concentration, with that produced by the same concentration of drug in methanol. Each measurement was taken as the average of two determinations (Table 30).

The recoveries at 0.5ug/ml concentration were extremely good and the extracts were sufficiently clean for direct injection onto the HPLC system,

Extraction of greyhound samples

Four greyhounds were each administered a single oral dose of flunitrazepam (2mg). Urine samples were freely collected at timed intervals following administration.

TABLE 30.

EXTRACTION EFFICIENCY FOR FLUNITRAZEPAM AND ITS METABOLITES FROM GREYHOUND URINE USING CN CARTRIDGES

Drug	Percentage recoveries
Flunitrazepam	99.8 ± 0.5%
Desmethylflunitrazepam	97.9 ± 3.6%
7-aminoflunitrazepam	96.3 ± 1.1%

The samples were extracted according to the procedure described and analysed by HPLC using the chromatographic system described above (C18 column; methanol-deionised water-ammonium acetate buffer (pH8)-acetonitrile (200:125:100:75)) as the mobile phase.

No parent flunitrazepam or any related compounds were detected.

Various literature reports agree with these findings: Following oral administration (2ml/kg), a higher level than in this administration, measurable levels of the parent drug in the plasma were low and erratic (Kaplan et al., 1974). Also, no measurable levels of the parent drug or its metabolites were seen in the urine after this administration, indicating extensive and complete biotransformation and possible alternative routes of excretion (Kaplan et al., 1974; Vree et al., 1977), even though the HPLC methods described in these publications detected flunitrazepam concentrations as low as 1ng/ml.

Desmethylflunitrazepam is not a urinary metabolite Of flunitrazepam, so this would not be expected to appear in the urine.

No further work was carried out on this drug, although the extraction method is efficient.

BENZODIAZEPINE SCREENING PROCEDURE

Extraction of standard benzodiazepines

Nineteen benzodiazepines (0.5 ug/ml standards) were subsequently extracted using the procedure described above and the percentage recovery of each was determined.

The recoveries for all these drugs from the urine of racing greyhounds, was determined by comparison with peak areas obtained from standard solutions of the drugs in methanol, with the peak areas obtained from an extract of a known concentration of the drug in urine (Table 31). Generally, the recoveries were excellent (over 90%) with the exception of loprazolam (75.8%). The nitrogen groups in loprazolam (Figure 24) are prevented from interacting with the charged silanol groups on the surface of the sorbent to some extent by the physical presence of a large heterocyclic group. This may account for the poor recovery due to inefficient retention of the drug on the sorbent. The other benzodiazepines studied do not possess this physical disadvantage and so are well retained and eluted.

The coefficient of variation of the extraction method over three days is also given. The extraction procedure was reproducible for all the drugs.

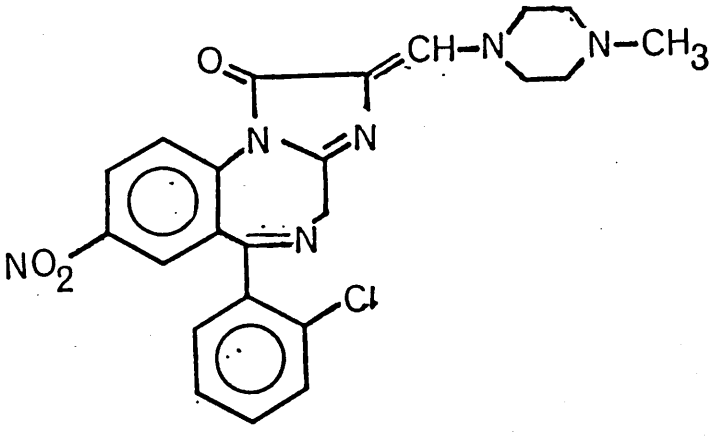
TABLE 31.

PERCENTAGE RECOVERIES OF BENZODIAZEPINES (0.5ug/ml) USING CYANOPROPYL COLUMNS AND ACIDIC ELUENT

Drug	Recovery
Alprazolam	101 ± 4.9%
Clobazam	96.1 ± 7.2%
Clonazepam	94.5 ± 9.9%
Desmethyldiazepam	105.8 ± 2.6%
Diazepam	99.8 ± 1.8%
Flunitrazepam	99.8 ± 0.5%
Desmethylflunitrazepam	97.9 ± 3.6%
7-aminoflunitrazepam	96.3 ± 1.1%
Flurazepam	96.2 ± 4.1%
Lorazepam	92.7 ± 5.4%
Lormetazepam	93.5 ± 3.6%
Loprazolam	75.8 ± 0.8%
Midazolam	100.8 ± 2.7%
Nitrazepam	97.8 ± 3.2%
Oxazepam	100 ± 2.2%
Temazepam	91.2 ± 9.4%
Triazolam	100 ± 2.3%
1-hydroxytriazolam	97.6 ± 3.4%
4-hydroxytriazolam	104.4 ± 10.6%

FIGURE 24.

The structure of loprazolam.



Conclusion

The results show that this extraction is feasible as a screening technique for benzodiazepines. The increased selectivity of polar sorbents makes this procedure preferable to non-polar extraction. However, removal of the wash stage reduced the cleanliness of the extracts, although they were still adequately clean for direct injection into the HPLC system.

2. Xylazine

Xylazine in greyhound urine samples

Urine samples in the range 10-1000 ng/ml were prepared by adding aliquots of a standard solution of xylazine in methanol (1mg/ml) to blank greyhound urine.

These were extracted according to the procedure outlined above with the addition of a wash stage: CN columns were conditioned with methanol and deionised water. The urine sample, buffered to pH 7 and diazepam standard was drawn through, and the columns were washed and dried. The drug was eluted with methanolic hydrochloric acid - acetonitrile (50 : 50 v/v).

The percentage xylazine recovery was determined from spiked greyhound urine samples, by comparing the peak area obtained after injection of an extract from a spiked urine sample of known concentration, with that produced by the same concentration of drug in methanol. Each measurement was taken as the average of two determinations.

The positive identification of xylazine was based on comparison with standards, absorbance maxima (225 nm) and retention time.

The extracts were clean and the recoveries from spiked urine samples were high (Table 32). No interference from endogenous compounds was noted.

The assay was consistent over three days (Table 32).

TABLE 32.

EXTRACTION EFFICIENCY FOR XYLAZINE FROM GREYHOUND URINE USING CN CARTRIDGES

Concentration of xylazine	Recovery
10 ng/ml	97.6%
100 ng.ml	99.8%
1000 ng/ml	98.4%

Consistency of assay over three days: 90.34 ± 4.6%

Extraction of actual samples

The extraction of the urine samples previously determined using non-polar cartridges, proved to be much more consistent using cyanopropyl bonded sorbents (Figure 25).

The peak excretion times (2 to 3 hours) was the same as previously determined, but far greater correlation between administrations and greyhounds was achieved with CN columns.

Average excretion values (Table 33) are consistent.

Three of the four greyhounds showed a peak excretion time of 2 hours and very similar excretion patterns. The fourth greyhound showed a peak excretion after 3 hours and a somewhat erratic excretion pattern. This occurred in all three dosing trials for that particular greyhound.

Unfortunately, samples could not be obtained from the greyhounds before 2 hours since they were sedated. This observation differs from that reported (Newkirk and Miles, 1974).

Using the average excretion values between the dogs, an excretion curve for unchanged xylazine was produced (Figure 26). From the excretion curve, an average peak excretion value of 0.29 ug/ml parent xylazine would be expected two hours after dosing.

FIGURE 25.

Xylazine extracted from greyhound urine -
2 hours after a single intramuscular dose -
using CN columns and HPLC analysis.

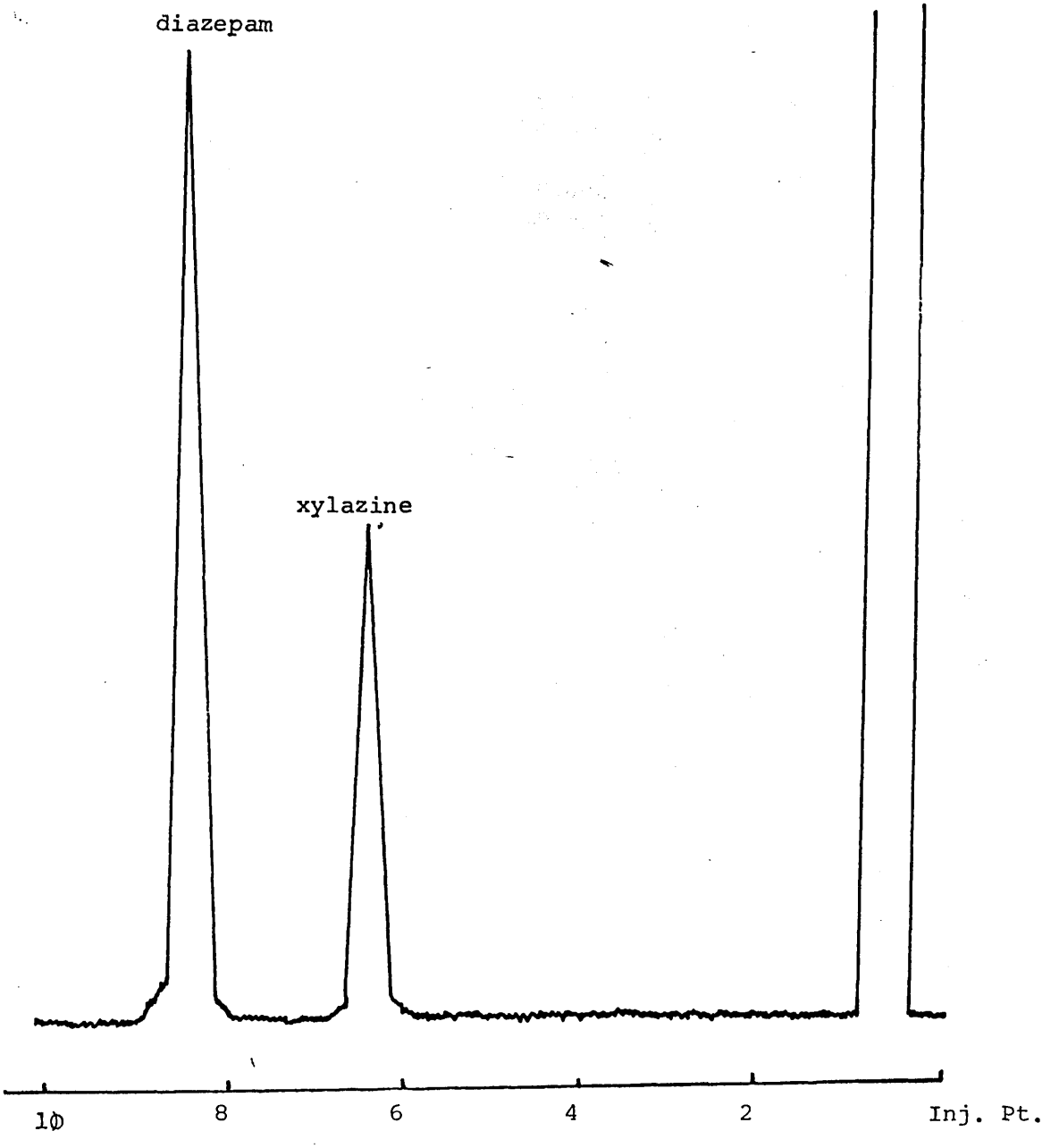


TABLE 33.

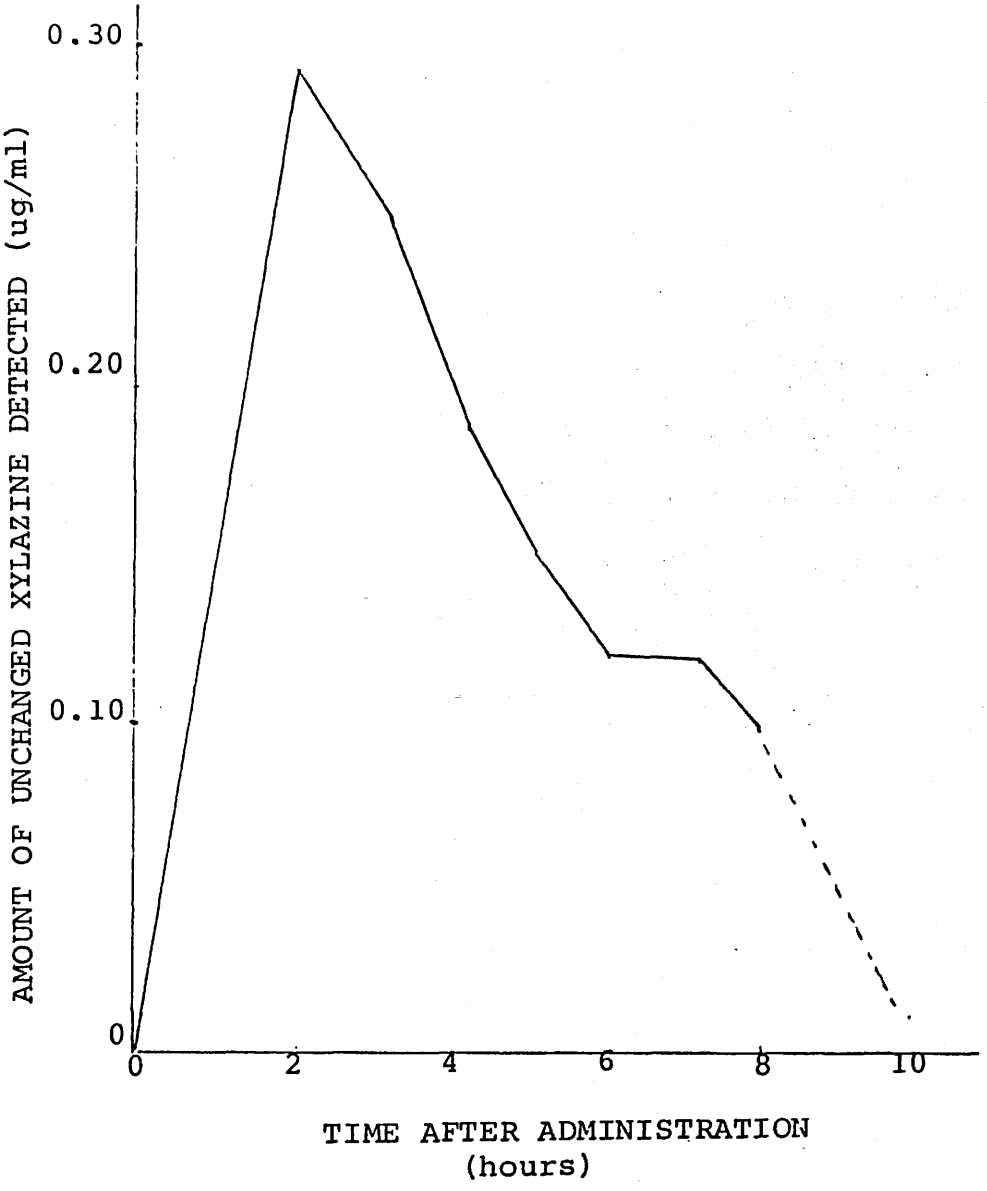
AMOUNT OF XYLAZINE EXCRETED DETERMINED BY CN EXTRACTION PROCEDURES AND HPLC ANALYSIS

Greyhound Number after dosing (hours)	1	2	3	4
	Xylazine excreted (ug/ml)			
2	0.217	0.309	0.216	0.370
	----	0.462	0.175	0.309
3	0.211	0.259	0.222	0.515
	0.203	0.183	0.126	0.247
4	0.206	0.197	0.120	0.451
	0.123	0.075	0.120	0.152
5	0.102	0.091	0.04	0.185
	0.204	0.077	----	0.149
6	0.077	----	----	0.209
	0.0987	0.019	----	0.483
7	0.05	----	----	0.181
	0.185	----	----	0.101
	Total xylazine excreted (ug)			
2	6.08	96.4	99.4	3.7
	----	56.4	44.7	24.7
3	17.5	64.8	6.67	12.36
	22.4	9.8	15.1	12.8
4	9.89	74.9	50.4	14.4
	7.4	4.36	18.0	7.89
5	3.59	27.3	1.87	5.56
	28.5	3.97	----	16.73
6	1.54	----	----	6.7
	4.74	1.73	----	54.1
7	0.607	----	----	1.81
	1.67	----	----	1.01

Results are mean values from three consecutive day extractions. Overall precision of extraction was 90.34%±4.6%.

FIGURE 26.

Average xylazine excretion from greyhound urine.



A total of 2.7% of the parent dose was excreted within eight hours and no xylazine was detected after this time. This is a far greater percentage of the dose excreted than observed using the non-polar extraction columns and supports the fact that the polar extraction method is much more efficient and robust than the non-polar extraction procedure.

A further comparison with the non-polar extractions showed this method to be more reproducible. Care was taken to use, where possible, the same batches of CN columns, and urine samples were diluted further with buffer in this extraction procedure so reducing the effect of salt content of the urine.

Pharmacokinetics

Figure 27 shows the semi-logarithmic plot of average xylazine concentrations in urine versus time after intramuscular dosage to the greyhounds.

The levels of drug peaked at 2 hours, then declined exponentially over the next 5 hours.

These data are well fitted by a one compartment open model with a rapid absorption phase followed by elimination. Table 34 shows that kinetic parameters calculated from the results, when compared with the published data for domestic dogs following a slightly higher intramuscular dose are similar (Garcia Villar et al., 1981).

FIGURE 27.

Semi-logarithmic plot of xylazine excretion from greyhound urine.

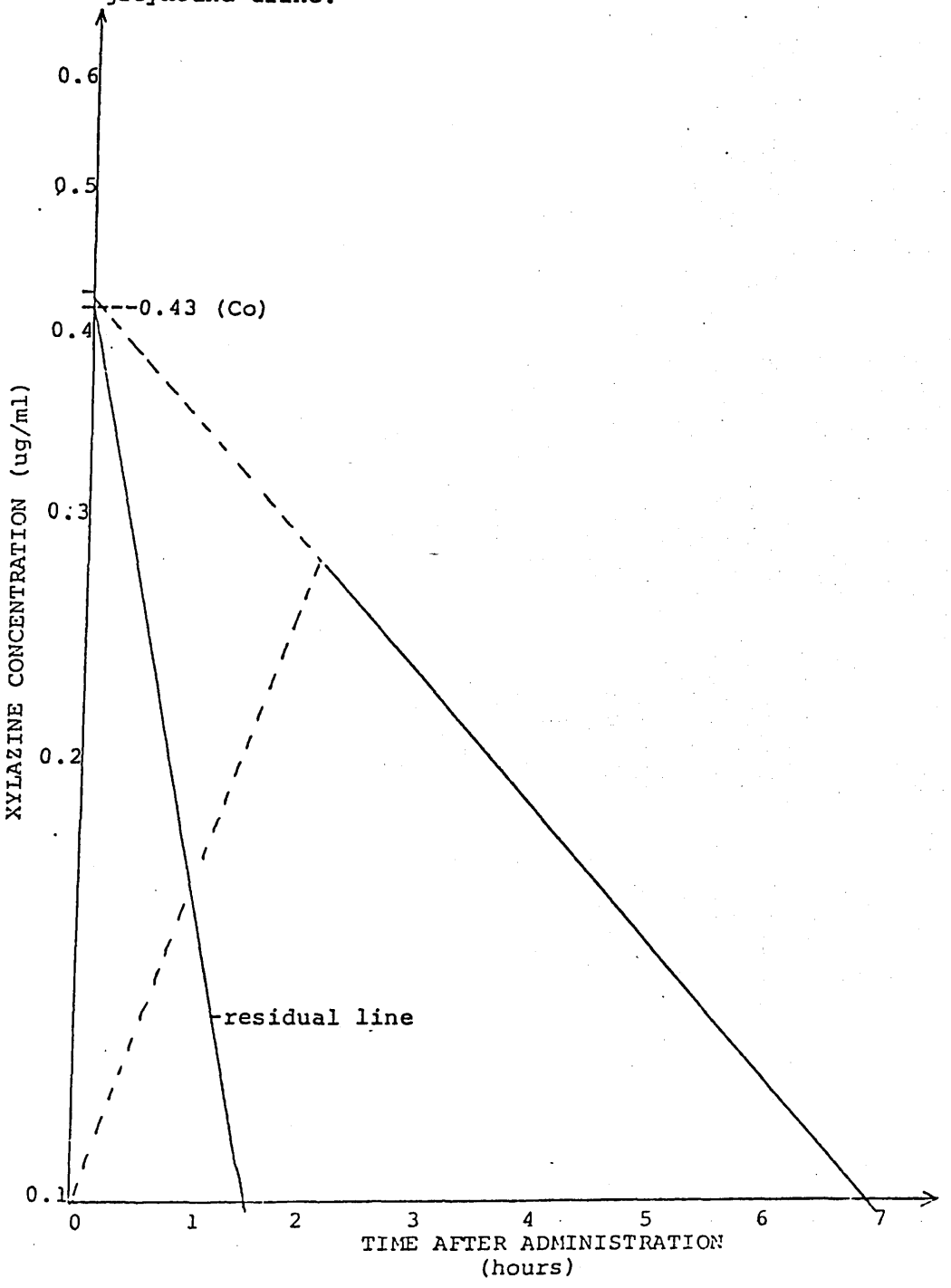


TABLE 34.

PHARMACOKINETIC PARAMETERS FOR XYLAZINE IN THE URINE OF RACING GREYHOUNDS

Dose=36000 ug

	Greyhounds (n=4)	Domestic Dogs (n=4)
Co (ug/ml)	0.43	0.432
$T_{0.5}$(hrs)	0.68	0.06
Vd (litres)	84	not given
k_{el}(hrs-1)	0.217	1.2
Clu (l/hr)	18	not given
AUC	1.98	not given
k_{ab}(hrs-1)	1.019	12.06
$T_{0.5}$ab(hrs)	3.20	0.60
1 (%)	78	73.9±17.89

Using 12.7 minutes as T_{max} (Garcia-Villar et al., 1981):
$T_{0.5}$ = 0.118 hours
k_{ab} = 5.83 hours-1

The apparent initial drug concentration in urine (Co) was calculated as 0.43 ug/ml. The published value for plasma concentration was 0.432 ug/ml. This suggests that the amount of xylazine available in plasma and urine at time zero is similar. Bioavailability of the drug was also consistent with published data (Table 34).

With the exception of these values, the other parameters are somewhat different for greyhounds than for domestic animals. Xylazine is absorbed and excreted from plasma in the domestic dog much more rapidly than in the urine of the racing greyhound.

In the greyhound, all parent xylazine is excreted within 7 hours whereas in the domestic dog, all parent xylazine has been excreted from the plasma within the first 2 hours.

Since the greyhounds in this study were sedated up to 2 hours after dosing, a maximum absorption time (Tmax) was not calculated and the absorbance rate constant (kab) had to be calculated using assumed residual values. However, using 12.7 minutes as Tmax (Garcia-Villar et al., 1981), a much faster rate of absorption and half-life of absorption (T 0.5abs) were calculated.

Since there are no reported studies of xylazine metabolism in the racing greyhound, comparison of data is difficult, but greyhounds would appear to eliminate a single dose much more slowly than domestic dogs.

Greyhound absorption of xylazine is probably as quick as in domestic dogs, but the period of analgesia apparent after a single therapeutic dose is much longer than has been reported (Newkirk and Miles, 1974).

Conclusion

The detection of parent xylazine up to eight hours after the intramuscular injection of a single therapeutic dose shows that this analytical procedure is suitable for the detection of the misuse of this drug in greyhound racing. Since any greyhound seen to deteriorate markedly on previous performances is sampled immediately post race, parent xylazine from an effective dose would be detected.

The method described, demonstrates quantitative recovery of the drug using the cyanopropyl columns. It is rapid (up to ten samples can be extracted simultaneously) and has minimal sample volume and solvent requirements.

The success of polar bonded sorbent extraction led quite naturally to considering the possibilities of exploiting the nitrogen groups within basic drugs as a means of retaining the drugs on sorbents. An extraction sorbent where the primary interaction is ionic rather than non-polar would further increase selectivity.

A dual interaction where both non-polar and ionic interactions are of major importance would then allow washing with organic solvents (for example, methanol, since the drug would be held to the sorbent through ionic interaction) and also, ionic and aqueous solvents (for example, acids since the drug would be held to the sorbent through non-polar interactions) so increasing vastly the cleanliness of the extract without reducing extraction efficiency. Strong cation exchange sorbents would appear to bridge this gap between selectivity and cleanliness, so ion-exchange interactions for a range of basic drugs were studied.

4.2.3. Extraction Methods exploiting Ion-exchange Sorbent Interactions

1. Basic Drugs

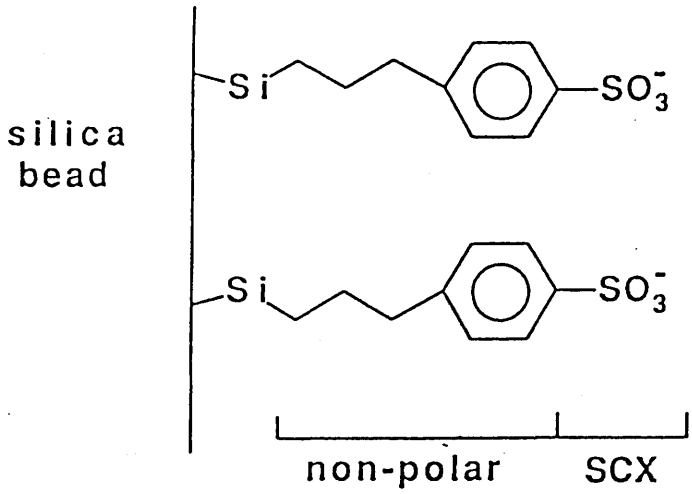
Cationic ion exchange interactions occur between an isolate molecule carrying an ionic charge (in this case, a positive charge) and a sorbent carrying an opposite charge (negative)(Figure 28). In order for effective retention of the isolate to occur, the matrix must be at a pH where both the isolate and the sorbent are charged. Also, the matrix must not contain high concentrations of strongly competing ionic species of the same charge as the isolate (Van Horne, 1985).

Thirty basic drugs at 1 and 5ug/ml concentrations were made up in methanol and extracted using SCX columns conditioned with methanol, water and 7mM phosphoric acid (pH3.4). Urine and 7mM phosphoric acid were applied to the column. The column was air dried, washed with 7mM phosphoric acid, 0.1N acetic acid and methanol. Ammoniacal methanol (1%) was passed through the column and collected.

An aliquot of the residue (20ul) was removed for analysis by HPLC with diode array detection so that the percentage recoveries of each drug could be determined by comparison with standard drug peak areas.

FIGURE 28.

Primary interactions of cation exchange sorbent.



The eluent was monitored at 200nm and full spectra were recorded from 190 to 400nm for each peak. The column was a Hibar Lichrospher 100 CH-8/II (25cm x 4.6mm).

The initial mobile phase composition was 10% acetonitrile in 0.05M pH3.2 potassium phosphate buffer. This was increased to 50% over 15 minutes, and the final composition was maintained for 5 minutes. A re-equilibration time of 5 minutes was required between injections (total cycle time 25 minutes).

Recoveries of standard drugs

The absolute drug recoveries obtained both by direct analysis of the eluent from the sorbent (1ml of 1% ammoniacal methanol), and also after a concentration (evaporation and reconstitution) step for the basic compounds were determined (Table 35). The linearity of the extraction method was determined for the range 1 to 5 ug/ml, and the correlation is also given.

Direct analysis of the eluent gave very good recoveries for all compounds except caffeine, theophylline and theobromine, for which the recovery was between 72 and 86%. Theophylline and theobromine have strong acid functions (pKa<1), and will not be fully protonated at the pH of extraction, (pH3), and so will not be completely retained on the ion exchanger.

TABLE 35.

RECOVERY DATA BEFORE AND AFTER EVAPORATION FOR BASIC DRUGS USING CATION EXCHANGE EXTRACTION.

Compound	Direct Analysis Recovery	Direct Analysis COV	After Evaporation Recovery	After Evaporation COV
Amphetamine	99.3	15	34.5	19
Benzoylecgonine	94.2	8	92.5	8.4
Caffeine	72.1	18.6	73.2	14
Cocaine	100	12	61.9	4.4
Codeine	100	2	99.4	1
Cyclizine	100	0.4	95.1	1.6
Dextromethorphan	97.2	5	94.1	3.2
Diazepam	93.7	3.2	78	5.5
Dihydrocodeine	95.7	6.3	96.1	4.2
Diphenhydramine	100	1.4	98.7	2.3
Ephedrine	100	4.3	96.2	4.0
Hydrocodone	99.8	2.4	98.7	3.7
Mazindol	100	3.9	94	1.4
Mesoridazine	92.1	2.9	87.1	4.1
Methadone	100	4.2	86.1	3.0
Methamphetamine	100	2.0	47.9	15
Metoprolol	94	2.0	93.3	3.2
Morphine	93.1	8.6	96.1	5.1
Nordiazepam	98.4	4.3	95	6.4
Norpropoxyphene	95.2	1.5	94.3	3.7
Oxazepam	96.4	9.0	92.1	7.8
Papaverine	100	1.0	100	1.0
Phencyclidine	85	10	80.6	4.7
PPA	97.8	2.6	95.2	1.0
Propoxyphene	90.8	6.5	97.9	1.4
Temazepam	94.5	2.3	87	6.7
Theobromine	76.5	1.1	59.8	13
Theophylline	81.6	3.5	63.5	11
Thioridazine	86.4	3.4	75.2	2.3

PPA = phenylpropanolamine

COV = coefficient of variation

Caffeine has an extremely high pKa value (pKa 14) and so will be strongly ionised at the pH of extraction. Therefore, it is retained very strongly on the sorbent and will not be completely removed at the pH of the eluting solvent (pH10).

Their recovery, however, while being significantly less than that obtained for the remainder of the compounds, is equal to what might be expected for a good liquid/liquid extraction procedure and so was quite acceptable for a screening method.

These high recoveries, impractical with a liquid/liquid partition extraction, are possible due to the use of this absorption/elution approach to extraction which is ideally binary in nature: the analytes are either all absorbed or all eluted, depending on the polarity of the surrounding medium.

The technique also has the advantage over liquid phase ion-paired extraction in that the immobilisation of the drug-ligand ion-pair on a solid support allows the removal of extraneous sample material by washing the support with an ionic solvent (phosphoric acid, acetic acid) to remove ionic material while the drugs are retained by non-polar interactions with the benzenesulphonylpropyl moiety. A subsequent wash with a relatively non-polar solvent (methanol) removes less polar material while the drugs are retained by ion-paired interactions.

This washing results in the production of a clean extract. The final eluent (1% ammoniacal methanol) disrupts both the ion-pair and the non-polar interactions and allows elution of all the drugs in a relatively small volume. A chromatogram of 11 basic drugs extracted from a spiked post-mortem urine sample (5ug/ml, 1ml sample) is shown (Figure 29).

Having demonstrated that the recoveries using cation exchange extraction were essentially complete, the effect of evaporating the solvent and reconstituting the residue was investigated in order to maximise the sensitvity of the method.

Evaporation and reconstitution

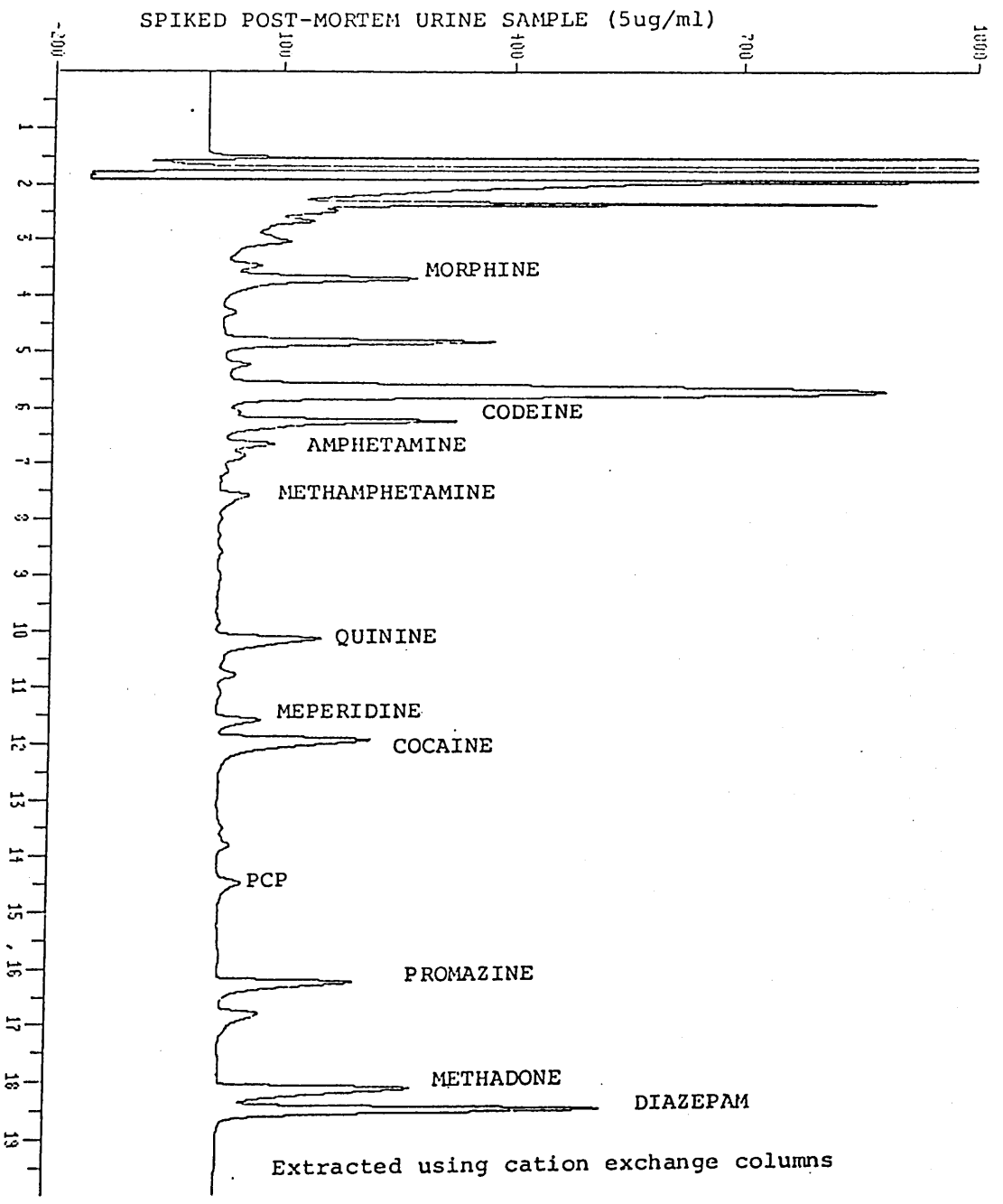
Extracts were reconstituted in 50ul, and 10ul was analysed, therefore a concentration factor of 20 was expected.

When the residues were reconstituted in acetonitrile, the apparent recoveries were low: between 5 and 12% for each of the compounds analysed.

This appeared to be a matrix effect due to the insolubility of the coextracted residue in acetonitrile.

Reconstitution in mobile phase (10% acetonitrile in 0.05M pH3.2 potassium phosphate buffer) improved recoveries considerably for all compounds (Table 35; Figure 30).

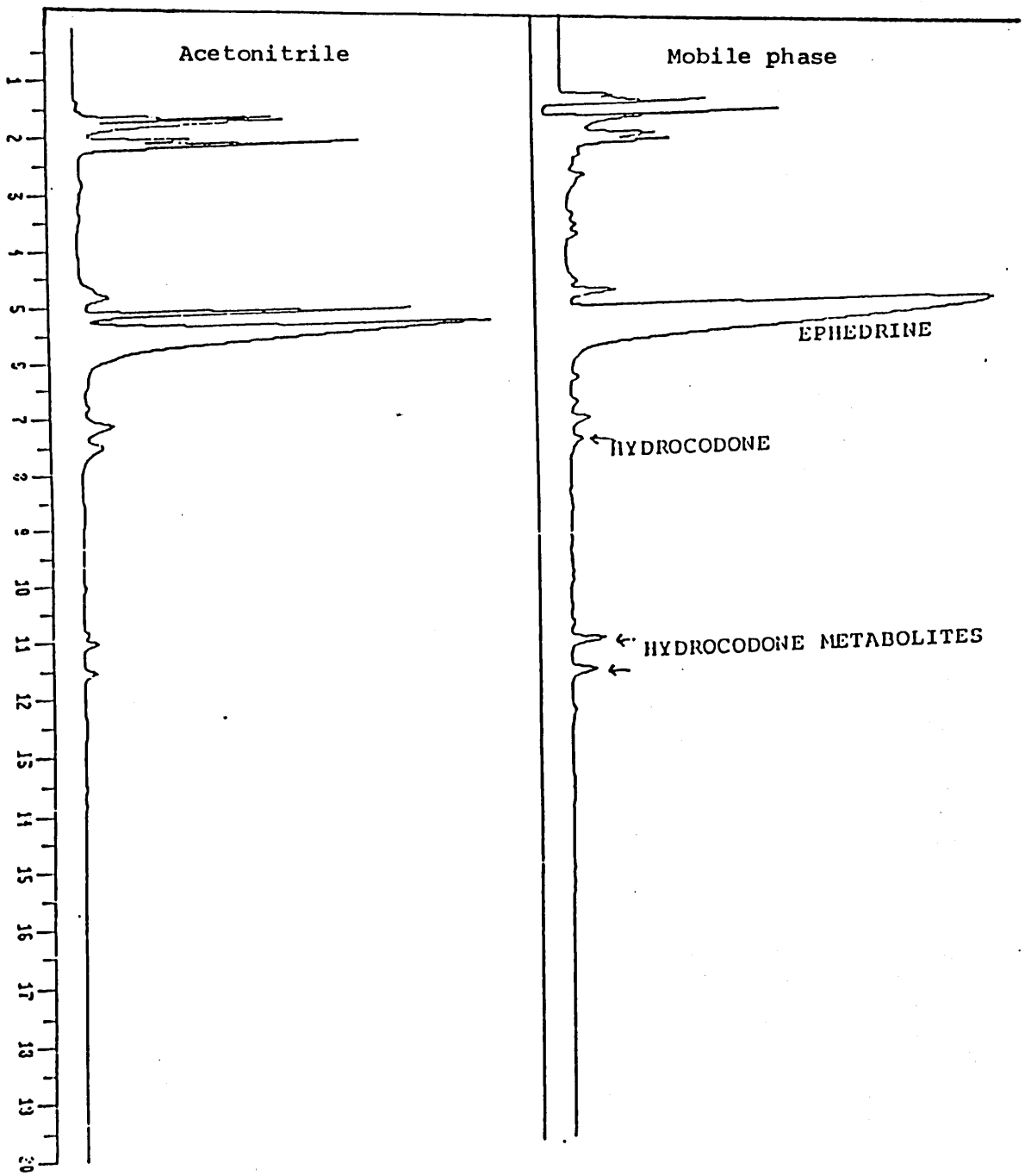
FIGURE 29.



End of plot. Time = 0.01 to 19.98 minutes Chart speed = 1.00 cm/min

FIGURE 30.

Comparison of ACN and MP reconstitution of a post-mortem urine sample extracted with SCX columns.



End of plot. Time = 0.01 to 19.99 minutes Chart speed = 0.90 cm/min

The importance of the reconstitution solvent is an important consideration in any analytical procedure where an evaporation/reconstitution step is involved.

Recovery of volatile drugs

Notably poor recoveries were obtained for amphetamine and methamphetamine, two compounds whose volatility is known to cause sample loss during evaporation. This was corrected by adding a small amount (4-5 drops) of 0.1N hydrochloric acid to the ammoniacal methanol before evaporation.

This increased recovery of the compounds to 94% and 92% respectively, but it was noted that the presence of the acid could cause the hydrolysis of some cocaine to benzoylecgonine and methylecgonine if cocaine was present in the extracted samples.

Amphoteric compounds

Of particular note in these results, was the high recoveries of benzoylecgonine and morphine, two amphoteric compounds which tend to be difficult to extract by liquid/liquid extraction methods (Horning et al., 1974).

Liquid/liquid extraction requires the conversion of the drug to an uncharged/neutral form and its preferential partition into an organic liquid.

The amphoteric nature of morphine and benzoylecgonine however, results in the formation of charged species at any pH other than their isoelectric points, thus making them water soluble/organic insoluble and poorly extracted requiring recourse to salting out procedures or ion paired extractions.

The use of a bonded ion-exchange solid-phase simplifies assays for these compounds considerably and allows them to be extracted efficiently as part of a general drug screen (Figure 31). The use of a diatomaceous earth procedure did not allow these compounds to be extracted (Figure 32).

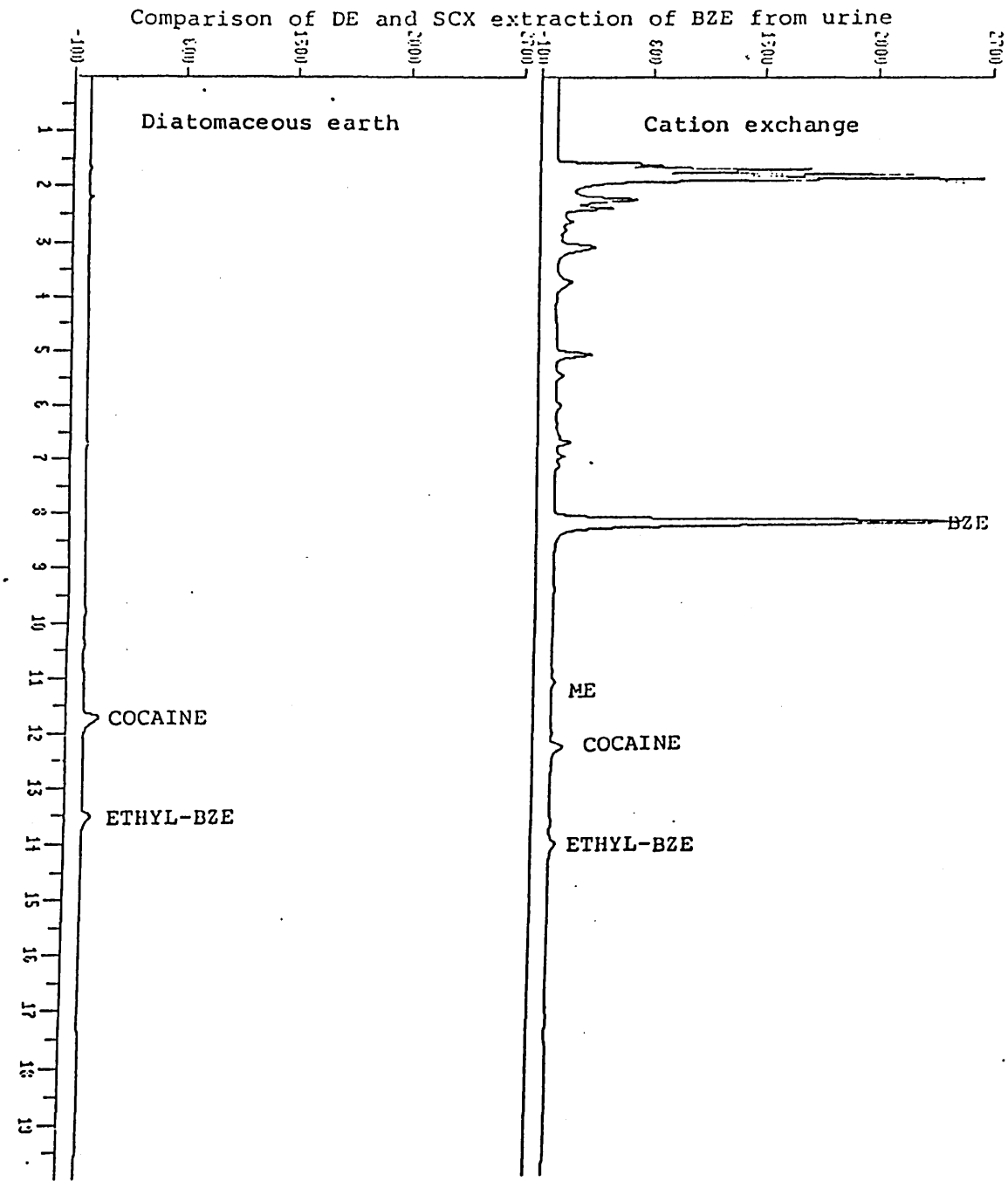
Method Validation

Human post-mortem urine samples

Twenty-four post-mortem urine samples were extracted by conventional solvent extraction methods (basic drug extraction into chloroform)(Foerster and Mason, 1974; Anderson and Stafford, 1983), in parallel with the cation exchange solid-phase extraction procedure previously described and a non-selective diatomaceous earth extraction:

Urine samples were diluted with 0.2M borate buffer (pH9;3ml), and applied to absorption extraction columns containing diatomaceous earth material. Solvent (n-butyl chloride) was applied and allowed to flow through until the required volume had been collected.

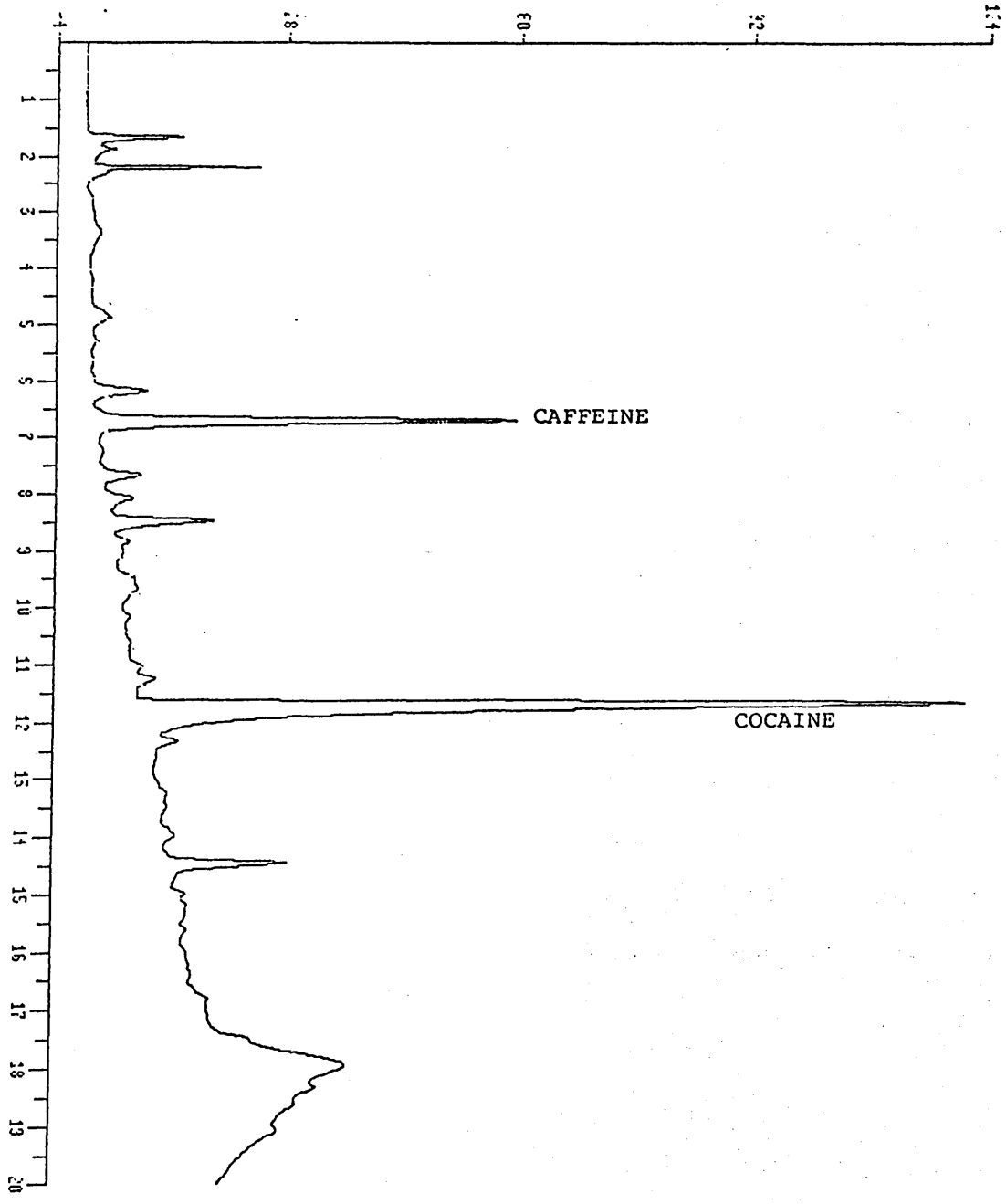
FIGURE 31.



End of plot. Time = 0.01 to 19.98 minutes Chart speed = 0.98 cm/min

FIGURE 32.

Extracted standards of cocaine, BZE and ME in urine using diatomaceous earth.



End of plot. Time = 0.01 to 19.99 minutes Chart speed = 1.00 cm/min

The eluting solvent was evaporated to dryness, reconstituted in mobile phase and analysed.

Results

The results of the comparison are given in table 36.

Solid-phase extraction/DAD HPLC method was able to confirm the presence of basic drugs indicated from routine gas chromatography-mass spectrometry routinely in use in all cases.

Generally, the SCX extractions were much more efficient than either the diatomaceous earth or the solvent extractions (Figure 33).

Diatomaceous earth, however, had the advantage of being extremely clean compared to the bonded sorbent extract (Figure 34) although the cation exchange extracts were sufficiently clean for direct injection onto either HPLC or GC-MS.

Also, for the analysis of caffeine and its metabolites (Figure 35), the diatomaceous earth was more efficient than SCX, again probably due to the difficulty of protonating theophylline and theobromine at pH3 and the difficulty in eluting caffeine itself from the bonded sorbent at pH10.

TABLE 36: COMPARISON OF LIQUID/LIQUID, CATION EXCHANGE AND DIATOMACEOUS EARTH EXTRACTION PROCEDURES.

Urine Sample #	Basic Drug Screen (L/L, GCMS)	Basic Drug Screen (SPE, HPLC/DAD)	Basic Drug Screen (DE, HPLC/DAD)
1	codeine, diazepam, nordiazepam	codeine, nordiazepam	codeine, diazepam, nordiazepam
2	caffeine, propoxyphene norpropoxyphene	caffeine, propoxyphene norpropoxyphene	caffeine, propoxyphene norpropoxyphene
3	cocaine, ME, homocaine	cocaine,BZE,ME,homocaine	cocaine
4	positive opiates (emit)	codeine	codeine
5	negative	negative	negative
6	ephedrine, dextromethorphan	ephedrine, dextrometh.	ephedrine, dextromethorphan
7	cocaine	cocaine, BZE	negative
8	negative	negative	negative
9	negative	negative	negative
10	cocaine metabolite	caffeine, BZE	caffeine
11	negative	negative	negative
12	negative	negative	negative
13	cocaine and metabolites	cocaine, ME	cocaine, ME
14	cocaine	cocaine,BZE,ME	cocaine, BZE, ME
15	negative	negative	negative
16	negative	negative	negative
17	negative	negative	negative

TABLE 36: Continued

Urine Sample #	Basic Drug Screen (L/L, GCMS)	Basic Drug Screen (SPE, HPLC/DAD)	Basic Drug Screen (DE, HPLC/DAD)
18	desmethyldiazepam	desmethyldiazepam	desmethyldiazepam
19	ephedrine,dihydrocodeine,hydrocod.	ephedrine, hydrocodone	ephedrine, hydrocodone
20	chlorpheniramine,metoprolol	chlorphen.,metoprolol	chlorpheniramine, metoprolol
21	negative	negative	negative
22	negative	negative	negative
23	+ve benzodiazepines (emit)	temazepam	negative
24	negative	phenylpropanolamine	negative

BZE = benzoylecgonine
ME = methylecgonine

FIGURE 33.

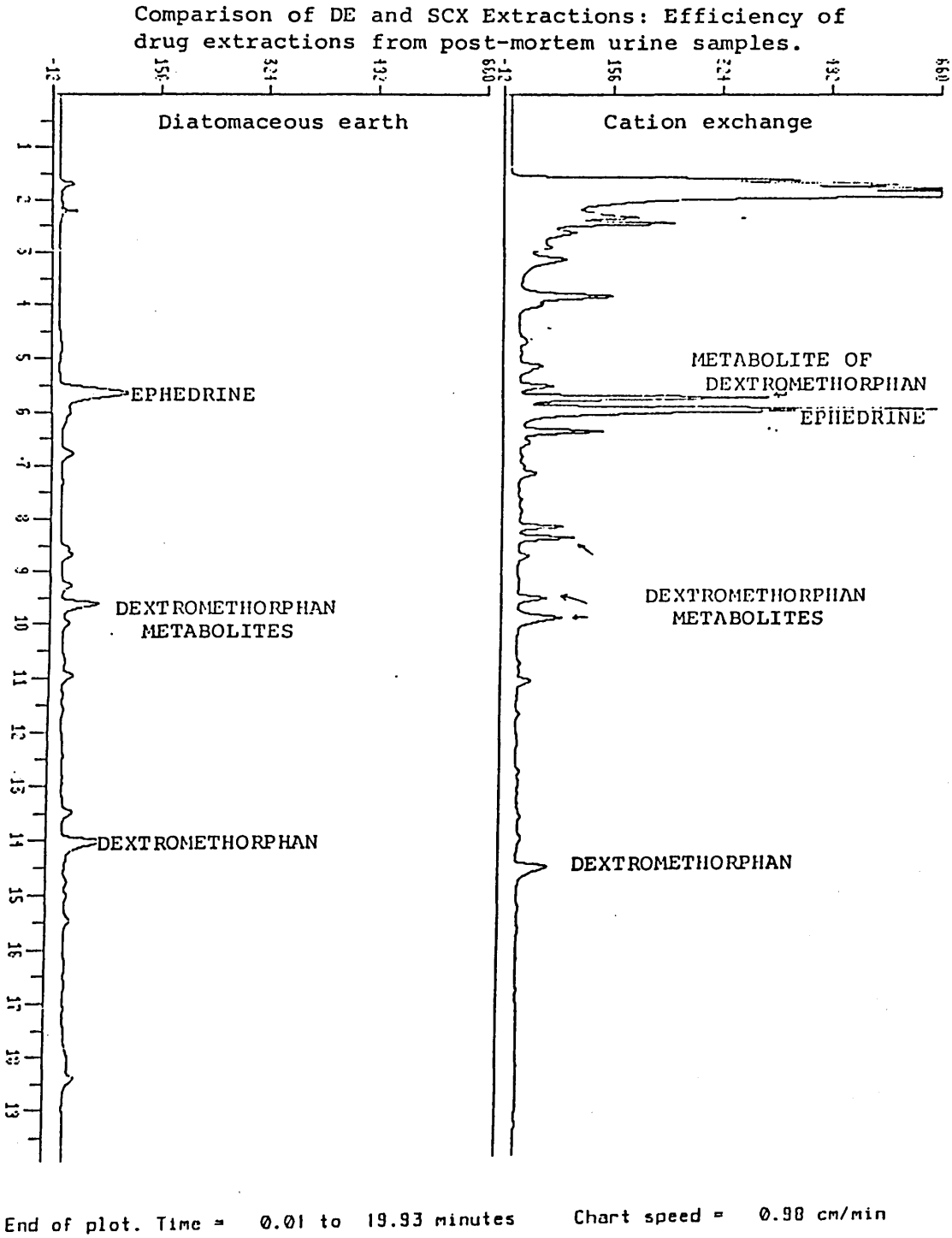

Comparison of DE and SCX Extractions: Efficiency of drug extractions from post-mortem urine samples.
Diatomaceous earth
Cation exchange
EPHEDRINE
DEXTROMETHORPHAN METABOLITES
DEXTROMETHORPHAN
METABOLITE OF DEXTROMETHORPHAN
EPHEDRINE
DEXTROMETHORPHAN METABOLITES
DEXTROMETHORPHAN
End of plot. Time = 0.01 to 19.93 minutes
Chart speed = 0.98 cm/min

FIGURE 34.

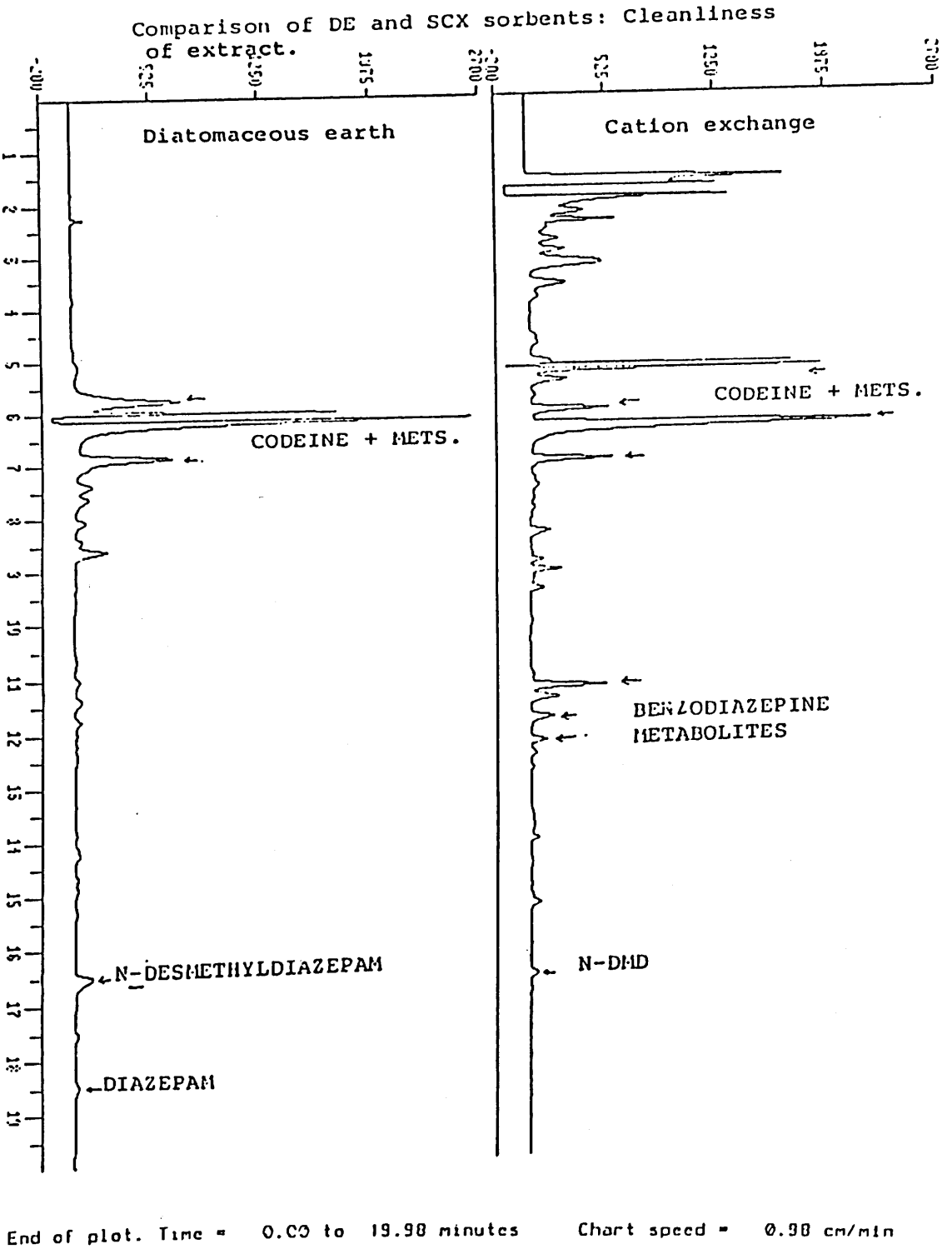
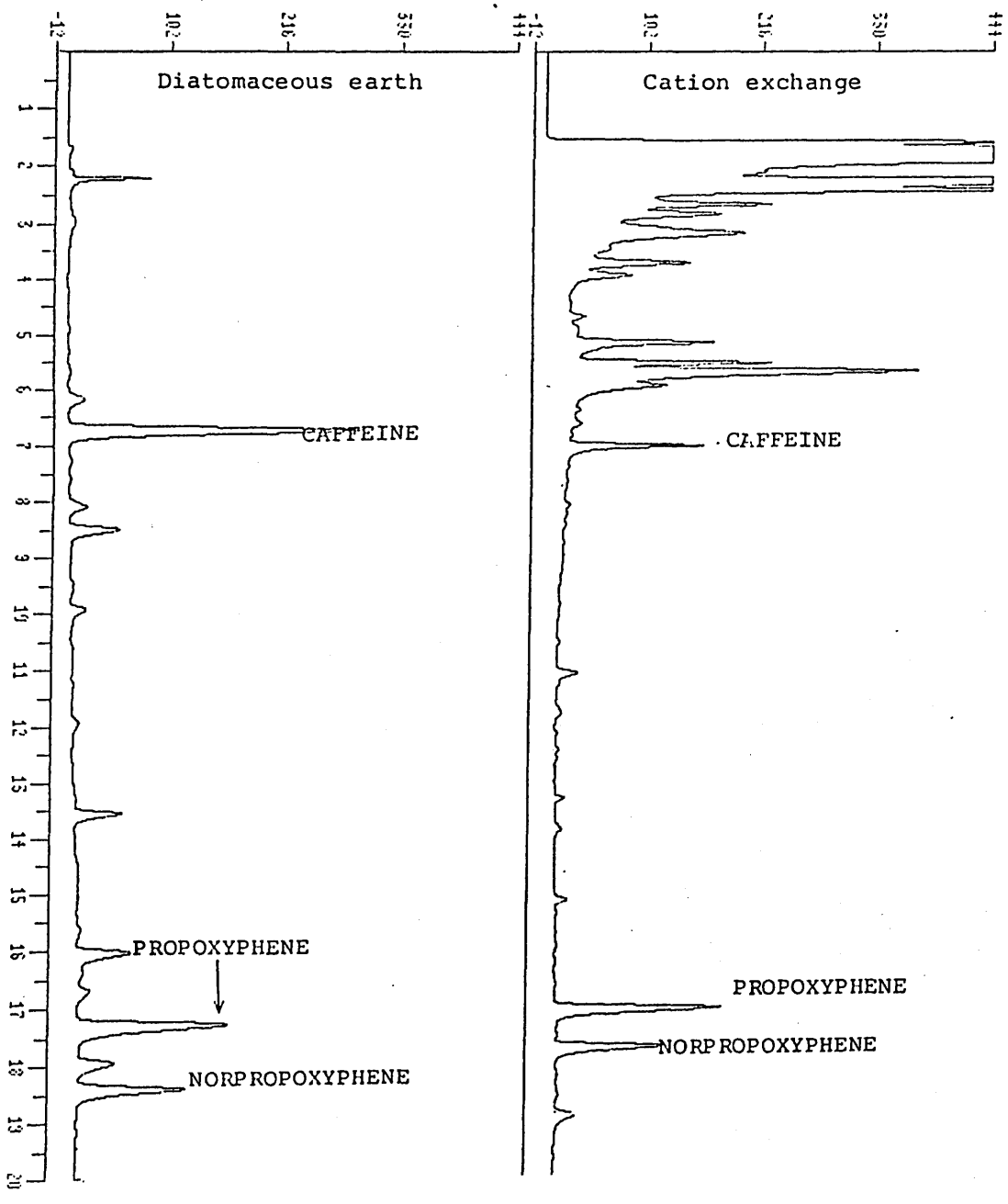

Comparison of DE and SCX sorbents: Cleanliness of extract.
Diatomaceous earth
Cation exchange
CODEINE + METS.
CODEINE + METS.
BENZODIAZEPINE METABOLITES
N-DESMETHYLDIAZEPAM
DIAZEPAM
N-DMD
End of plot. Time = 0.00 to 19.98 minutes
Chart speed = 0.98 cm/min

FIGURE 35.

Comparison of DE and SCX sorbents: Efficiency of caffeine extraction from post-mortem samples.



End of plot. Time = 0.01 to 19.99 minutes Chart speed = 0.98 cm/min

In addition, the cation exchange method detected temazepam and phenylpropanolamine in post-mortem samples which had appeared negative by the liquid/liquid extraction/GC method and the diatomaceous earth/HPLC DAD method.

Overall, the cation exchange method was the quickest, most efficient and most robust of the procedures. Extracts were sufficiently clean for direct injection onto HPLC-DAD or GC-MS depending on the original nature of the sample.

Potential for automation

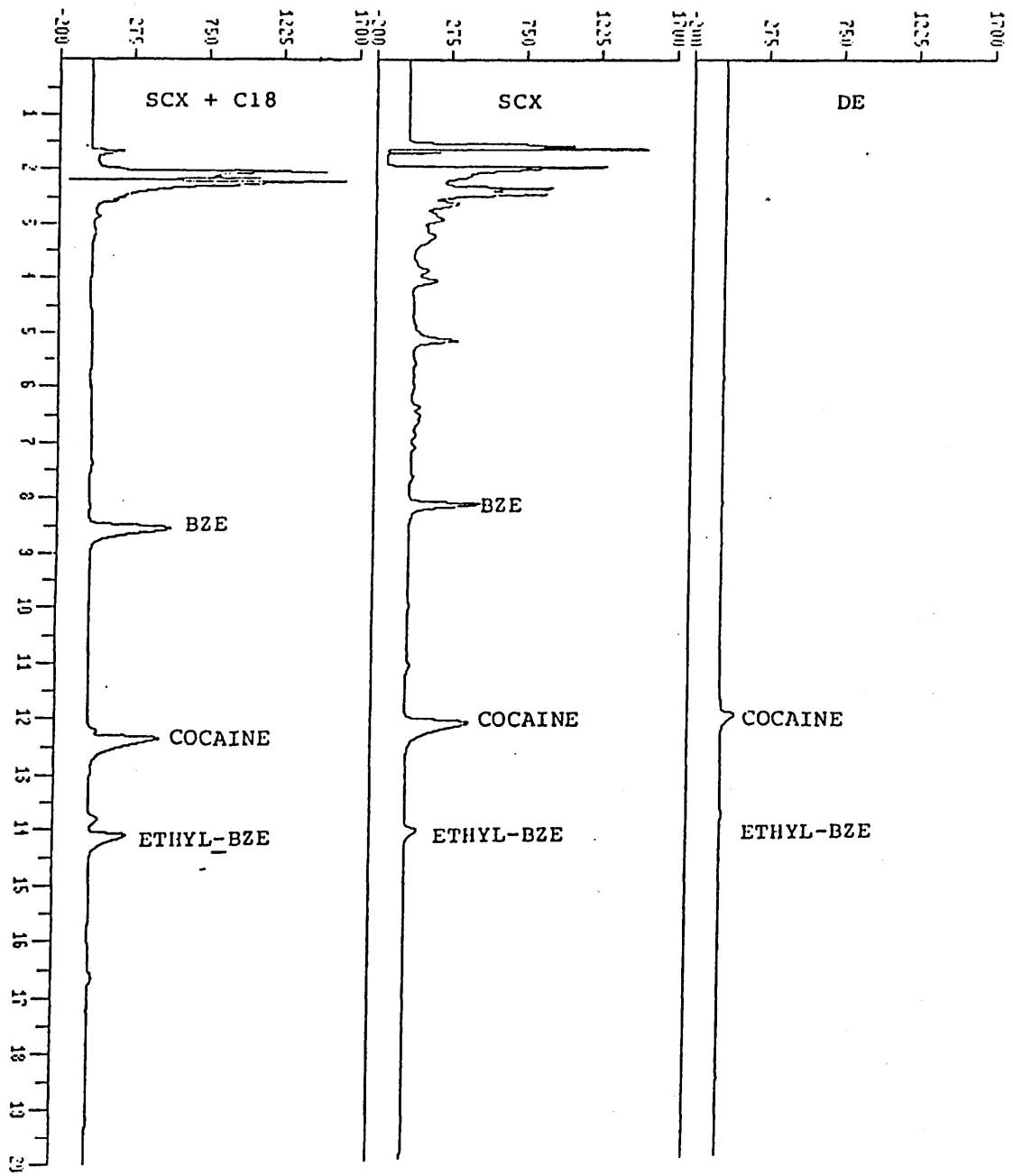
In the extraction system described, the analyte is in a basic medium prior to analysis. This is fine for sequential separate analysis, but for automation, the sample eluent must be compatible with the mobile phase used in the HPLC system (in this case, pH 3.2).

Experimental

Non-polar C18 columns (1ml capacity) were conditioned with one column volume of methanol and one of deionised water. The eluent from the cation exchange extraction (ammoniacal methanol, 1ml) was passed through the sorbent. The column was washed with deionised water (0.5ml) and the adsorbed drugs were directly eluted onto the HPLC/DAD system using mobile phase (acetonitrile-0.05M potassium phosphate buffer, pH3.2)(Figure 36).

FIGURE 36.

Post-mortem sample extracted by SCX followed by C18 sorbent extraction in an automation attempt.



This had the effect of cleaning the extract further, but, of the basic drugs studied, morphine and benzoylecgonine did not re-extract well. This was probably due to their amphoteric nature and their insolubility in the mobile phase at pH3.2.

Basic drugs in greyhound urine samples

The cation-exchange procedure described was carried out on spiked greyhound urine samples containing twenty-five basic drugs at 1ug/ml concentration, in order to determine the percentage recoveries.

The eluent from the extraction was not directly injected onto HPLC, but was evaporated to dryness and reconstituted in papaverine solution (0.5mg/ml, 25ul) for GC analysis.

The recoveries were above 95% (Table 37) in all cases except caffeine, theophylline and theobromine. The same reasons given previously apply again.

Method Validation

Greyhound urine samples

For many years, the extraction of basic drugs from the urine of racing greyhounds has been carried out using conventional thin-layer chromatography-based pre-race screening procedures (Clarke, 1986).

TABLE 37.

DRUG RECOVERIES FROM SPIKED GREYHOUND URINE

Drug	Recovery
1. Codeine	100 ± 2.0%
2. Dihydrocodeine	95.7 ± 6.3%
3. Hydrocodone	98.7 ± 3.7%
4. Morphine	94.1 ± 2.6%
5. Methadone	100 ± 3.2%
6. Mazindol	100 ± 3.9%
7. Promazine	97.5 ± 5.3%
8. Cocaine	98.1 ± 2.0%
9. Quinine	100 ± 2.3%
10.Meperidine	95.2 ± 0.5%
11.Diazepam	95.5 ± 2.1%
12.Temazepam	98.5 ± 3.4%
13.N-Desmethyldiazepam	97.4 ± 6.2%
14.Oxazepam	95.4 ± 2.9%
15.Thioridazine	92.6 ± 8.4%
16.Ephedrine	100 ± 8.6%
17.Metoprolol	97.1 ± 3.0%
18.Caffeine	70.1 ± 8.6%
19.Theophylline	82.6 ± 4.2%
20.Theobromine	76.5 ± 1.9%
21.Propoxyphene	95.8 ± 4.5%
22.Norpropoxyphene	95.2 ± 1.5%
23.Cyclizine	95.1 ± 1.6%
24.Papaverine	100 ± 1.0%
25.Diphenhydramine	98.7 ± 2.3%

For the purpose of testing the feasibility of using solid-phase extraction for basic drug screening in greyhound racing, thirty urine samples from greyhound racing tracks in the UK, received between December 1988 and January 1989 were extracted using cation exchange solid-phase extraction in parallel with the existing liquid/liquid method:

Greyhound urine (pH basic with 3.5M ammonia solution) was extracted with ethyl acetate. The aqueous layer was discarded and the organic layer was extracted with 2M sulphuric acid. The acidic layer was made alkaline with 3.5M ammonia solution and extracted into ethyl acetate. The aqueous layer was discarded and the organic layer dried over anhydrous sodium sulphate.

The extract was evaporated to dryness and reconstituted in papaverine solution (0.5mg/ml, 25ul), as an external standard, for analysis by gas chromatography (GC).

Of the thirty samples analysed, three were found to be positive. The drugs detected: quinine, cyclizine and procaine, were extracted by both methods, analysed by GC and were confirmed by gas chromatography-mass spectrometry (GC-MS).

Figure 37(a) shows the GC trace of the sample positive for cyclizine by solvent extraction (SE), and figure 37(b) shows the same sample using solid-phase extraction (SPE).

The solid-phase extract was much cleaner then the solvent extract, and the cation exchange extraction the more efficient of the two techniques.

The two procedures can be compared and summarised as shown in the following table:

Comparison (see Appendix 3)

(per 10 extractions)

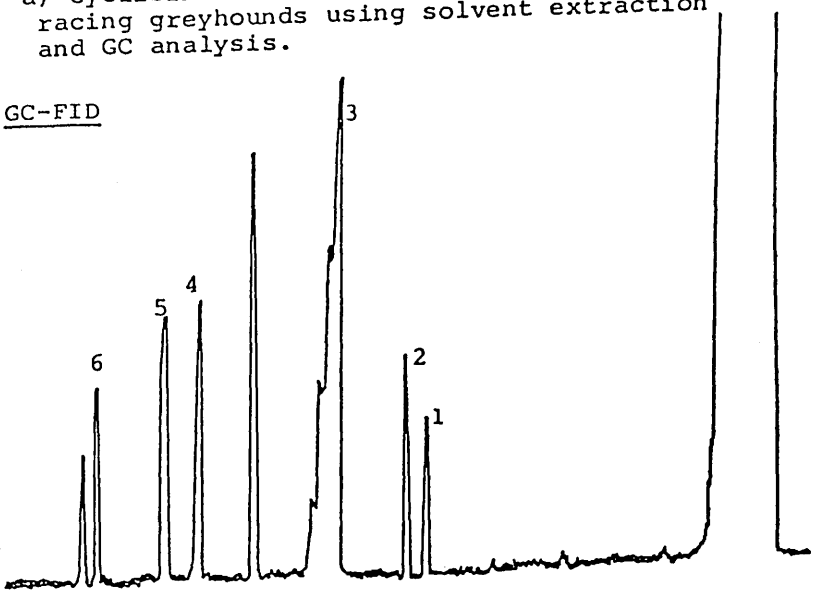
	SPE	SE
Sample volume required	10ml	200ml
Solvent cost	8 pence	£4.72
Extraction time	10 minutes	2 hours
Automation Potential	Yes	No
Glassware requirement	Minimal	Extensive
Extract quality	Good	Acceptable

As explained earlier, this solid-phase extraction method exploits the basic nature of the drug, hence it's usefulness as a screening technique. The basic character of the drug is exposed by the protonation of the nitrogen groups in the molecule in acid conditions.

FIGURE 37.

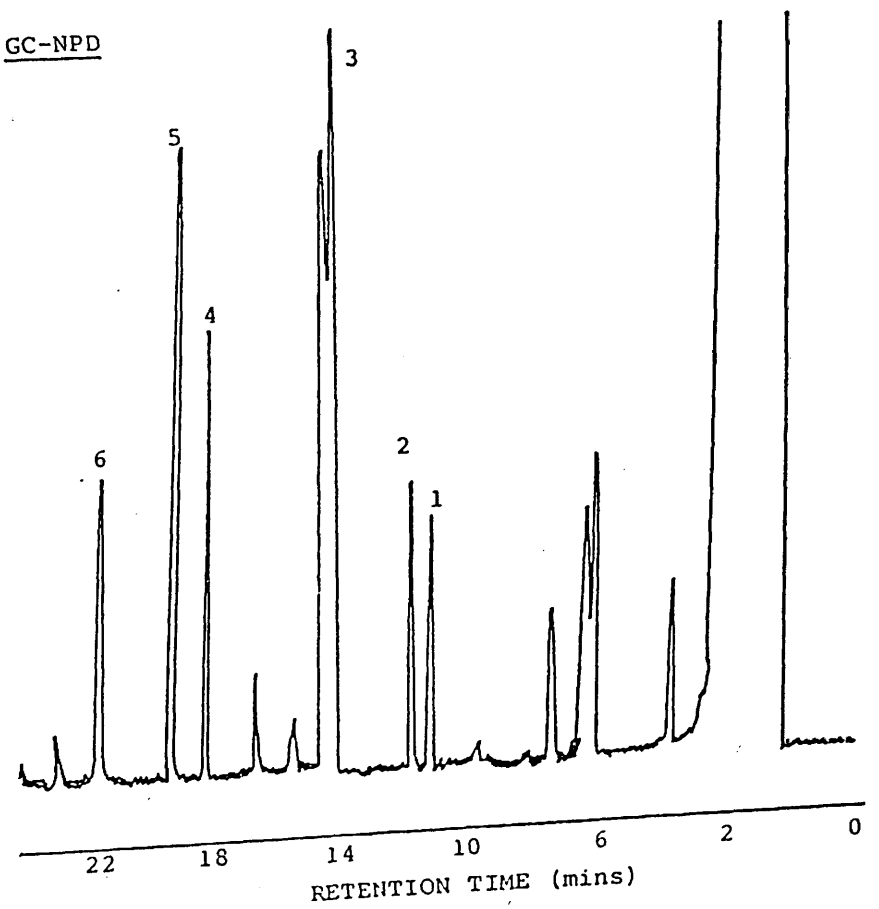
a) Cyclizine extracted from the urine of racing greyhounds using solvent extraction and GC analysis.

GC-FID



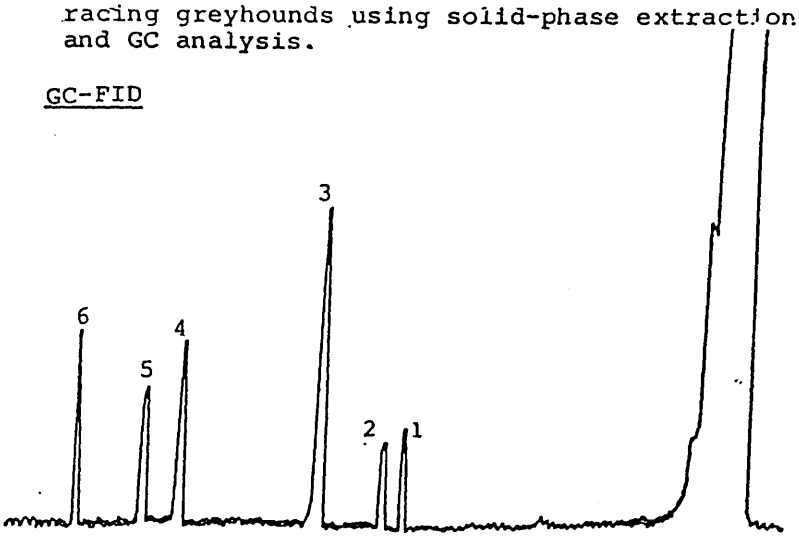
1,2,4,5:metabolites of cyclizine
3:cyclizine 6:papaverine

GC-NPD



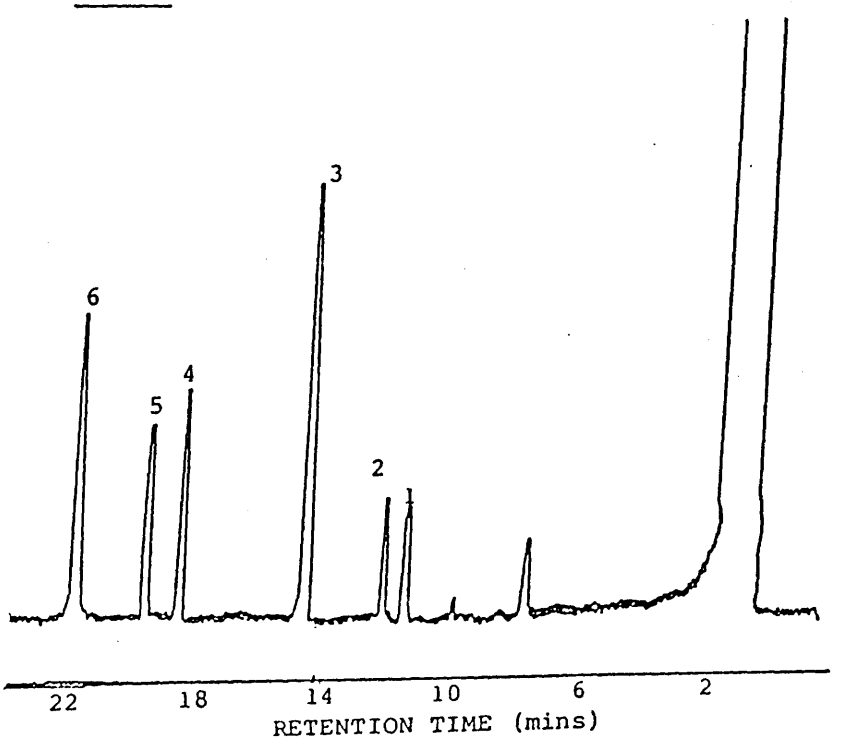
b) Cyclizine extracted from the urine of racing greyhounds using solid-phase extraction and GC analysis.

GC-FID



1,2,4,5:metabolites of cyclizine
3:cyclizine 6:papaverine

GC-NPD



2. Mazindol

Mazindol in racehorse urine samples

Horse urine samples were prepared by adding microlitre aliquots of a standard solution of the drug (1mg/ml) in methanol to urine (1ml). Solutions containing 100 ng to 10 ug/ml of drug were prepared in this manner, mixed by vortexing and kept at $4^{o}C$ until required for analysis.

The spiked samples were extracted as previously described, with slight modifications. The larger capacity SCX columns were used (3ml) and 10ml of sample, buffered to pH3.5 with 7mM phosphoric acid (5ml) was applied to the column.

For HPLC analysis, the sample was directly injected into the system, and the percentage recovery determined from the spiked samples, but for GC-MSD analysis, the extract was evaporated to dryness and reconstituted in chloroform (25ul) prior to injection.

Recovery of mazindol from spiked urine using the outlined extraction procedure and HPLC analysis was found to be 99.4 ± 7.7% for urine between 0.1 and 10 ug/ml (Table 38).

This is a simple and rapid technique which allows 100% recovery of low levels of drug from urine. The extract is sufficiently clean so as to allow its direct injection onto an HPLC or GC-MS (Figures 38, 39 respectively).

TABLE 38.

RECOVERY OF MAZINDOL FROM RACEHORSE URINE USING SCX COLUMNS

Concentration (ng/ml)	Recovery (%)
100	98.1
200	103.6
500	96.8
1000	93.0
5000	106.0
10000	98.8
	99.4

FIGURE 38.

Mazindol extracted from the urine of racehorses- 4-6 hours after a single dose- using cation exchange columns and HPLC analysis.

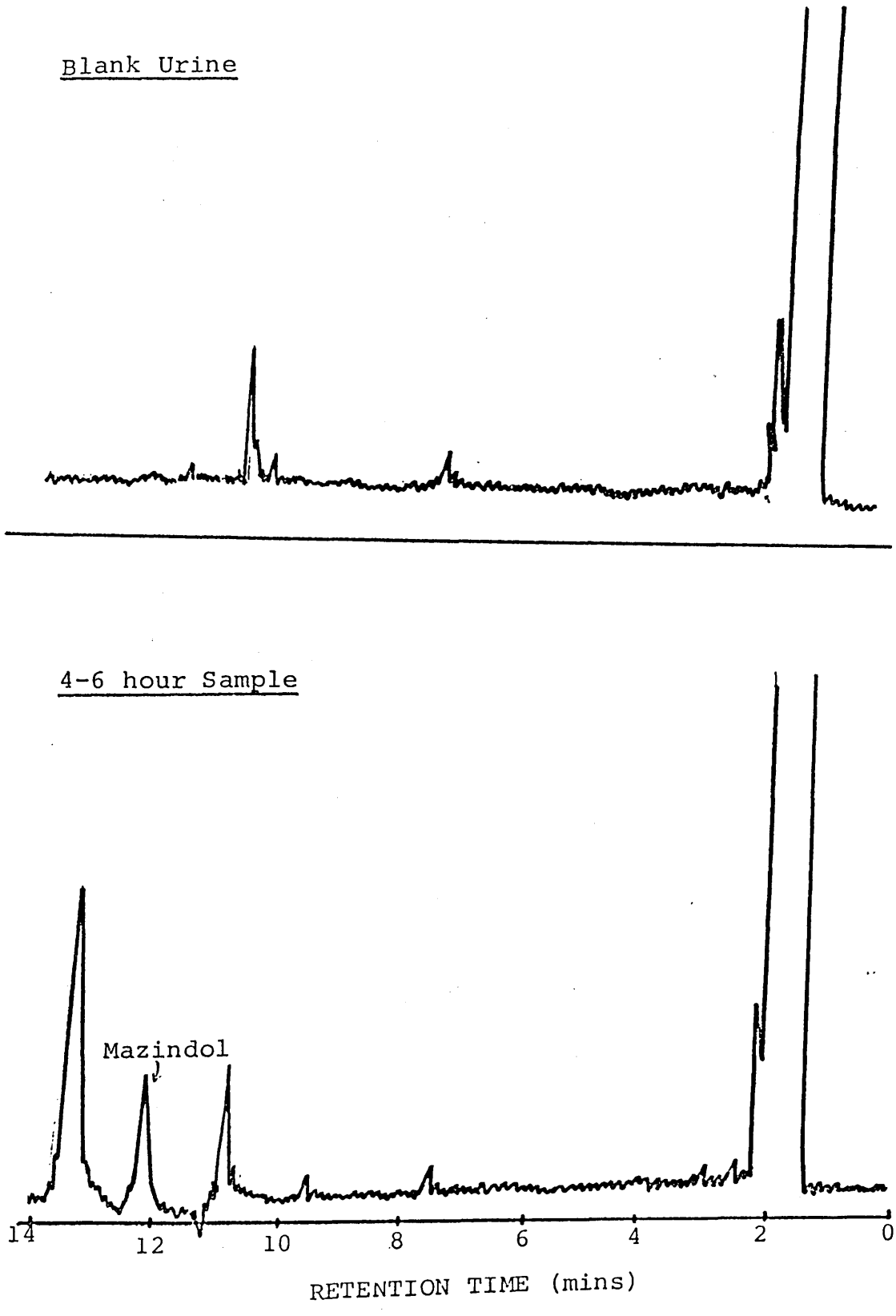
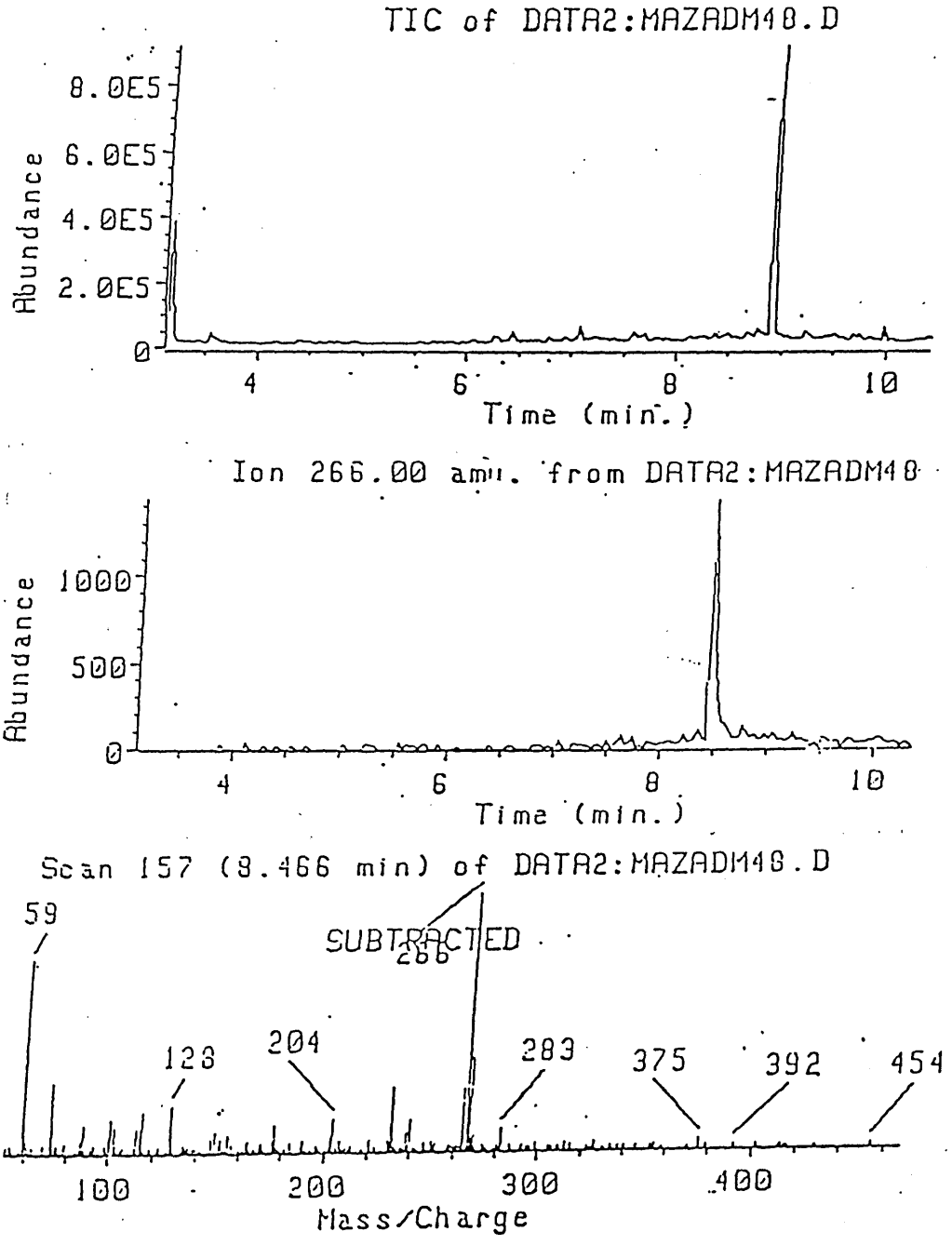


FIGURE 39.

Mazindol extracted from the urine of racehorses-4-6 hours after a single oral dose-using cation exchange columns and GC-MS analysis.



The advantages of this method are particularly apparent in the analysis of mazindol, where very little of the parent drug is present in the biological fluid.

Extraction of racehorse samples

Three racehorses were each given an oral dose of 50mg of mazindol, and urine samples were taken at timed intervals after administration.

This was repeated following an oral dose of 5mg of mazindol.

Mazindol was readily identified in the urine of racehorses having received an oral dose of 50mg (Table 39; Figure 40).

These 50mg administration results show good agreement with those reported (Timmings et al., 1985) regarding the excretion pattern of parent mazindol in the horse. The peak excretion time of between 4 and 6 hours is in agreement, but mazindol levels quantified by this method were somewhat higher than those reported.

This can be accounted for by comparing the percentage recoveries of the two methods (Timmings: 50%).

No parent mazindol was detected after 28 hours.

Analysis of 5mg dosings gave negative results on both HPLC and GC-MS.

TABLE 39.

MAZINDOL LEVELS IN HORSE URINE AFTER 50MG ADMINISTRATION

Hours After Administration	Mazindol Concentration* (ng/ml)
0→1	43.85
1→2	164.40
2→4	216.81
4→6	313.20
6→8	180.72
28	negative
52	negative

* Average of three determinations

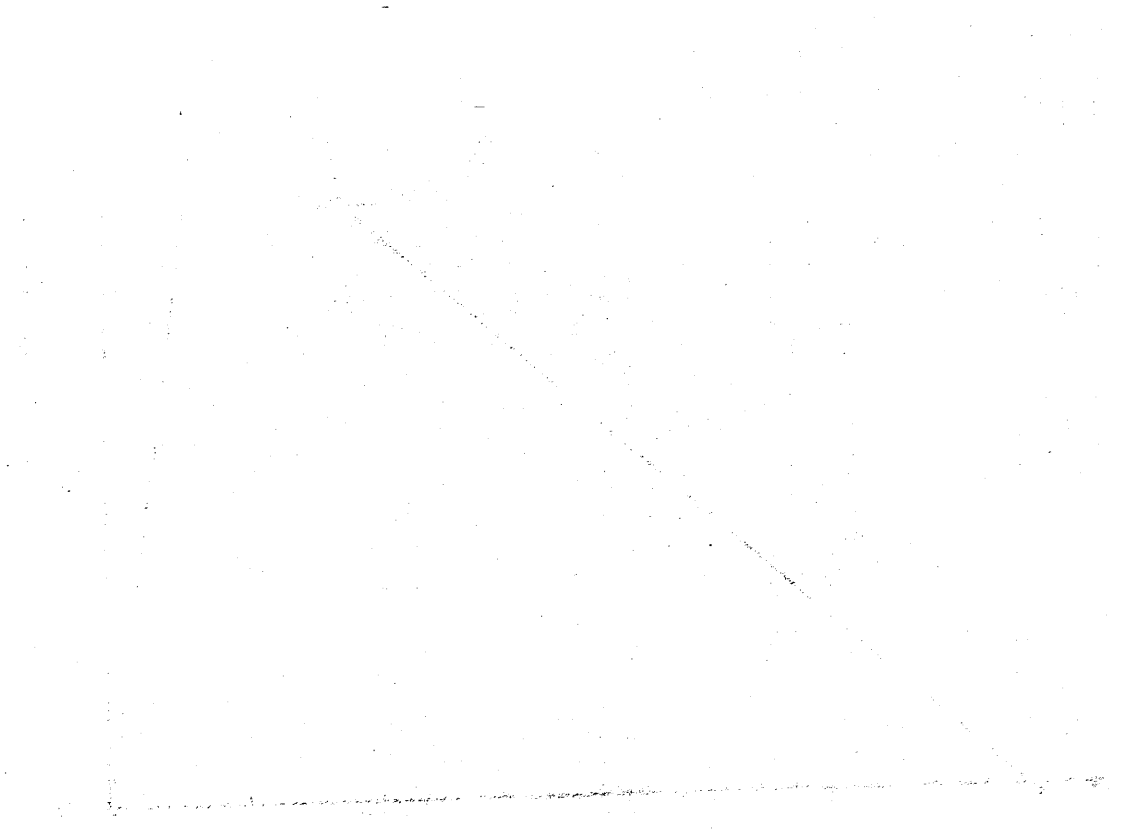
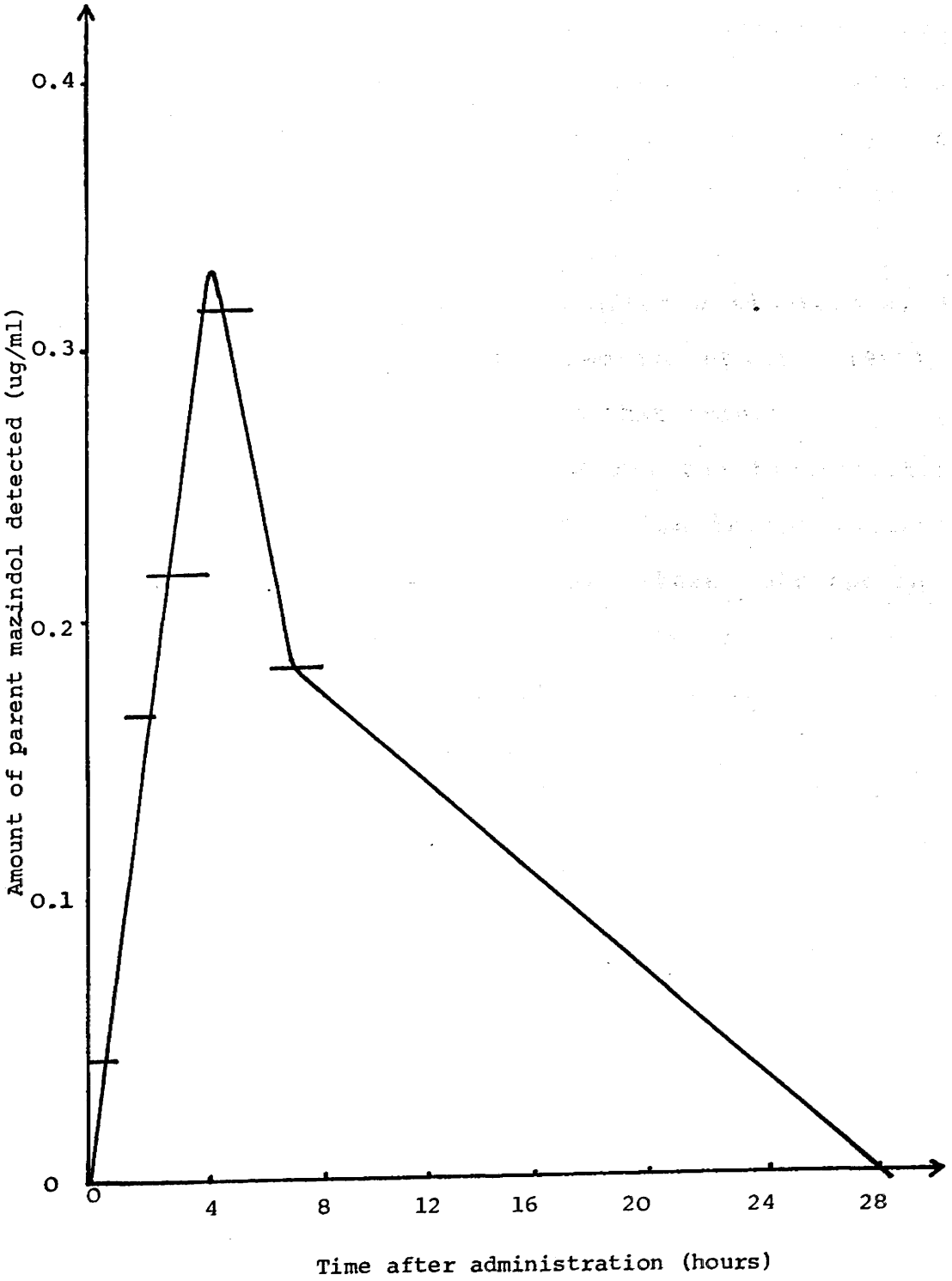


FIGURE 40.

Average mazindol excretion from racehorse urine.



Pharmacokinetics

Figure 41 shows a semi-logarithmic plot of mazindol concentration versus time after administration. No literature regarding mazindol pharmacokinetic parameters for the racehorse was available, but overall calculated data (Table 40) suggest that mazindol is fairly slowly eliminated from the horse (elimination half-life: 3 hours).

Mazindol was detected 12 hours after a single oral dose in the urine of racehorses (Timmings et al., 1985) but no parameters were calculated in that report.

The rate of absorption is slow and the bioavailability of 51% suggests incomplete absorption in the racehorse, differing from dog and man, where absorption is prolonged, but complete (Dugger et al., 1977). The elimination rate in racehorses is slow and this is also the case for both dog and man.

FIGURE 41.

Semi-logarithmic plot of mazindol excretion from racehorse urine.

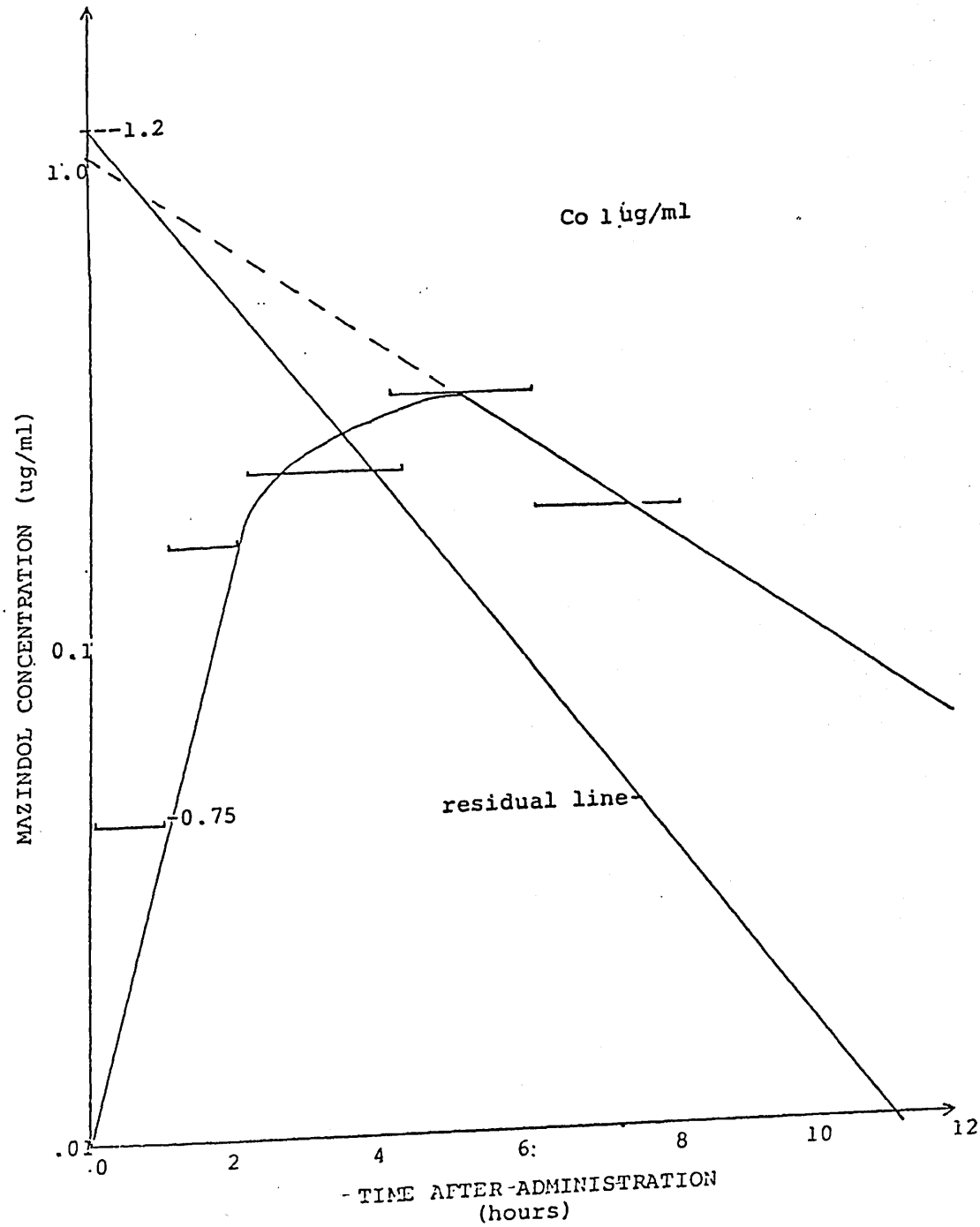


TABLE 40.

PHARMACOKINETIC PARAMETERS FOR MAZINDOL IN THE URINE OF RACEHORSES

Co = 1.0 ug/ml

$T_{0.5}$ = 3.0 hours

Vd = dose/Co = 50000/1.0 = 50 litres

k_{el} = 0.231/hour.

Clu = 11.55 litres/hour.

AUC = 4.33

k_{ab} = 0.469/hours

$T_{0.5}ab$ = 1.48 hours.

F (bioavailability) = 0.51 (51%).

Conclusion

Using cation exchange extraction, the identification of parent mazindol in the racehorse is possible 12 hours after administration.

A level of 800pg of mazindol using this method was detected which should be sufficient to detect the drug following administration of an effective dose.

There is some debate as to the effective dose required to excite a horse.

Even with doses of 100mg, given intravenously, veterinarians' have observed only a slight increase in irritability and respiratory rate and no change in pulse rate (Veterinarians Reports, Illinois Racing Board, Elgin, Illinois). It is therefore unlikely that a dose as low as 5mg would improve the racing performance of a horse.

5. CONCLUSIONS

Analysis

Benzodiazepines are normally determined, by HPLC, using reversed-phase columns and incorporating phosphate buffers in the mobile phases.

In this study, an HPLC system which separated nineteen benzodiazepines was developed, although not all were chromatographically resolved from each other. The system did not incorporate phosphate buffers, so removing the possibility of column blocking and subsequently removing the time-consuming process of column flushing and washing.

Further, the system was LC-MS compatible so that, if necessary, the positive identification of any interfering peaks could be carried out.

The system was sufficiently sensitive for the determination of diazepam and its metabolites, triazolam and its metabolites, and flunitrazepam and its metabolites in biological fluids at therapeutic levels.

Of particular note in this study, was the use of HPLC determination using diode array detection. The system was able to identify all samples analysed without recourse to further identification procedures. The analysis system was fully automated and allowed determination of at least one hundred basic drugs.

Reversed-phase HPLC systems for the determination of the basic drugs xylazine and mazindol in biological fluids, are not reported in the literature.

In this study, such systems were successfully developed.

Several methods were tried, and it was ultimately shown that suitable chromatography could be achieved for these drugs. The methods were sufficiently sensitive to determine the amount of drug in urine after a single administration of a therapeutic dose to greyhounds and racehorses respectively.

Extraction

Basic drugs are usually extracted from biological fluids using liquid/liquid or non-selective solid-phase extraction. In this study, the extraction of basic drugs from urine was explored using bonded sorbents, and the drugs were successfully isolated.

Sorbents exhibiting non-polar, polar and ion-exchange interactions were studied, and it was ultimately shown that the extraction of basic drugs from biological fluids could be achieved successfully and efficiently using any of the three types of cartridges.

Non-polar sorbents were shown to be highly efficient for the extraction of substances with a high degree of non-polar character, from urine. They were used successfully to extract oxazepam and xylazine from the urine of racing greyhounds.

Polar sorbents were successfully used for the more selective, highly efficient extraction of nineteen benzodiazepines and xylazine from human and greyhound urine.

Finally, ion-exchange sorbents were used to extract a wide range of basic drugs from racehorse urine, human post-mortem urine and greyhound urine.

Of particular note, using ion-exchangers, was the successful extraction of the amphoteric drugs, benzoylecgonine and morphine.

These two compounds are particularly difficult to extract using liquid/liquid extraction or non-selective solid-phase extraction.

The success of this technique showed that ion-exchange sorbents were particularly suitable for basic drug screening procedures especially when combined with determination with HPLC/DAD detection.

Authentic samples

The bonded sorbent methods were applied to authentic urine samples, and were shown to be suitable for the extraction of therapeutic levels of oxazepam, triazolam and its metabolites, and xylazine from greyhound urine; mazindol from racehorse urine; and a wide range of basic drugs from human post-mortem urine.

Detection after administration

Oxazepam was detected in the urine of racing greyhounds thirty hours after the administration of a single oral dose of diazepam; unchanged mazindol was detected twelve hours post-administration of a single therapeutic dose; and triazolam and its metabolites and unchanged xylazine were detected eight hours after dosing. The presence of drugs and their metabolites in the urine of greyhounds or racehorses administered with these drugs shows that the use or misuse of these drugs at therapeutic levels would be detected and confirmed in a racing situation.

Pharmacokinetic parameters

The metabolic routes of many drugs in racing greyhounds are not widely reported, therefore pharmacokinetic parameters for racing greyhounds are not widely available.

Preliminary pharmacokinetic studies carried out using the extraction and analysis procedures developed, showed that greyhound metabolism is markedly different from the metabolism of domestic dogs. These procedures can be subsequently applied to many basic drugs to allow the study of metabolism in racing greyhounds.

Financial requirements

Liquid/liquid extraction is a time-consuming, tedious procedure, often requiring several extraction stages, evaporation and derivatisation steps prior to analysis.

The solid-phase extraction procedures developed are quicker, more efficient, cheaper and easier to perform than conventional solvent extraction.

Several samples can be simultaneously extracted and the glassware, sample and solvent requirements are considerably less than with liquid/liquid extraction.

APPENDIX 1.

Technical Details

1.1. Drugs

Alprazolam (Xanax®), triazolam (Halcion®), 1-hydroxy triazolam and 4-hydroxytriazolam were gifts from Upjohn Ltd., Crawley, Sussex.

Clobazam (Frisium®) was a gift from Albert Products, a division of Hoescht UK Ltd., Hounslow, Middlesex.

Clonazepam (Rivotril®), diazepam (Valium®), desmethyldiazepam, flunitrazepam (Rohypnol®), 7-aminoflunitrazepam, desmethylflunitrazepam, midazolam (Hypnovel®) and nitrazepam (Mogadon®) were gifts from Roche Products Ltd., Welwyn Garden City, Hertfordshire.

Tritiated diazepam was purchased from Amersham International, Amersham, Hertfordshire.

Lorazepam (Ativan®), lormetazepam, oxazepam (Serenid®) and temazepam (Normison®) were donated by Wyeth Laboratories, Maidenhead, Berkshire.

Loprazolam (Dormonoct®) was donated by Roussel Laboratories Ltd., Wembley, Middlesex.

Prazepam (Centrax®) was purchased from William R. Warner & Co., Eastleigh, Hampshire.

Xylazine (Rompun®) was a gift from Bayer UK Ltd., Bury St. Edmunds, Suffolk.

Mazindol (Sanorex®) was purchased from Sandoz Pharmaceuticals Ltd., New Jersey, U.S.A.

Codeine and methadone hydrochloride were purchased from The Sigma Chemical Company, Missouri, U.S.A.

Morphine sulphate was purchased from Merck, Sharp and Dohme, Pennsylvania, U.S.A.

Robitussin A-C, containing codeine (2mg/ml), was purchased from A.H.Robins, Virginia, U.S.A.

Paregoric (Parepectolin), containing opium (0.38mg/ml), was purchased from W.H.Rorer, Pennsylvania, U.S.A.

Methajade, containing methadone (0.3mg/ml) was purchased from Merck, Sharp and Dohme, Pennsylvania, U.S.A.

1.2. Solvents

All the solvents used were of HPLC grade and were purchased from Rathburn Chemicals, Walkerburn, Scotland and BDH Chemicals, Poole, Dorset.

1.3. Chemicals

Disodium hydrogen orthophosphate dihydrate (Sorensons salt), sodium acetate, sodium hydrogen carbonate and ammonium acetate were purchased from BDH Chemicals, Poole, Dorset.

Disodium tetraborate and pentane sulphonic acid were purchased from The Sigma Chemical Company, Missouri, U.S.A.

Ecoscint® (biodegradable liquid scintillant) was purchased from National Diagnostics, Edinburgh.

Tetramethyl ammonium hydroxide pentahydrate (TMAH) and B-glucuronidase (Helix Pomatia Juice) were purchased from The Sigma Chemical Company, Poole, Dorset.

1.4. Extraction Equipment

The Vac-Elut® manifold was purchased from Crawford Scientific (Analytichem International), Strathaven, Lanarkshire.

The extraction columns were donated by and purchased from various suppliers:- Bond-Elut® cartridges from Crawford Scientific (Analytichem International), Strathaven, Lanarkshire; Spe-ed® cartridges from Laboratory Impex Ltd. (Applied Separations Inc.), Teddington, Middlesex; SPE™ cartridges from J.T.Baker UK, Hayes, Middlesex.

1.5. Buffers

a) HPLC phosphate buffer (pH8): 1.7g/l Sorensons salt.

b) Phosphate buffer (pH10.25): (a) + sodium hydroxide to the required pH.

c) 0.1M sodium acetate buffer (pH5): 13.6g/l sodium acetate adjusted to pH5 with acetic acid.

d) 0.01M ammonium acetate (pH7): 0.77g/l ammonium acetate.

e) 7mM phosphoric acid: 1 drop of 85% phosphoric acid in deionised water (10ml).

f) 0.2M borate buffer (pH9): 7.6g disodium tetraborate in deionised water (100ml)

1.6. Eluents

a) Methanolic hydrochloric acid: Methanol (50ml) was added to 36% w/w hydrochloric acid (3ml).

b) Methanolic ammonium hydroxide: Methanol (99ml) was added to concentrated ammonium hydroxide (1ml).

APPENDIX 2.

Calculation of Pharmacokinetic Parameters (Clark and Smith, 1986; Bourne et al., 1986).

	Units
Co = Apparent value for urine concentration at zero time. This is calculated by extrapolation of the semi-logarithmic plot of drug concentration .v. time to time zero.	ug/ml
$T_{0.5}$ = Time taken for the concentration of drug in urine to decline to half its original value. This is calculated from the same graph and is equal to Co/2.	hours
Vd = Volume of distribution = dose/Co.	litres
k_{el} = Rate of elimination = 0.693/ $T_{0.5}$.	hours-1
Clu = Clearance rate = Vd x k_{el}.	l/hr
AUC = Area under curve = Co/k_{el}.	
k_{ab} = Rate of absorption = residual line slope x 2.303	hours-1
$T_{0.5}ab$ = Half-life of absorption = 0.693/k_{ab}.	hours
Fraction of oral dose absorbed, F, (bioavailability) F = ((Co/k_{el}) - (Co/k_{ab}))/AUC.	%

APPENDIX 3.

Cost of chemicals and solvents for the financial comparison of solvent extraction with solid-phase extraction.

Solvent	Cost	Cost/10 extns.
Ethyl acetate	£7.90/2.5 litres	£3.16
Sulphuric acid	£5.20/2.5 litres	0.2p
Ammonium hydroxide	£3.50/2.5 litres	2p
85% phosphoric acid	£56.50/0.5 litre	0.06p
1N acetic acid	£20.50/carton of 6	1.8p
Methanol	£6.50/2.5 litres	6.5p
Acetonitrile	£14.20/2.5litres	1.5p
Chemicals		
Anhydrous Na_2SO_4	£7.70/500g	£1.54

Total Cost per 10 extractions (polar extractions):

Solvent extraction: £3.16 + 2p + 0.2p + £1.54 = £4.72

Solid-phase extraction : 6.5p + 0.06p + 1.8p + 1.5p = 10p

Total Cost per 10 extractions (ion-exchange extractions):

Solid-phase extraction : 6.5p + 0.06p + 1.8p = 8p

7. REFERENCES

Adams, W.J. (1979), Specific and sensitive HPLC determination of alprazolam or triazolam. Analytical Letters, 12(B6), pp.657-671.

Akbari, A., Gordon, B.J., Bush, P.B. and Moore, J.M. (1988), Determination of xylazine in blood components using high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 426, pp. 207-211.

Alvinerie M. and Toutain, P.L. (1981), Determination of xylazine in plasma using high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 222, pp.308-310.

Anderson, W.H. and Stafford, D.T. (1983), Applications of gas chromatography in routine toxicological analysis. Journal of High Resolution Chromatography and Chromatographic Communications, 6, pp. 247-253.

Axelson, M., Sahlberg, B.L. and Sjovall, J. (1981) Analysis of profiles of conjugated steroids in urine by ion exchange separation and gas chromatography-mass spectrometry. Journal of Chromatography, 224, pp.355-370.

Barcza, S. and Houlihan, W.F. (1975), Structure determination of the anorexic agent, mazindol. Journal of Pharmaceutical Science, 64, pp.829-831.

Baselt, R.C., Stewart, C.B. and Franch, S.J. (1977), Toxicological determination of benzodiazepines in biological fluids and tissues by FI-GC. Journal of Analytical Toxicology, 1, pp.10-13.

Baktir, G., Bircher, J., Fisch, H.U. and Karlaganis, G. (1985), Capillary GLC determination of the benzodiazepine triazolam in plasma using a retention gap. Journal of Chromatography, 339(1), pp.192-197.

Bellantuono, C., Reggi, G., Tognoni, G. and Garattini, S. (1980), Benzodiazepines: Clinical pharmacology and therapeutic use. Drugs, 19, pp.195-219.

Bogusz, M., Klys, M., de Zeenw, R.A. and Franke, J.P. (1985) Applicability of diode array spectrophotometric detector in toxicological analysis. A preliminary report. In Proceedings of the 22nd Meeting of The International Association of Forensic Toxicologists (TIAFT), Switzerland, ed. Brandenberger, H.and R., pp.125-134.

Bourne, D.W.A., Triggs, E.J. and Eadie, M.J. (1986), Pharmacokinetics for the non-mathematical. Lancaster, England, MTP Press Ltd..

Breiter, J., Helger, R. and Lang, H. (1976), Evaluation of column extraction: A new procedure for the analysis of drugs in body fluids. Forensic Science, 7, pp.131-140.

Brodie, R.R., Chasseaud, L.F. and Taylor, T. (1978), HPLC determination of benzodiazepines in human plasma. Journal of Chromatography, 150, pp.361-366.

Brooks, M.A., Hackman, M.R., Weinfeld, R.E. and Macasieb, T. (1977), Detemination of chlorazepate and its major metabolites in blood and urine by EC-GLC. Journal of Chromatography, 135(1), pp. 123-131.

Bugge, A. (1976), Quantitative HPLC of diazepam and desmethyldiazepam in blood. Journal of Chromatography, 128(1), pp.111-116.

Carlucci, G. (1988), A high-performance liquid chromatographic method for the determination of doxazepam in human plasma using a solid-phase extraction column. Journal of Liquid Chromatography, 11(7), pp.1559-1568.

Carruthers,S.G., Nelson, M., Wexler, H.R. amd Stiller, C.R. (1979), Xylazine hydrochloride (Rompun) overdose in man. Clinical Toxicology, 15, pp.281-285.

Chiarotti, M., de Giovanni, N. and Fiori, A., (1986), Analysis of benzodiazepines. I. Chromatographic identification. Journal of Chromatography, 358, pp.169-178.

Christensen, H. (1984), Methodological alterations affecting the recovery of drugs from biological material using liquid-liquid extraction procedure as Extrelut®-programme in forensic chemistry. In Proceedings of the 21st Meeting of The International Association of Forensic Toxicologists (TIAFT), U.K., ed. Dunnett, N. and Kimber, J. pp.51-58.

Clark, B. and Smith, D.A. (1986), An introduction to pharmacokinetics. 2nd Edition, Oxford, England. Blackwell Scientific Publications.

Clarke, (1986), Isolation and identification of drugs. ed. Moffat, A.C., The Pharmaceutical Press, London.

Clarke, K.W. and Hall, L.W. (1969), Xylazine. A new sedative for horses and cattle. Veterinary Record, 85, pp.512-517.

Clifford, J.M. and Smyth, W.F. (1974), Determination of some 1,4, benzodiazepines and their metabolites in body fluids - a review. The Analyst, 99(1178), pp.241-272.

Coassolo, Ph., Aubert, C. and Cano, J.P. (1983), Simultaneous assay of triazolam and it's main hydroxy metabolite in plasma and urine by capillary gas chromatography. Journal of Chromatography (Biomedical Applications), 274, pp.161-170.

Cotler, S., Puglisi, C.V. and Gustafson, J.H. (1981), Determination of diazepam and it's major metabolites in man and in the cat by high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 222, pp.95-106.

Daenens, P., Bruneel, N. and Van Buren, M. (1980), GC method for the determination of chloralose in biological samples. Toxicological Aspects, In Proceedings of the 9th International Congress of the European Association of Poison Control Centres and the European Meeting of The International Association of Forensic Toxicologists (TIAFT), Greece, ed. Kovatsis, A.N., pp.204-211.

De-Gier, J.J. and Hart, B.J. (1979), Sensitive GC method for determination of diazepam and N-desmethyldiazepam in plasma. Journal of Chromatography, 163(3), pp.304-309.

De-Groot, G. and Grotenhuis-Mullenders, A.M.L. (1983), Combined bonded-phase/liquid-liquid extraction as a rapid specific sample pre-treatment technique for the determination of benzodiazepines in plasma by RP-HPLC. Topics in Forensic and Analytical Toxicology, 20, pp.95-103. In Proceedings of the Annual European meeting of The International Association of Forensic Toxicologists, W. Germany.

Delbeke, F.T. and Debackere, M. (1978), Detection of sympathomimetric central nervous stimulants with special reference to doping. III. Column extraction with Extrelut®. Journal of Chromatography, 161, pp.360-365.

De-Silva, J.A. and Bekersky, I. (1974), Determination of clonazepam and flunitrazepam in blood by EC-GLC. Journal of Chromatography, 99, pp.447-460.

Dhar, A.K. and Kutt, H. (1978), Monitoring diazepam and desmethyldiazepam concentrations in plasma by gas-liquid chromatography, with use of nitrogen-sensitive detector. Clinical Chemistry, 25, pp.137-140.

Dixon, W.R., Earley, J. and Postma, E. (1975), Radioimmunoassay of chlordiazepoxide in plasma. Journal of Pharmaceutical Science, 64, pp.937-939.

Dordain, G., Puech, A.J. and Simon, P. (1981), Triazolam compared with nitrazepam and oxazepam in insomnia: Two double blind crossover studies analyzed sequentially. British Journal of Clinical Pharmacology, 11, pp.43S-49S.

Dugger, H.A. and Heider, J.G. (1979), Biotransformation of mazindol. II. Absorption and excretion in the dog and man. Drug Metabolism and Disposition, 7(3), pp.129-131.

Dugger, H.A., Coombs, R.A., Schwarz, H.J., Migdalof, B.H. and Orwig, B.A. (1976), Drug Metabolism and Disposition, 4, pp.262-268.

Dugger, H.A., Madrid, V.O., Talbot, K.C., Coombs, R.A. and Orwig, B.A. Biotransformation of mazindol. III. Comparison of metabolism in rat, dog and man. Drug Metabolism and Disposition, 7(3), pp.132-137.

Eberts, F.S.Jr. (1974), Comparative metabolism of triazolam, a new sedative-hypnotic in rat, dog and man. Pharmacologist, 16, pp.196- .

Eberts, F.S.Jr. (1977), Disposition of triazolam (8-chloro-6-(o-chlorophenyl)-1-methyl-4H-s-triazolo[4,3-a][1,4]-benzodiazepine) in the dog. Drug Metabolism and Disposition, 5, pp.547-555.

Eberts, F.S.Jr. (1979), Disposition of 11-C triazolam, a short-acting hypnotic in man. The Pharmacologist, 21, pp.168-175.

Faber, B.D., Kok, R.M. and Dijk, E.M. (1977), Quantitative gas chromatographic analysis of flunitrazepam in human serum with electron capture detection. Journal of Chromatography, 133(2), pp.319-326.

Fami, L., Lim, C.K. and Peters, T.J. (1987), Solid phase extraction and direct HPLC separation of bilirubin and it's conjugates in plasma. Chromatographia, 24, pp.637-638.

Ferslew, K.E., Hagardorn, A. and McCormick, W.F., (1989), Post-mortem determination of the biological distribution of sufentanil and midazolam after an acute intoxication. Journal of Forensic Sciences, 34(1), pp.249-257.

Fillingim, J.M. (1979), Double-blind evaluation of the efficacy and safety of temazepam in outpatients with insomnia. British Journal of Clinical Pharmacology, 8, pp.73S-77S.

Foerster, E.H. and Mason, M.F. (1974), Preliminary studies on the use of n-butyl chloride as an extractant in a drug screening procedure. Journal of Forensic Sciences, 19, pp.155-162.

Ford, B., Vine, J. and Watson, T.R. (1983), A rapid extraction method for acidic drugs in hemolyzed blood. Journal of Analytical Toxicology, 7, pp.116-119.

Fraser, A.D. (1987), Urinary screening for alprazolam, triazolam and their metabolites with EMIT d.a.u. benzodiazepine metabolite assay. Journal of Analytical Toxicology, 11(6), pp.263-266.

Frethold, J. and Sunshine, I. (1980), The use of Bond-Elut columns for plasma preparation in anti-epileptic and theophylline HPLC analysis. Clinical Chemistry, 26, pp.1054.

Frings, C.S. and Cohen, P.S. (1971), Rapid colorimetric method for the quantitative determination of librium in serum. American Journal of Clinical Pathology, 56, pp.216-219.

Gallanosa, A.G., Spyker, D.A., Shipe, J.R. and Morris, D.D. (1981), Human xylazine overdose : A comparative review of clonidine, phenothiazines and tricyclic antidepressants. Clinical Toxicology, 18, pp.663-678.

Garcia-Villar, R., Toutain, P.L., Alvinerie, M. and Ruckebusch, Y. (1981), The pharmacokinetics of xylazine hydrochloride : an interspecific study. Journal of Veterinary Pharmacological Therapeutics, 4, pp.87-92.

Gault, M.H. (1985), Combined liquid chromatography/radioimmunoassay with improved specificity for serum digoxin. Clinical Chemistry, 31(8), pp.1272-1277.

George, K.A. and Dundee, J.W. (1977), Relative amnesic actions of diazepam, flunitrazepam and lorazepam in man. British Journal of Clinical Pharmacology, 4, pp.45-50.

Gill, R., Law, B. and Gibbs, J.P. (1986), HPLC systems for the separation of benzodiazepines and their metabolites. Journal of Chromatography, 356(1), pp.37-46.

Gonnet, C. and Rocca, J.L. (1976), Separation de produits pharmaceutiques par chromatographie en phase liquide a haute performance. Influence de l'eau.II. Journal of Chromatography, 120, pp.419-433.

Good, T.J. and Andrews, J.S. (1981), The use of bonded-phase extraction columns for rapid sample preparation of benzodiazepines and metabolites from serum for HPLC analysis. Journal of Chromatographic Science, 19, pp.562-566.

Greenblatt, D.J. (1978), Simultaneous gas chromatographic analysis for diazepam and it's major metabolite, desmethyldiazepam, with use of double internal standardisation. Clinical Chemistry, 24, pp.1838-1841.

Greenblatt, D.J. (1978), Determination of desmethyldiazepam in plasma by electron-capture GLC : Application to pharmacokinetic studies of chlorazepate. Journal of Pharmaceutical Science, 67, pp.427-429.

Greenblatt, D.J. and Divoll, M. (1981), Plasma concentrations of temazepam, a 3-hydroxy benzodiazepine determined by electron-capture gas liquid chromatography. Journal of Chromatography, 222, pp.125-128.

Greenblatt, D.J., Divoll, M. and Abernathy, D.R. (1983), Reduced clearance of triazolam in old age:relation to antipyrine oxidising capacity. British Journal of Clinical Pharmacology, 15, pp.303-309.

Greenblatt, D.J., Divoll, M., Moschitto, L.J. and Shader, R.I (1981), Electron-capture detector-gas chromatographic analysis of the triazolobenzodiazepines, alprazolam and triazolam. Journal of Chromatography, 225(1), pp.202-207.

Greenblatt, D.J., Ochs, H.R. and Loyd, B.L. (1980), Entry of diazepam and it's major metabolite into cerebrospinal fluid. Psychopharmacology, 70, pp.89-93.

Greenblatt, D.J., Shader, R.I., Divoll, M. and Harmatz, J.S. (1981), Benzodiazepines: A summary of pharmacokinetic properties. British Journal of Clinical Pharmacology, 11, pp.11S-16S.

Hailey, D.M. (1974), Chromatography of the 1,4 benzodiazepines. Journal of Chromatography (Chromatographic Reviews), 98, pp.527-568.

Hama, K., Akane, A., Maseda, C., Tanabe, K. and Fukui, Y. (1987), Analysis of etizolam, triazolam, nitrazepam and maprotiline in autopsy materials by HPLC and GC. Nippon Hoigaku Zasshi, 41(1), pp.45-51.

Harkey, M.A. and Stolowitz, M.L. (1984), Solid phase extraction techniques for biological specimens. In Advances in Analytical Toxicology. ed. Baselt, R.E., Ch.9, pp.255-269, Foster City, California.

Harrison, P.M., Tonkin, A.M., Dixon, S.T. and McLean A.T. (1986), Determination of alpha-naphthoxlactic acid, a major metabolite of propanolol in plasma by HPLC. Journal of Chromatography, 374, pp.223-225.

Haver, V.M., Porter, W.H., Dorie, L.D. and Lea, J.R. (1986), Simplified HPLC method for the determination of clonazepam and other benzodiazepines in serum. Therapeutic Drug Monitoring, 8(3), pp.352-357.

Hill, D.W., Kelley, T.R., Matiuck, S.W., Langner, K.J. and Phillips, D.E. (1982), Single extraction for the recovery of basic, neutral and weakly acidic drugs from greyhound dog urine. Analytical Letters, 15(B2), pp. 193-204.

Higuchi, S., Urabe, H. and Shiobara, Y. (1979), Simplified determination of lorazepam and oxazepam in biological fluids by GC-MS. Journal of Chromatography, 164(1), pp.55-61.

Hirayama, H. and Kasuya, Y. (1983), High-pressure liquid chromatographic determination of bromazepam in human plasma. Journal of Chromatography (Biomedical Applications), 277, pp.414-418.

Horning, M.G., Gregory, P., Nowlin, J., Stafford, M., Lertratanangkoon, K., Butler, C., Stillwell, W.G. and Hill, R.M. (1974), Isolation of drugs and drug metabolites from biological fluids by use of salt-solvent pairs. Clinical Chemistry,, 20, pp.282-287.

Horton-McCurdy, H., Slightom, E.L. and Harrill, J.C. (1979), Diazepam and desmethyldiazepam quantitation in whole blood by nitrogen sensitive detector after derivatisation. Journal of Analytical Toxicology, 3, pp.195-197.

Horton-McCurdy, H., Lewellen. L.J., Cagle, J.C. and Solomons, E.T. (1981), A rapid procedure for the screening and quantitation of barbiturates, diazepam, desmethyldiazepam and methaqualone. Journal of Analytical Toxicology, 5, pp.253-256.

Horvath, C. and Melander, W. (1977), Liquid chromatography with hydrocarbonaceous bonded phases: Theory and practice of reversed-phase chromatography. Journal of Chromatographic Science, 15, pp.393-404.

Hulshoff, A. and Perrin, J.H. (1976), A comparison of the determination of partition coefficients of 1,4 benzodiazepines by high-pressure liquid chromatography and thin-layer chromatography. Journal of Chromatography, 129, pp.263-276.

Hyde, P.M. (1985), Evaluation of drug extraction procedures from urine. Journal of Analytical Toxicology, 9, pp.269-272.

Inoue, T. and Suzuki, S-I. (1987), HPLC determination of triazolam and metabolites in human urine. Journal of Chromatography (Biomedical Applications), 422, pp.197-204.

Jatlow, P., (1972) Ultraviolet spectrophotometric measurement of chlordiazepoxide in plasma. Clinical Chemistry, 18, pp.516-518.

Jochemsen, R. and Breimer, D.D. (1981), Assay of triazolam by capillary gas chromatography. Journal of Chromatography, 223, pp.438-444.

Jochemsen, R. and Breimer, D.D. (1982), Assay of flunitrazepam, temazepam and desalkylflurazepam in plasma by capillary gc-ecd. Journal of Chromatography, 227(1), pp.199-206.

Kabra, P.M., Nelson, M.A., and Marton, L.J. (1983), Simultaneous very fast LC analysis of ethosoximide, primidone, phenobarbital, phenytoin and carbamazepine in serum. Clinical Chemistry 29(3), pp.473-476.

Kabra, P.M. and Nzekwe, E.U. (1985), Liquid chromatographic analysis of clonazepam in human serum with solid-phase Bond-Elut® extraction. Journal of Chromatography (Biomedical Applications), 341, pp.383-390.

Kabra, P.M., Stevens, G.L. and Marton, L.J. (1978), High-pressure liquid chromatographic analysis of diazepam, oxazepam and N-desmethyldiazepam in human blood. Journal of Chromatography, 150, pp.355-360.

Kaempe, B. (1983), Evaluation of quantitative determination of basic drugs isolated from blood with Extrelut®. Topics in Forensic and Analytical Toxicology, 20, pp.87-93. In Proceedings of the Annual European meeting of The International Association of Forensic Toxicologists, W. Germany.

Kaithsa, K.K. (1977), Guide to urine testing in drug abuse prevention and multi modality treatment programs. Journal of Chromatography, 141, pp.145-196.

Kaithsa, K.K. and Tadrus, R. (1978), Thin-layer detection of diazepam and/or chlordiazepoxide or in combination with major drugs of abuse in drug abuse urine screening programs. Journal of Chromatography, 154, pp.211-218.

Kangas, L. (1977), Comparison of two GLC methods for the determination of nitrazepam in plasma. Journal of Chromatography, 136(2), pp.259-270.

Kaplan, S.A., Alexander, K., Jack, M.L., Puglisi, C.V., de Silva, J.A.F., Lee T,K. and Weinfeld, R.E. (1974), Pharmacokinetic profiles of clonazepam in dog and humans and of flunitrazepam in dog. Journal of Pharmaceutical Science, 63(4), pp.527-532.

Kitigawa, H., Esumi, Y., Kurosawa, S., Sekine, S. and Yokoshima, T. (1979a), Metabolism of (8-chloro-6-(o-chlorophenyl)-1-methyl-4H-s-triazolo[4,3-a][1,4]-benzodiazepine), triazolam, a new central depressant. I Absorption, distribution and excretion in rats, dogs and monkeys. Xenobiotica, 9, pp.415-428.

Kitigawa, H., Esumi, Y., Kurosawa, S., Sekine, S. and Yokoshima, T. (1979b), II. Identification and determination of metabolites in rats and dogs. Xenobiotica, 9, pp.429-439.

Klockowski, P.M. and Levy, G. (1987), Simultaneous determination of diazepam and its active metabolites in rat serum, brain and cerebrospinal fluid by high-pressure liquid chromatography. Journal of Chromatography (Biomedical Applications), 422, pp.334-339.

Klotz, U. (1981), Determination of bromazepam by GLC and its application for pharmacokinetic studies im man. Journal of Chromatography, 222(3), pp.501-506.
Ko, H., Royer, M.E., Hester, J.B. and Johnston, K.T. (1977), Radioimmunoassay of triazolam. Analytical Letters, 10(13), pp.1019-1040.

Koch, S. and Levine, J. (1971), The determination of opium alkaloids in anti-diarrheal preparations. Microgram, 4(8), Number 18.

Koenigbauer,M.J., Assenza, S.P., Willoughby, R.C. and Curtis, M.A. (1987), Trace analysis of diazepam in serum using microbore high-performance liquid chromatography and on-line preconcentration. Journal of Chromatography (Biomedical Applications), 413, pp.161-169.

Komiskey, H.L., Rahman, A., Heisenburger, W.P., Hayton, W.L., Zobrist, R.H. and Silvius, W. (1985), Extraction, separation and detections of 14C-diazepam and 14C-metabolites from brain tissue of mature and old rats. Journal of Analytical Toxicology, 9, pp.131-133.

Koves, G. and Wells, J. (1986), The quantitation of triazolam in post mortem blood by gas chromatography/ negative ion chemical ionization mass spectrometry. Journal of Analytical Toxicology, 10, pp.241-244.

Laitem, L., Bello, I., and Gaspar, P. Gas chromatographic determination of tranquiliser residues in body fluids and in the meat of slaughtered animals. Journal of Chromatography, 156(2), pp.327-329.

Lau, C.E., Dolan, S. and Tang, M. (1987), Microsample determination of diazepam and it's three metabolites in serum by reversed-phase high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 416, pp.212-218.

Lensmeyer, G.L. (1986), Extraction of cyclosporin and antidepressant drugs from whole blood with the cyanopropyl solid-phase sorbent: Optimization process, separation mechanisns and sorbent quality. Analytichem Applications Note, Analytichem International, California.

Lewis, S. and O'Calaghan, C.L.P. (1983), Clinical curio : Self-medication with xylazine. British Medical Journal, 287, 1369.

Lindley, T.N. (1979), Temperature dependence of electron-capture detector responses in the analysis of chlorpromazine, diazepam, and clonazepam. Journal of Analytical Toxicology, 3, pp.18-20.

Lloyd, J.B.F. and Parry, D.A. (1988), Detection and determination of common benzodiazepines and their metabolites in blood samples of forensic science interest. Microcolumn cleanup and high-performance liquid chromatography with reductive electrochemical detection at a pendant mercury drop electrode. Journal of Chromatography, 449, pp.281-297.

Locniskar, A., Greenblatt, D.J. and Ochs, H.R. (1985), Simplified GC assay of underivatized nitrazepam in plasma. Journal of Chromatography, 337, pp.131-135.

Logan, B.K. (1988), Personal Communication, Health Science Centre, University of Tennessee, Memphis, Tennessee, U.S.A.

MacLeod, N. (1981), Triazolam: Monitored release in the U.K. British Journal of Clinical Pharmacology, 11, pp.51S-53S.

MacKichen, A.J., Jusko, W.J., Duffner, P.K. and Cohen, M.E. (1979), Liquid chromatographic assay of diazepam and its metabolites in serum. Clinical Chemistry, 25(6), pp.856-859.

Masahura, K., Yoshio, M. and Kentaro, H. (1982), Simultaneous determination of triazolobenzophenone (2,5-dichloro-2-3-glycylaminomethyl-5-methyl-4H-1,2,4-triazol-4-yl) benzophenone (TB) and its major blood metabolite, triazolam (TZ), in monkey plasma by EC-GLC. Journal of Chromatography, 229(2), pp.355-363.

Mascher, H., Nitsche, V. and Schutz, H. (1984), Separation, isolation and identification of optical isomers of 1,4-benzodiazepine glucuronides from biological fluids by reversed-phase HPLC. Journal of Chromatography, 306, pp.231-239.

Mattila, M.A.K. and Larni, H.M. (1980), Flunitrazepam: A review of it's pharmacological properties and therapeutic use. Drugs, 20, pp.353-374.

McDonald, I.A. (1985), An improved method for extracting catecholamines from body fluids. Journal of Neuroscientific Methods, 13, pp.239-248.

McNicholas, L.F., Martin, W.R. and Pruitt, T.A. (1985), N-desmethyldiazepam physical dependence in dogs. Journal of Pharmacology and Experimental Therapeutics, 235, pp.368-376.

Metzler, C.M., Ko, H., Royer, M.E., Veldkamp, W. and Linet, O.I. (1977), Bioavailability and pharmacokinetics of orally administered triazolam in normal subjects. Clinical Pharmacology and Therapeutics, 21, pp.111-112.

Minder, E.I., Schaubhut, R., Minder, C.E. and Vonderschmitt, D.J. (1987), Identification of drugs in human serum by high-performance liquid chromatography with photodiode array detection and a search algorithm for ultraviolet spectra. Journal of Chromatography (Biomedical Applications), 419, pp.135-154.

Moffett, R.B. (1976), New benzodiazepine analogues: Synthesis and CNS activity. Journal of Heterocyclic Chemistry, 3(Suppl.), pp.S123-S130.

Moore, B., Nickless, G., Hallett, C. and Howard, A.G. (1977), Analysis of nitrazepam and it's metabolites by high-pressure liquid chromatography. Journal of Chromatography, 137, pp. 215-217.

Moore, C.M. and Tebbett, I.R. (1987), Rapid extraction of anti-inflammatory drugs in whole blood for HPLC analysis. Forensic Science International 34, pp.155-158.

Mura, P., Piriou, A., Fraillon, P., Papet, Y. and Reiss, D. (1987), Screening procedure for benzodiazepines in biological fluids by high-performance liquid chromatography using a rapid-scanning multichannel detector. Journal of Chromatography (Biomedical Applications), 416, pp.303-310.

Newkirk, H.L. and Miles, D.G. (1974), Xylazine as a sedative analgesic for dogs and cats. Modern Veterinary Practice, 55, pp.677-680.

Nicholson, A.N. and Stone, B.M. (1980), Activity of the hypnotics flunitrazepam and triazolam in man. British Journal of Clinical Pharmacology, 9, pp.187-194.

Nolan, L. and O'Malley, K. (1988), Patients, prescribing, and benzodiazepines. European Journal of Clinical Pharmacology, 35, pp.225-229.

Osselton, D.M., Hammond, M.D. and Twitchett, P.J. (1977), The extraction and analysis of benzodiazepines in tissues by enzymatic digestion and high-performance liquid chromatography. Journal of Pharmacy and Pharmacology, 29, pp.460-462.

Osselton, D.M. (1979), The use of proteolytic enzymes to release high levels of drugs from biological materials submitted for toxicological analysis. Veterinary and Human Toxicology, 21 (supplement), pp.177-179.

Pakes, G.E., Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S. (1981), Triazolam: A review of it's pharmacological properties and therapeutic efficacy in patients with insomnia. Drugs, 22, pp.81-110.

Pape, B.E. and Ribick, M.A. (1977), Analysis of medazepam, diazepam and metabolites in plasma by GLC with ECD. Journal of Chromatography, 136(1), pp.127-139.

Peat, M.A., Kopjak, L. (1979), The screening and quantitation of diazepam, flurazepam, chlordiazepoxide and their metabolites in blood and plasma by electron-capture gas chromatography and high pressure liquid chromatography. Journal of Forensic Sciences, 24, pp.46-54.

Poklis, A., Mackell, M. and Case, M.E.S. (1985), Xylazine in human tissues and fluids in a case of fatal drug abuse. Journal of Analytical Toxicology, 9, pp.234-236.

Purpura, R.P. (1981), Approaches in the evaluation of hypnotics: Studies with triazolam. British Journal of Clinical Pharmacology, 11, pp.37S-42S.

Putter, J. and Sagner, G. (1973), Chemical studies to detect residues of xylazine hydrochloride. Veterinary Medicine Reviews, 73(2), pp.145-149.

Randall, L.O. and Kappell,B. (1973), "The Benzodiazepines". Garattini, S., Mussini,E. and Randall,L.O. Eds., Raven Press, New York, N.Y. pp.27-51.

Rao, S.N., Dhar, A.K., Kutt, H. and Okamoto, M. (1982), Determination of diazepam and it;s pharmacologically active metabolites in blood by Bond Elut™ column extraction and reversed-phase high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 231, pp.341-348.

Ratnaraji, N., Goldberg, V.D., Glyas, A. and Lascelles, P.T. (1981), Determination of diazepam and it's major metabolites using HPLC. The Analyst, 106(1266), pp.1001-1004.

Richardson, F.J. and Manford, M.L.M. (1979), Comparison of flunitrazepam and diazepam for oral premedication in older children. British Journal of Anaesthesia, 51, pp.313-319.

Rizzi, R., Butera, G., Lion, P. and Pesarin, F. (1975), Flunitrazepam, a new benzodiazepine compound in general anaesthesia. Clinical and statistical considerations on the first thousand cases. Anaesthesia, Resuscitation and Intensive Therapy, 3, pp.339-356.

Rogstad, A.and Yndestad, M. (1981), Analysis of xylazine in biological material by gas chromatography using packed and capillary columns. Journal of Chromatography (Biomedical Applications), 216, pp.350-354.

Sample, R.H.B., Chulk, A., Kolanows, C. and Baenzige, J.C. (1986), Analysis of benzodiazepines using a bonded-phase extraction system. Clinical Chemistry, 32(6), pp.1055-

Sams, R., (1978), Pharmacokinetic and metabolite considerations as they apply to clinical pharmacology in equine pharmacology. Eds. Powers, J.D. and T.E., pp.17-33. American Association of Equine Practitioners. Golden, Colorado.

Samuels, R.W. (1977), A rapid screening test for diazepam in serum. Journal of Analytical Toxicology, 1, pp.208-209.

Sandoz Pharmaceuticals (1980), Sanorex (mazindol), product monograph. Sandoz Pharmaceuticals, Dorval, Quebec, 1980.

Schwartz, M.A., Koechlin, B.A., Postma, E., Palmer, S. and Krol, G. (1965), Metabolism of diazepam in rat, dog and man. Journal of Pharmacology and Experimental Therapeutics, 149, pp.423-435.

Scott, C.G. and Bommer, P. (1970), The liquid chromatography of some benzodiazepines. Journal of Chromatographic Science, 8, pp.446-448.

Shader, R.I. and Greenblatt, D.J. (1981), Benzodiazepines: A clinical review. British Journal of Clinical Pharmacology, 11, pp.3S-9S.

Shaw, W., Long, G. and McHahn, J. (1983), An HPLC method for the analysis of clonazepam in serum. Journal of Analytical Toxicology, 7, pp.119-122.

Skellern, G.G., Meier, J., Knight, B.I. and Whiting, B. (1978), The application of HPLC to the determination of some 1,4 benzodiazepines and their metabolites in plasma. British Journal of Clinical Pharmacology, 5, pp.483-487.

Smith, F.M. and Nuessle, N.O. (1982), HPLC method for determination of diazepam injection. Analytical Letters, 15(B4), pp.363-371.

Smith, R.B., Divoll, M., Gillespie, W.R. et al., (1983), Effect of subject age and gender on the pharmacokinetics of oral triazolam and temazepam. Journal of Clinical Psychopharmacology, 3, pp.172-176.

Smith, R.B., Kroboth, P.D. and Phillips, J.P. (1986), Temporal variation in triazolam pharmacokinetics and pharmacodynamics after oral administration. Journal of Clinical Pharmacology, 26, pp.120-124.

Smith, R.M., Hurdley,T.G., Gill,R. and Osselton, M.D. (1987), Retention reproducibility of basic drugs on a silica column with a methanol ammonium nitrate eluent. The effect of mobile phase and operating conditions. Journal of Chromatography, 398, pp.73-84.

Smith, R.M., Murilla, G.A. and Burr, C.M. (1988), Alkyl aryl ketones as a retention index scale with acetonitrile or tetrahydrofuran containing eluents in reversed-phase HPLC. Journal of Chromatography, 388, pp.37-45.

Sohr, C.J. and Buechel, A.T. (1982), Separation of parent benzodiazepines and their major metabolites by reverse-phase ion-pair chromatography. Journal of Analytical Toxicology, 6, pp.286-289.

Sperling, A.R. (1971), Quantitative analysis of methadone in orange juice. Microgram, 4(5), Number 16.

Stanworth, D. (1984), The analysis of benzodiazepines in blood using bonded-phase capillary gas chromatography with electron capture detection. In Proceedings of the 21st Meeting of The International Association of Forensic Toxicologists (TIAFT), U.K., ed. Dunnett, N. and Kimber, J. pp.287-296.

Stewart, J.T., Reeves, T.S. and Honigberg, I.L. (1984), A comparison of solid-phase extraction techniques for assays of drugs in aqueous and human plasma samples. Analytical Letters, 17, pp.1811-1826.

Steyn, J.M. and Hundt, H.K. (1975), Quantitative GC determination of diazepam and it's major metabolite in human serum. Journal of Chromatography, 107(1), pp.196-200.

Stubbs, R.J., Chiou, R.and Bayne, W.F. (1986), Determination of codeine in plasma and urine by reversed-phase HPLC. Journal of Chromatography, 377, pp.447-453.

Stovner, J., Edresen, R.and Osterud, A. (1973), Intravenous anaesthesia with a new benzodiazepine. Ro 5-4200. Acta Anaesthesiologica Scandinavica, 17, pp.163-169.

Strojny, N., Puglisi, C.V. and deSilva, J.A.F. (1978), Determination of chlordiazepoxide and its metabolites in plasma by high-pressure liquid chromatography. Analytical Letters, B11(2), pp.135-160.

Sumirtapura, Y.C., Aubert, C., Coassolo, P. and Cano, J.P. (1982), Determination of 7-aminoflunitrazepam (Ro 20-1815) and 7-aminodesmethylflunitrazepam (Ro 5-4650) in plasma by HPLC and fluorescence detection. Journal of Chromatography (Biomedical Applications), 232(1), pp.111-118.

Suzuki, O., Seno, H. and Kumazawa, T. (1988), Rapid isolation of benzodiazepines with Sep-Pak® C18 cartridges. Journal of Forensic Sciences, 33, pp.1249-1253.

Szczerba, T.J., Glunz, L.J., Rateike, J.D., Patel, S.R. and Perry, J.A. (1986), HPLC column finds drugs in serum. Research and Development, The Regis Chemical Company, pp.84-86.

Theis, D.L. and Bowman, P.B. (1983), Development of a liquid chromatographic method for the determination of triazolo-benzodiazepines. Journal of Chromatography, 268, pp.92-98.

Timmings, S., Beaumier, P., Sutherland, D.and Nikolajev,J. (1985), Metabolism and elimination of mazindol by the horse after oral administration. Proceedings of the 6th International Conference of Racing Analysts and Veterinarians, Hong Kong, 1985.

Tippens, B. (1987), Selective sample preparation of endogenous biological compounds using solid-phase extraction. American Biotechnical Laboratory, 5(1), pp.25-31.

Tippens, B. (1987), Solid-phase extraction: Sample preparation for HPLC, GC, NMR, FTIR and MS. American Laboratory News, 8.

Tjaden, U.R., Meeles, M.T.H.A., Thys, C.P. and Van Der Kaay, M. (1980), Journal of Chromatography (Biomedical Applications), 181, pp.227-241.

Twitchett, P.J., Gorvin, A.E.P. and Moffat, A.C. (1976), High-pressure liquid chromatography of drugs: II. An evaluation of a microparticulate cation exchange column. Journal of Chromatography, 120, pp.359-368.

Ubbink, J.B., Schnell, A.M.and Rapley, C.H. (1988) Quantification of urinary xanthurenic acid excretion by anion exchange solid-phase extraction and HPLC. Journal of Chromatography, 425, pp.182-186.

Van der Merwe, P.J. and Steyn, J.M. (1978) Thin-layer chromatographic method for determination of diazepam and it's major metabolite, N-desmethyldiazepam, in human serum. Journal of Chromatography, 148, pp.549-552.

Van Horne, K.C. (ed.) (1985), Sorbent extraction technology handbook, Analytichem International, Harbor City, CA.

Vasiliades, J.V. and Sahawneh, T. (1982), Midazolam determination by GC, LC and GCMS. Journal of Chromatography, 228, pp.195-203.

Vessman, J., Johansson, M., Magnusson, P. and Stromberg, S. (1977), Determination of intact oxazepam by electron capture gas chromatography after an extractive alkylation reaction. Analytical Chemistry, 49(11), pp.1545-1549.

Violon, C. and Vercruysse, A. (1980), Screening procedure for therapeutic benzodiazepines by HPLC of their benzophenones. Journal of Chromatography, 189(1), pp.94-97.

Violon, C., Pessemier, L. and Vercruysse, A. (1982), HPLC of benzophenone derivatives for the determination of benzodiazepines in clinical emergencies. Journal of Chromatography, 236(1), pp.157-168.

Vree, T.B., Baars, A.M., Hekster, Y.A., van der Kleijn, E. and O'Reilly, W.J. (1979), Simultaneous determination of diazepam and its metabolites, N-desmethyldiazepam, oxydiazepam and oxazepam in plasma and urine of man and dog by HPLC. Journal of Chromatography, 162(4), pp.605-614.

Vree, T.B., Lenselink, B. and Van der Kleijn, E. (1977), Determination of flunitrazepam in body fluids by means of high-performance liquid chromatography. Journal of Chromatography (Biomedical Applications), 143, pp.530-534.

Wahlund, K.G. (1981), Separation of acidic drugs in the ug/ml range in untreated blood plasma by direct injection onto liquid chromatographic columns. Journal of Chromatography, 218, pp.671-679.

Weijers-Everhard, J.P., Wijker, J., Verrijk, R., Van Rooij, H.H. and Soudijn, W. (1986), Improved qualitative method for establishing flunitrazepam abuse using urine samples and column liquid chromatography with fluorimetric detection. Journal of Chromatography (Biomedical Applications), 374, pp.339-346.

Westerink, B.H.C., Bosker, F.J. and O'Hanlon, J.F. (1982) Use of alumina, Sephadex G10 and ion-exchange columns to purify samples for determination of epinephrine, norepinephrine, dopamine, homovanillic acid and 5-hydroxyindoleacetic acid in urine. Clinical Chemistry, 28, pp.1745-1748.

Wilson, I.D. (1986), A rapid method for the isolation and identification of drug metabolites from human serum using solid-phase extraction and proton NMR spectroscopy. Journal of Pharmaceutical and Biomedical Analysis, 4(5), pp.663-665.

Wong, A.S. (1983), An evaluation of HPLC for the screening and quantitation of benzodiazepines and acetaminophen in post mortem blood. Journal of Analytical Toxicology, 7, pp.33-36.

Wong, R.J. (1984), The determination of the triazolobenzodiazepine, triazolam in post-mortem samples. Journal of Analytical Toxicology, 8, pp.10-13.

Wu, A.H.B., and Gornet, T.G. (1985) Preparation of urine samples for LC determination of catecholamines: Bonded phase phenylboronic acid, cation exchange resin and alumina adsorbents compared. Clinical Chemistry, 31, pp.298-302.

Zbinden, G. and Randall, L.O. (1967), Pharmacology of benzodiazepines: Laboratory and clinical correlations. Advances in Pharmacology, 5, pp.213-291.

Zilli, M.A. and Nisi, G. (1986), Simple and sensitive method for determination of clobazam, clonazepam and nitrazepam in human serum by HPLC. Journal of Chromatography, 378, pp.492-497.