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Chiral and achiral analysis of benzodiazepine and anti-anginal drugs in Forensic Toxicology

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

Doctor of Philosopy

by

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November 1996

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ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor, Dr Robert Anderson for his kind support and guidance throughout. I would also like to thank Professor Hamilton Smith, Dr John Oliver and Professor Peter Vanezis for words of wisdom and advice.

My thanks also go to Mrs Liz Doherty for typing the tables of contents, Mr Jeff Cockburn for assistance with computing and all the staff and fellow students at the Department of Forensic Medicine and Science, University of Glasgow for making the working environment happy over the last four years.

In addition Professor Nick Price, University of Stirling must be thanked for his help and assistance in obtaining the circular dichroism spectra.

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SUMMARY

The study in this thesis has three main parts important to the subject of modern forensic toxicology. The first is the extraction and chromatographic separation of nifedipine and its metabolites from whole blood, the second is an evaluation of a β-cyclodextrin high performance liquid chromatography column and the third is the chiral analysis of temazepam enantiomers from human plasma samples.

Nifedipine is a drug used to treat angina and the analysis of this drug has proved difficult in the past. One of the reasons for this is that the drug is photolabile (decomposes in light) and where possible extraction and analysis must be carried out with the exclusion of light.

Several methods for the extraction of nifedipine and its metabolites were evaluated, these being liquid-liquid extraction, solid phase extraction and a combination of protein precipitation and solid phase extraction.

Liquid-liquid extraction was found to be a poor method of extraction because many co-eluting interferences were extracted from whole blood masking drug peaks. This was true of six solvents with a range of polarities.

Solid phase extraction was attempted with the following materials : polar Extrelut® and non-polar C2 (ethylsilica), C8 (octylsilica), C18 (octadecylsilica), CH (cyclohexylsilica) and PH (phenylsilica). The Extrelut® sorbent gives poor extracts with many interferences from whole blood.

Several different eluting solvent mixtures were attempted on all the non-polar silica phases with 5 or 10% butan-1-ol in dichloromethane on C8 (octylsilica) or C18 (octadecylsilica) sorbents giving the highest recoveries.

The percentage recovery of nifedipine from C8 and C18 is 61 and 81% respectively when eluted with 5% butan-1-ol in dichloromethane. The C8 sorbent was used as the material of choice because nifedipine, nitrendipine (internal standard) and all three metabolites were extracted, while only nifedipine, nitrendipine and one metabolite were extracted on C18. A problem encountered with approximately one in three solid phase extraction columns was blockage with blood particulates, even from diluted blood.

Protein precipitation was used as an extraction step prior to solid phase extraction for removal of blood particulates. This was achieved by addition of the blood to Extrelut® or methanol. Poor recoveries (9-37% depending on eluant) were obtained from Extrelut® protein precipitation plus solid phase extraction on CH sorbent. Protein precipitation in methanol plus solid phase extraction on C8 sorbent gives 48% recovery for nifedipine, with all other compounds of interest extracted, and this was chosen as the extraction method of choice.

The linearity of the extraction method was proved by construction of calibration curves for nifedipine and nicardipine (another common anti-angina drug related chemically to nifedipine). The method was found to be clean, robust with no degradation of drug material and is used in the routine laboratory in the analysis of post-mortem blood samples for nifedipine and nicardipine.

The second part of this thesis involved evaluation of a β-cyclodextrin column for separation of enantiomers of four common chiral benzodiazepines (temazepam, oxazepam, lorazepam and lormetazepam) over several solvent conditions. The solvent conditions consisted of mixtures of water with methanol, ethanol, propan-1-ol and acetonitrile plus phosphate buffer with methanol and acetonitrile. Phosphate buffer : acetonitrile (95 : 5) was also evaluated at different pH's (3.5, 6.8, 7.35) for temazepam and oxazepam.

It was found that lormetazepam separated poorly with all mobile phases, no separations of lormetazepam enantiomers were observed, while oxazepam and temazepam enantiomers separated well on most mobile phases. The most suitable mobile phases for oxazepam and temazepam were 5 or 10% acetonitrile in phosphate buffer at pH 7.35.

The third part of this thesis involved evaluation of direct chiral analysis for the purposes of forensic toxicology. It has been known for some time that enantiomers of chiral drugs may differ in pharmacokinetics and pharmacodynamics, and over the past 5 years regulatory bodies such as the EC have put in place draft regulations for dealing with mixtures of isomers. These regulations affect the pharmaceutical industry most presently, but will become more important to forensic toxicologists in the near future.

Two studies focused on temazepam, a chiral drug of forensic interest, and involved a healthy male volunteer consuming 10 or 20 milligrammes temazepam, with blood samples collected over 23 or 60 hours respectively. After collection the blood was separated and plasma samples frozen. A solid phase extraction method with C8 sorbent was used to isolate temazepam

enantiomers and analysis was carried out on a β-cyclodextrin high performance liquid chromatography column with mobile phase consisting of 5% acetonitrile in phosphate buffer (pH 7.35).

Both studies showed that the ratio of enantiomers was 1:1 until 12-13 hours. The first study showed that the concentration of the first eluting enantiomer falls slower than the second up to 23 hours with the second study confirming this observation up to 60 hours. This suggests that the differences in concentration between the two temazepam enantiomers is due to stereoselective binding rather than stereoselective metabolism, although it is certainly possible that both could occur.

It can be concluded here that direct chiral separations can be applied to forensic toxicological analysis but requires careful planning of extraction strategies, because the materials used in direct chiral separations are not as robust as the materials used in nonchiral separations, and many of the materials analysed have the potential for racemisation.

Section 1- Introduction

Chapter 1 : General Introduction and aims

1.1 Forensic Toxicology

Toxicology is the study of poisons and the impact they have on biological systems. This includes physical and chemical properties of toxic substances, their physiological and clinical effects, and quantitative or qualitative methods of analysis in biological and non-biological materials. Two basic types of exposure in toxicology are defined as acute and chronic. Acute exposure applies to a single episode where a harmful amount of substance enters the organism, and chronic exposure applies to repeated events in which absorption of the substance occurs. Both of these are important to forensic toxicology.

Since forensic means "pertaining to the courts of law " then forensic toxicology literally means the application of the science of toxicology within the legal system. In this context the word poison is used to indicate any chemical entity capable of having injurious effect on a living organism.

This subject is a vital component of society today because of the varied circumstances it benefits and clarifies. For example, a toxicological investigation may give insight into a death resulting from an overdose or from impairment of senses. This can be described as acute exposure.

Chronic exposure is also important and forensic issues resulting from this include drug screening for employment or road traffic incidents. Monitoring of individual exposure to potentially hazardous substances is also of interest to the forensic toxicologist, in which the subject overlaps with environmental toxicology. Examples of this include farm worker exposure to

pesticides, inhalation of lead vapour during soldering processes by employees in electronics manufacturing industries or the exposure of the general public to anabolic agents which have been used illegally in beef production. The important connection between forensic and environmental toxicology is the use of results of the study or investigation in legal proceedings, either civil or criminal.

In future forensic toxicology will increasingly be involved with drug and substance abuse, as well as new problems. These could include female hormones in the environment and in food chains resulting from contraceptives and human/animal exposure to crude oil products from the oil industry, for example, tanker spillages and leaks in pipelines.

1.2 Current substance abuse problems

Presently an assortment of chemical compounds are abused including illegal drugs, prescription-only medicines, over-the-counter medicines, veterinary medicines and various volatiles.

The illegal drugs include cannabis, amphetamines, opiates, cocaine and LSD. Cannabis has been found less addictive than other abused substances but at high levels of intake still causes toxicity. Amphetamine use seems to be on the rise in parallel with the dance and rave scene and deaths are on the increase. Heroin is the most widely used opiate and is a significant factor in many drug-related deaths in Britain today. Cocaine is not a serious problem in Scotland today, but its prevalence in England is increasing and it is anticipated that these problems will gradually spread to the rest of the U.K. Attributing deaths to LSD is often difficult because very small quantities ($\approx$60 μg) of drug are required and death is usually caused by an accident resulting from delusions rather than from an overdose of the drug.

Prescription drugs which are abused often have psychopharmacological effects and include barbiturates and benzodiazepines. Methadone is also a commonly abused prescription drug even though controls are tight to make sure the drug is consumed according to the recommended regime. One reason for this is that often the patient will try and keep the methadone to sell on the street. Another problem encountered with prescription drugs results from the patient accidentally or deliberately consuming the wrong dosage. This could include almost any class of drug.

Veterinary medicines are also abused, mostly in rural areas and are usually tranquillisers or pain killers for animals. This was very rare until a few years ago, but there is now a steady increase of this type of abuse.

Volatiles include solvents, hydrocarbon gases and glues. Examples are halogenated ethane isomers, propane/butane and toluene/xylene.

Due to the wide range of substances available for abuse there is a constant need to develop new methods and to review old methods. Until recently most regulations concerning drug analysis did not require any stereochemical analysis, but this is gradually changing as more is learned about the stereochemical consequences of optically-active drugs in living systems.

1.3 New legislation - chiral medicinal products

An important aspect of the need for new methods could be the forthcoming legislation concerning chiral discrimination. A recently published paper [1] reviews current draft guidelines for Canada, EC, Japan and the United States. Most of the guidelines are similar, with only minor differences. Presently, committees for the EC and the other nations are trying to unify their proposals. These guidelines have come about due to the

rapid development of stereospecific separations and also due to the need to separate enantiomers as more is learned about their differing biological activities.

The draft guidelines look at three situations which are : new single enantiomer, new racemate or a single enantiomer developed from a racemate.

The first situation treats a new single enantiomer as a new active substance and an application must contain all the quality, safety and efficacy investigations reserved for a pure substance. The only difference from a symmetrical molecule is that *in vivo* conversion and subsequent biotransformation should be treated as such in development and safety evaluation.

The next case is concerned with a new racemate. Currently, it is considered as a single new active substance, although it is composed of two enantiomers. However, both enantiomers cannot be assumed automatically to show the same pharmacodynamic effects in an asymmetrical biological environment. The question arises as to how much evidence is required to be produced by a pharmaceutical manufacturer in order to safeguard public health. A balance acceptable to society must be obtained between a "no expense spared" approach and preserving a commercially viable workload for the drug licence applicant.

Two possible situations have been suggested. The first arises if the racemate shows rapid interconversion (racemisation), especially *in vivo*. The two enantiomers will not be considered separately under these circumstances and only the racemate will be investigated. This is mentioned in the EC and Canadian guidelines.

The other situation occurs when interconversion is slow or non-existent, allowing both enantiomers to exert their own effects. This leads to the following requirements during drug evaluation :

Pharmacodynamics - the main effects should be studied in animals and humans with the racemate and both individual enantiomers.

Pharmacokinetics - the racemate should be studied using enantiomer-specific (enantiospecific) analytical methods in animals and humans, estimating separate exposures and deriving animal and human stereospecific pharmacokinetics. The interconversion should then be analysed *in vivo*.

Toxicology - the study should primarily focus on the racemate, but in cases where the toxicity is not predictable from the pharmacokinetics, the studies concerned should be repeated with individual enantiomers. For the development strategy it is important to know whether any toxicity involved is caused by one or both enantiomers.

Pharmacotherapy - therapeutic studies should be conducted primarily with the racemate and pharmacokinetic monitoring should be carried out with enantiospecific methods where necessary.

The last situation is a single enantiomer developed from a racemate. It makes a difference if the change of product happens during development or after approval of the racemate. The first case has received emphasis in the FDA (Food and Drug Administration) document, and the latter in the EC draft guidelines. It suggests that each enantiomer in a racemic mixture should be monitored individually making this case differ little from the development of a new single enantiomer. Regulatory problems will arise when switching after approval of the racemate or in a late phase of development : formally the EC requires a dossier on the enantiomer as a new

active substance. This is considered an extreme requirement, even by the regulators.

The subject of chiral drug production has already been discussed at the national and international level and a conference for world harmonisation is being planned.

1.4 Aims of this work

The work in this thesis had two main aims within the context of method development. The first was to develop a method suitable for the routine analysis of the drug nifedipine in the forensic toxicology laboratory and the second was to evaluate the applications of chiral chromatography in forensic toxicological analyses.

The development of a new method for nifedipine is very important because of the nature of the drug, i.e. prescription-based and used to treat angina. This is obviously very relevant for work based in the West of Scotland, which has a death rate from heart disease amongst the highest in the world.

Chiral chromatography is a rapidly developing subject and should be evaluated for forensic toxicology. The recommendations in Paragraph 1.3 above will be implemented first in the pharmaceutical industry but will have an impact on forensic toxicology in the future. Interpretation of results may change as differences in the pharmacology and toxicology of enantiomers is studied and methods of analysis for these will be required.

Section II: Nifedipine

Chapter 2 : Introduction

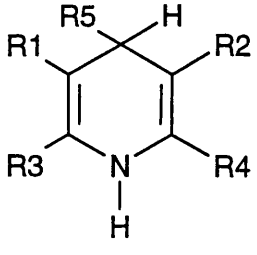
2.1 General

Nifedipine is a commonly prescribed drug used in the management of angina and several other heart related illnesses. Over the past couple of years it has become important to develop a suitable assay, due to the drug's increasing importance in medico-legal cases.

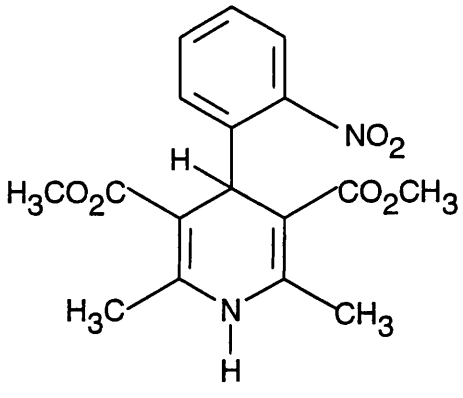
2.2 Chemistry

2.2.1 Structure

Nifedipine, along with several related compounds, is based on a substituted dihydropyridine ring as shown below in structure 1.



structure 1 : substituted dihydropyridine ring



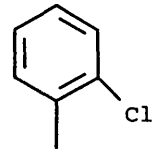
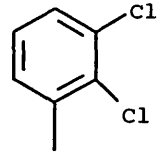
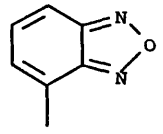
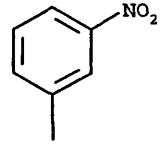
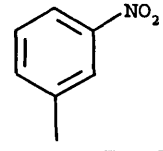
structure 2 : nifedipine

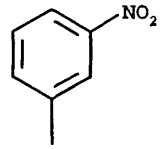
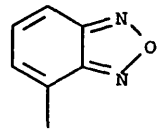
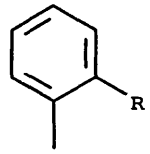
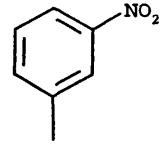
Often these compounds contain several common substituent features. These can be summarised as follows :

1. R1, R2 are ester groups
2. R3, R4 are alkyl groups
3. R5 = nitro or chlorinated benzene, but not always

Nifedipine (structure 2) does have R1=R2 and has no chiral centre unlike many of the others. Some common dihydropyridine structures are summarised in table 1.

Table 1: Common Dihydropyridines

name	R1	R2	R3	R4	R5
amlodipine	MeO_2C	CO_2Et	Me	$CH_2(CH_2)_2NH_2$	
felodipine	MeO_2C	CO_2Et	Me	Me	
isradipine	MeO_2C	$CO_2CH(Me_2)_2$	Me	Me	
nimodipine	Me_2CHO_2C	$CO_2(CH_2)_2OMe$	Me	Me	
nitrendipine	MeO_2C	CO_2Et	Me	Me	

nisoldipine	MeO_2C	$CO_2CH_2CHMe_2$	Me	Me	
darodipine	EtO_2C	CO_2Et	Me	Me	
lacidipine	EtO_2C	CO_2Et	Me	Me	 $R=CHCHCO_2C(Me)_3$
nilvadipine	Me_2CO_2C	CO_2Me	Me	CN	

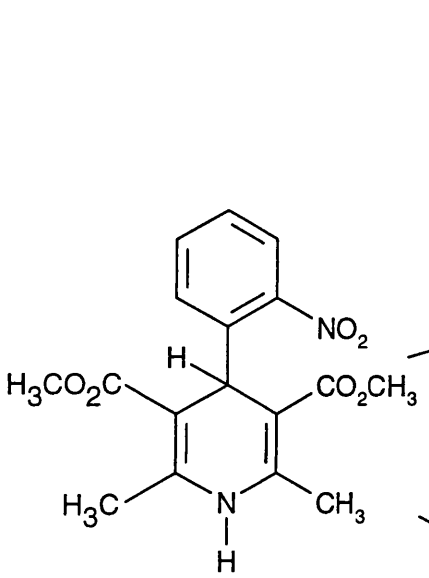
2.2.2 Chemical and Physical properties

Physically nifedipine is a yellow powder with m.pt. 171-175°C. It is soluble in acetone and chloroform, partially soluble in methanol and ethanol, and practically insoluble in water.

The drug itself is very sensitive to light and more especially towards UV wavelengths. Exposure to UV light causes a loss of H_2 giving dehyro-nifedipine (structure 3), and exposure to daylight gives dehydro-nitrosonifedipine (structure 4) with loss of H_2O [2]. It should be noted dehydronifedipine is also the first metabolite of nifedipine. This metabolite and all others are photostable. Also the dimer (structure 5) has been suggested as a possible degradation product [3].

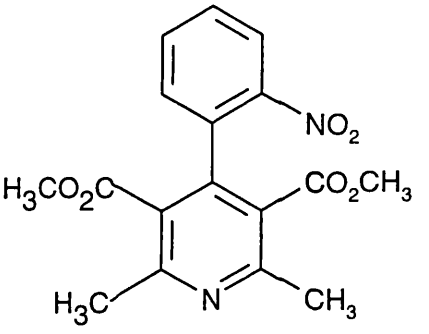
This scheme is shown in diagram 1

Diagram 1 : Photodecomposition of nifedipine



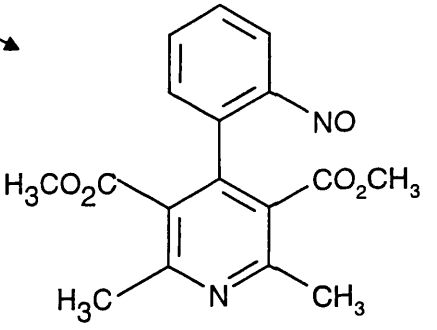
structure 2 : nifedipine

UV light

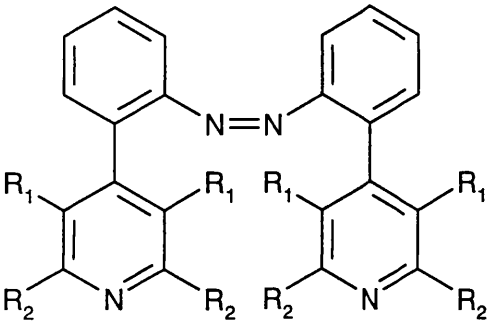


structure 3 : dehydronifedipine

daylight



structure 4 : dehydronitrosonifedipine



structure 5 : dimer

$R_1 = CO_2CH_3$

$R_2 = CH_3$

2.3 Pharmacology

Nifedipine is the prototype of dihydropyridine based compounds, and all are pharmacologically similar. The drug is a calcium antagonist, which inhibits the entry of calcium through the slow channel in the cell membrane of cardiac and smooth muscle cells. Calcium ions play an important role in the regulation of muscle contraction. At rest calcium ions are pumped into the sarcoplasmic reticulum (network of membrane bound tubules and vesicles) so that calcium ion concentration around the muscle fibres is very low. Excitation of the sarcoplasmic reticulum membrane by a nerve impulse leads to a sudden release of calcium ions in large amounts, which triggers muscle contraction i.e. calcium ions are the intermediary between the nerve impulse and muscle contraction [4]. As well as this inhibition of calcium ion influx nifedipine also binds to intracellular calcium binding proteins.

Because nifedipine and other dihydropyridines inhibit calcium movement they act as arterial dilators. The effects are seen more on vascular smooth muscle as these are mostly dependent on calcium movement. The effect is seen less on cardiac muscle cells as they are dependent on sodium and calcium ion influx for contraction. Thus nifedipine has a selective effect as a dilator of arterial vessels, namely the pulmonary, coronary and peripheral arteries [5].

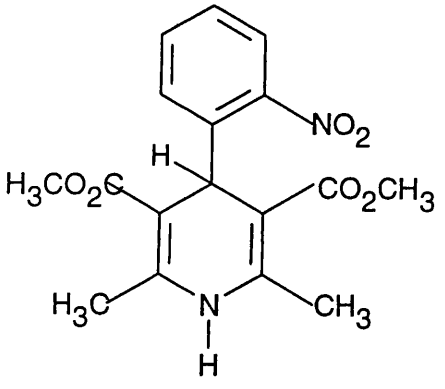
The drug is given for the treatment of angina pectoris and hypertension in doses of 15-80 mg/day in divided dose form. It is usually administered orally but can be given intravenously if required.

Absorption has been observed over the whole length of the gastrointestinal tract, with 90% of the oral dose absorbed largely in the

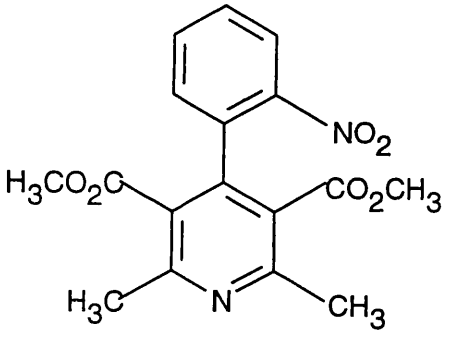
proximal small intestine[6]. However the measured mean bioavailability is between 45 and 70%. Following administration by mouth peak blood concentrations are reported to occur 30-70 minutes after ingestion, with a half life of 2-5 hours [7]. The minimal plasma concentration required to produce cardiovascular effects is 15 μg/L with a typical 10 mg oral dose producing plasma levels around 160 μg/L [5].

The metabolism of nifedipine appears to be hepatic oxidation to three metabolites which are excreted in the urine (80%) and faeces (20%). The pharmacological activity of nifedipine is due to the dihydropyridine ring structure. However the metabolites are present in the dehydrogenated form (i.e. based on a pyridine ring) and are thus pharmacologically inactive. A scheme for the metabolism is outlined in Diagram 2 and involves oxidative dehydrogenation to dehydronifedipine (BAY B 4759, structure 3), followed by demethylation to the pyridine mono-carboxylic acid metabolite (BAY O 2820, structure 6), then oxidation of the 2-methyl group to the primary alcohol (BAY H 2228, structure 7). This metabolite undergoes pH dependent cyclisation to the lactone (BAY O 4160, structure 8) [8].

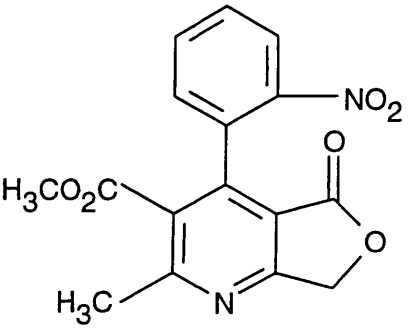
Diagram 2 : Metabolism of nifedipine



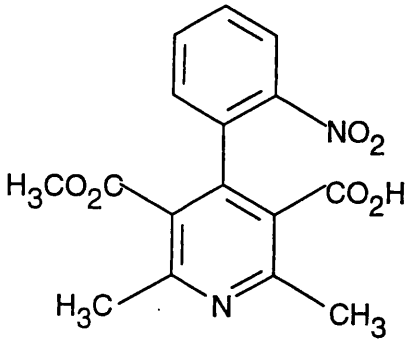
structure 2 : nifedipine



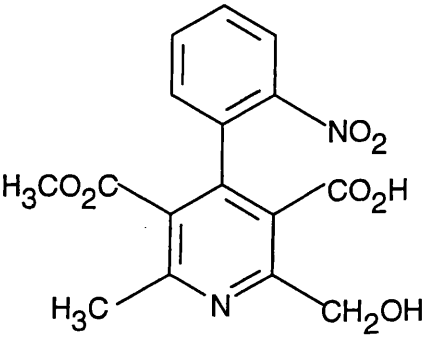
structure 3 : BAY B 4759



structure 8 : BAY O 4160



structure 6 : BAY O 2820



structure 7 : BAY H 2228

Adverse effects associated with nifedipine include dizziness, flushing, headache, hypotension and peripheral oedema. All of these are associated with the vasodilatory action. Gastro-intestinal disturbances, increased micturation frequency, lethargy, pain, and mental depression have also been noticed. Hypersensitivity reactions including rashes, fever and abnormalities in liver function have been reported as well as gingival hyperplasia which is reversible on drug withdrawal [7, 9].

To date, nifedipine related deaths have not been reported in the literature, but several overdose cases have been published [10-16]. In these reports various doses have been taken (200-1000mg) with two involving unknown alcohol consumption [10, 11]. The clinical effects are similar in all and include hypotension, hypoglycaemia and central nervous system alterations such as dizziness and weakness. The treatment involves IV fluids, calcium and dopamine or thiamine. Two of the cases tried gastric lavage with activated charcoal [11, 12]. Although all patients recovered complications upon recovery included pulmonary oedema and bradyarrhythmia.

Drug-drug interactions of nifedipine with alcohol [17] and cimetidine [18] have been noted, with both of these inhibiting the metabolism of nifedipine. Ranitidine, another antihistamine similar to cimetidine, has little effect on nifedipine metabolism, probably due to its lower binding affinity to cytochrome P-450 as compared to cimetidine [18]. Nifedipine interaction with digoxin has been suggested, but it is thought nifedipine does not cause significant increases of serum digoxin, although patients on both should be monitored carefully [19].

2.4 Extraction

The majority of papers published for the analysis of nifedipine from blood, plasma, serum or urine involve liquid-liquid extraction. A wide range of solvents has been used and procedures are highlighted in a recent review paper [20].

Approximately six methods using solid phase extraction have been published to date. Two of these methods involve on-line pre-column extraction from human plasma [21, 22], another combines SPE on octadecylsilica (C18) and basic solvent extraction from serum and plasma [23] and extraction of nifedipine from serum on Bond-Elut® (BE 6001) non-polar columns has been recorded [24]. Extractions from fetal calf serum using octylsilica(C8) [25] and from human plasma using octadecylsilica(C18) [26] have also been published.

2.5 Chromatographic methods

Several methods have been used for the chromatographic analysis of nifedipine and include GC and HPLC with various detectors. Approximately 190 papers have been published involving chromatographic analysis. A recent review summarised 50 papers prior to 1990 [20].

Gas chromatography has been used for analysis of nifedipine and also dehydronifedipine, which is produced by heat or UV light degradation. Some of the first GC methods were based on oxidation of the sample to dehydronifedipine [27, 28]. The procedure involves adding aqueous hydrochloric acid and sodium nitrite to the sample. This approach was used to overcome the thermal degradation of nifedipine on column. However

these methods lack specificity since the oxidation product is also the first metabolite in man and this metabolite is formed quickly with a half life of 2-5 hours [7]. The methods of detection for dehydronifedipine are electron capture detection [27] and mass spectrometry [28]. Another mass spectrometry method measures plasma nifedipine with no appreciable oxidation [29].

Several ECD methods have been described[24, 30-32] and this seems to be the detection method of choice for GC analysis.

HPLC is the most common analytical method in the literature for nifedipine assay. UV detection is by far the most common although other methods of detection have been tried including a carbon fibre flow-through amperometric detector [21].

For separation, reversed phase systems are most common, especially octadecylsilica [22, 23, 30, 33, 34] and, to a lesser extent, octylsilca [25, 26]. Few normal phase separations are recorded with silica [35, 36] being the most popular.

Most of the methods in the literature separate and detect nifedipine and one or two of its metabolites.

Chapter 3 : Experimental

3.1 General Introduction

One of the aims of the work for this thesis was to find a suitable method of extraction and chromatographic analysis for nifedipine and its metabolites from post-mortem whole blood samples. Several extraction approaches were tried including solvent extraction, solid phase extraction and sample pre-treatment before solid phase extraction.

A study was also completed investigating the photodegradation of nifedipine under two conditions : daylight and UV light.

3.2 Materials

3.2.1 Chemicals

Dichloromethane, methanol, butan-1-ol, chloroform, acetonitrile and propan-2-ol were of HPLC grade from BDH, Merck Ltd, Poole, UK. Hexane and pentane were purchased as HPLC grade from Aldrich Chemical Company, Gillingham, UK. Hexadecyltrimethylammonium bromide (cetrimide) was purchased as a normal laboratory reagent from Sigma Chemical Company Ltd., Poole, UK. The Bond-Elut solid phase extraction columns C18 (octadecyl), C8 (octyl), C2 (ethyl), CH (cyclohexyl) and PH (phenyl) were supplied by Varian, Harbour City, CA, USA and Extrelut® granules were obtained from E. Merck, Darmstadt, Germany.

3.2.2 Drug Standards and Biological Specimens

The reference drug standards of nifedipine, nitrendipine and metabolites BAY-B-4759, BAY-H-2228 and BAY-O-4160 were kindly donated by Bayer (Germany) Ltd.

Two stock standards were prepared, the first being nifedipine and metabolites and the second being the internal standard nitrendipine. Concentrations were as follows :

Stock solution 1 : nifedipine 6.1mg/L, BAY-B-4759 3.6mg/L, BAY-H-2228 7.1mg/L and BAY-O-4160 5.8mg/L.

Stock solution 2 : nitrendipine 7.9mg/L.

The specimens of post-mortem blood were obtained at routine autopsies in the Glasgow area. Blank blood was obtained from the blood transfusion service.

3.3 Ultraviolet Spectra

The UV spectra of nifedipine, BAY-B-4759, BAY-O-4160, BAY-H-2228 and nitrendipine were recorded in a 50:50 water : acetonitrile solution with 20mM cetrimide. This is the mobile phase used in the HPLC analysis. The instrument used was a Hewlett Packard 8451A diode array spectrophotometer.

3.4 Chromatography

The HPLC system consisted of a solvent pump, LDC/Milton Roy Constametric 3000 (LDC/Milton Roy, Stone, Staffs., UK), external injection valve with a 20µl loop (Rheodyne model 7725) , column 250x4.6mm i.d., ODS HL5 (Capital HPLC, Broxburn, West Lothian, UK), Waters 490-MS UV detector (Millipore UK Ltd / Waters Chromatography division, Harrow, Middlesex) and a pen recorder (Linseis NRE-68). The mobile phase used was 50:50 water : acetonitrile solution with 20mM cetrimide.

3.5 Photodegradation

An investigation of the photostability of nifedipine was carried out using a standard solution of nifedipine (5mg/L) dissolved in methanol. Two experimental conditions were used, namely daylight and UV light. The daylight exposure was carried out approximately 1m distance away from a basement window with full daylight, but no direct sunlight. The time of year was summer between 1-4:30pm in the afternoon.

The UV exposure was carried out in a darkened room with no windows. The UV lamp used for the photodegradation experiment was a Spectroline® UV lamp, λ= 254nm. The lamp was positioned approximately 10cm from the sample.

The products of photolysis were analysed by HPLC using the system outlined in section 3.4.

3.6 Extraction

3.6.1 Solvent Extraction

Most of the extraction methods for nifedipine in the literature are based on solvent extraction. Six solvents of different polarities were evaluated for the extraction method for whole blood. The solvents and polarities are summarised in Table 2 [37].

Table 2 : Polarity index of solvents

solvent	polarity index [37]
carbon tetrachloride	1.7
diethyl ether	2.9
dichloromethane	3.4
ethyl acetate	4.3
chloroform	4.4
methanol	6.6

The procedure for solvent extraction was as follows. To separate aliquats were added 100 µL of stock standard solutions and 900µL of blank blood. The samples were then mixed thoroughly and left to stand at room temperature for 1 hour. After this each blood sample was added dropwise to 10ml of solvent and mixed (rock and roll) for 15 minutes. Following centrifugation (2400rpm, 15minutes), separation and evaporation of the

organic layer, the extract was reconstituted in mobile phase and analysed by HPLC.

3.6.2 Solid Phase Extraction

Direct loading of blood on to a solid phase material was attempted with Extrelut® and Bond-Elut C18, C8, C2, CH and PH materials.

For the Extrelut® extraction a 5ml plastic syringe barrel was filled to the 3ml level (approximately 0.7-0.75g) with extrelut® granules. The granules had been washed prior to use with dichloromethane and dried. To 900µL aliquats of blood, 100µL of each stock solution were added, with thorough mixing thereafter. The blood was left to stand for 1 hour. After this the spiked blood sample was added to the top of the column and allowed to absorb for 5 minutes. The elution step involved passing 10ml of methanol or chloroform through the cartridge followed by evaporation of the solvent and reconstitution in mobile phase. Analysis was carried out by HPLC.

For the Bond-Elut C18, C8, C2, CH and PH solid phases the following procedure was used -

1. Conditioning : 1 column volume methanol
   1 column volume water or phosphate buffer
2. Sample application : whole blood was added and allowed to flow through the column very slowly, over 3-5 minutes.
3. Washing : 1 column volume water
   1 column volume hexane
   1 column volume pentane
4. Elution : 2 column volumes of eluting solvent.

The solvents were evaporated and the extract reconstituted in mobile phase. Eluting solvents tried were dichloromethane, chloroform, 5% butan-1-ol in dichloromethane (v/v) and 10% butan-1-ol in dichloromethane (v/v).

3.6.3 Protein Precipitation and Solid Phase Extraction

Protein precipitation: 900µl of blood containing 100µl of stock solution 1 and 100µl of stock solution 2 was added dropwise to 5ml of methanol and mixed for 15 minutes. After centrifugation (2400rpm, 15 minutes), separation and evaporation of the methanol, the extract was reconstituted in 1ml water and applied to a Bond-Elut PH solid phase extraction column.

Extrelut®: 1ml of blood spiked with standards was added to Extrelut® as described earlier and left for 5 minutes. Elution was tried with 10 ml of chloroform, 20% methanol in chloroform and 40% methanol in chloroform. After the solvent had been evaporated the extract was reconstituted in 1ml water and applied to a Bond-Elut C8 solid phase extraction column.

Procedure for Bond-Elut PH and C8 extraction :

1. Conditioning - 1 column volume of methanol
   - 1 column volume of water
2. Application of sample - the sample was allowed to flow through the column very slowly over 3-5 minutes
3. Rinsing - 1 column volume of water
   - 1 column volume of hexane
   - 1 column volume of pentane
4. Elution - 2 column volumes of 10% butan-1-ol in dichloromethane.

The solvent was evaporated and the extract was reconstituted in mobile phase. Analysis was carried out by HPLC.

Chapter 4 : Results and Discussion

4.1 Ultraviolet Spectra

The UV analysis carried out established suitable detector wavelengths for the assay. The data is summarised in Table 3.

Table 3 : UV Spectral data of nifedipine and its metabolites

compound	wavelength (nm)	molar extinction coefficient
nifedipine	214	19630
	236	22551
nitrendipine	208	27039
	238	36074
BAY-B-4759	212	2968
	268	10352
BAY-O-4160	212	14087
	274	3982
BAY-H-2228	224	101748
	276	55849

From the table it can be seen that the 236-238nm range has the highest molar extinction coefficient for nifedipine and nitrendipine.

4.2 Chromatography

The chromatography of nifedipine was based on an HPLC method published in the literature, with several modifications. The paper suggested UV detection at 275nm, but after acquiring UV spectra of the compounds in the final mobile phase, 238nm was used instead.

The mobile phase suggested was 55:45 water:acetonitrile and 25mM cetrimide with a flow rate of 0.5ml/minute. The ratio of water to acetonitrile was changed to 50:50, decreasing retention times, and the flow rate was increased to 1.0ml/minute which sharpened peak shapes.

The cetrimide concentration was dropped to 20mM with no marked effect on the chromatography. This did however decrease the amount of crystallisation of the salt in the HPLC apparatus. The C18 column suggested in the paper was used throughout.

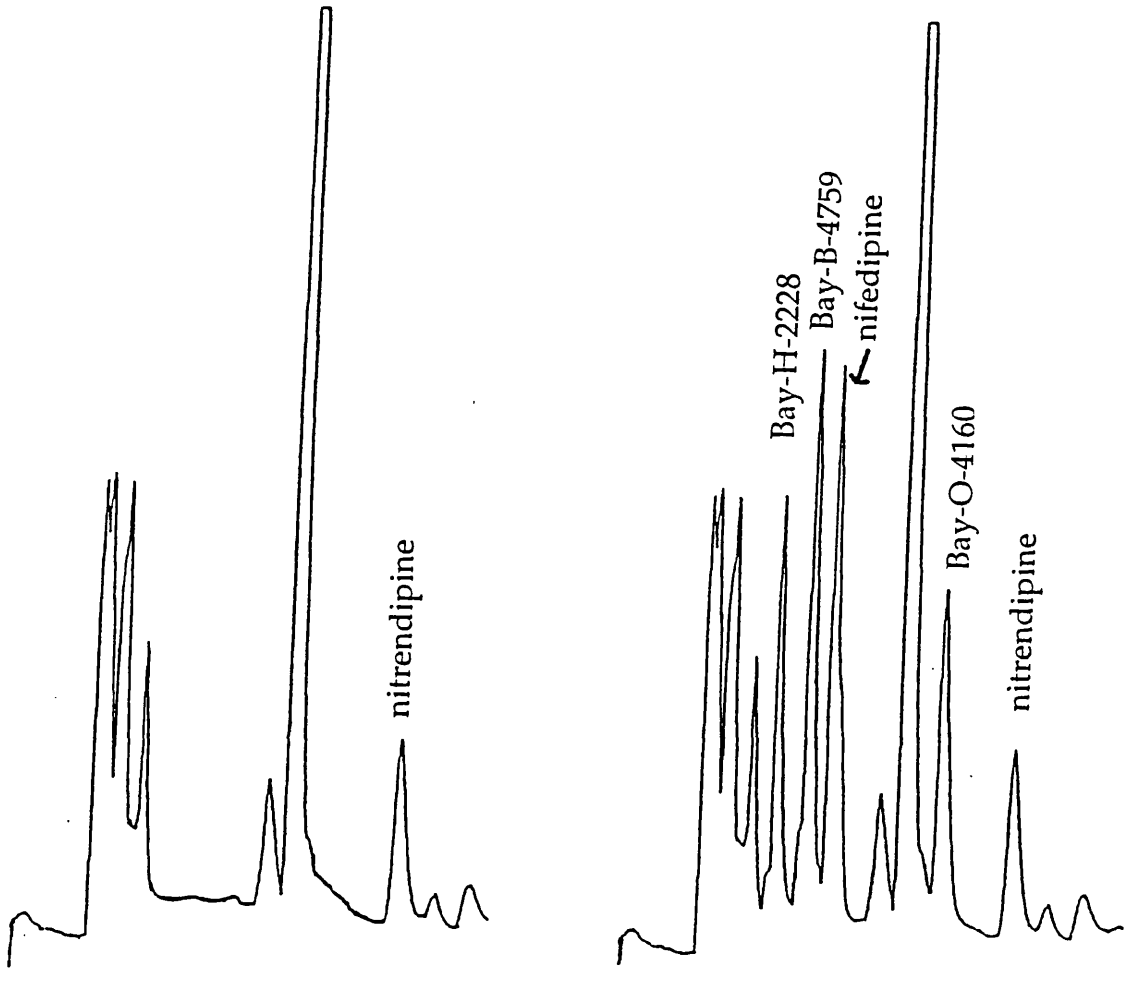
The capacity factors of the drugs in the final system are shown in Table 4.

Table 4 : Capacity factors (k') of nifedipine and metabolites

compound	capacity factor (k')
BAY-H-2228	0.69
BAY-B-4759	1.08
nifedipine	1.50
nitrendipine	2.85
BAY-O-4160	5.35

The chromatograms for a nifedipine extract and blank blood extract are shown in Diagram 3 and the chromatograms of the nifedipine photoproducts (discussed in section 3.5) are shown in Diagram 4.

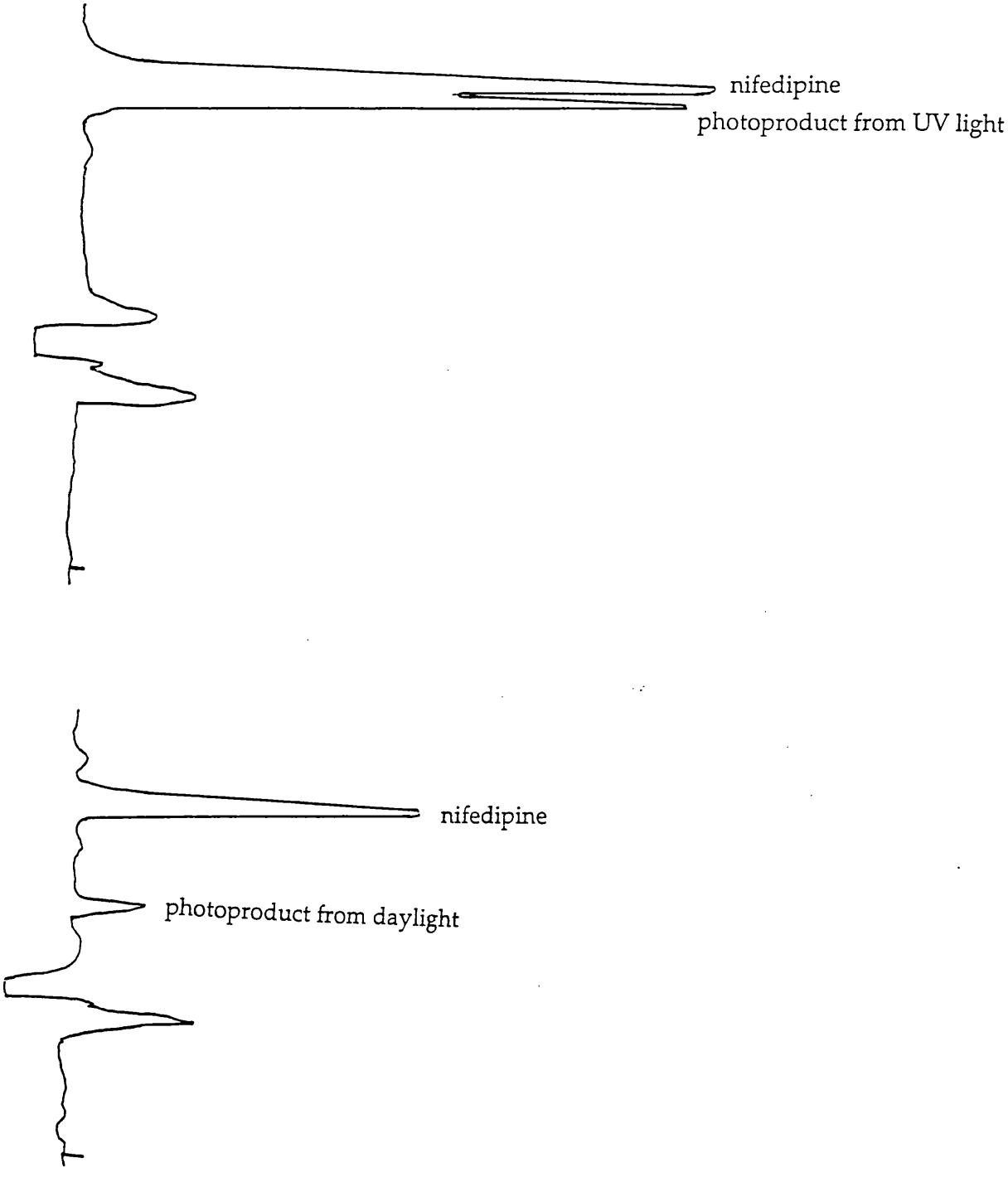
Diagram 3 : Chromatograms from nifedipine extraction



Blank blood

Spiked blood

Diagram 4 : Chromatograms of photoproducts from nifedipine



4.3 Photodegradation

Since nifedipine is sensitive to light and might decompose during analysis it was important to investigate this photosensitivity. Two experiments were carried out, in daylight and UV light, with the results shown in Diagram 5.

Diagram 5 : Photosensitivity of nifedipine

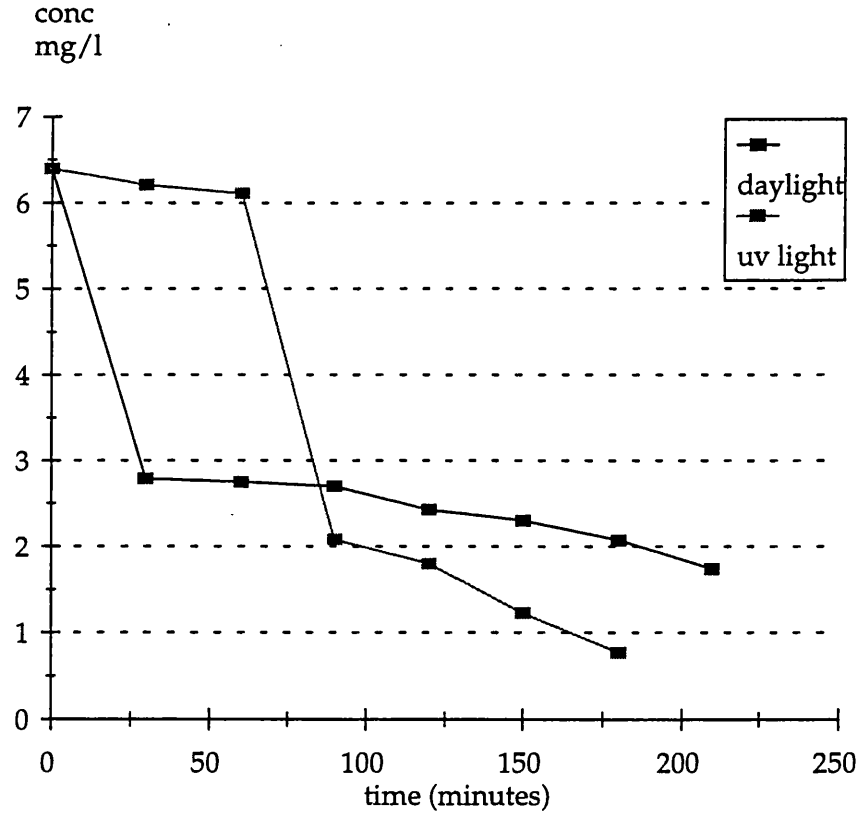


Diagram 5 shows nifedipine in daylight decomposed quickly within the first hour and then more slowly thereafter. In UV light nifedipine decomposed slowly until 90 minutes when the concentration dropped by 60% over a time of 30 minutes. After 120 minutes, the decomposition was slow. The diagram also shows that in the first 60 minutes nifedipine was more stable in UV light than daylight. However from 90 minutes onwards, nifedipine was less stable in UV light than daylight. The photolysis products were analysed by HPLC. The capacity factors in the system used were 1.37 and 0.73 from UV light and daylight respectively. However the capacity factor of the photoproduct (k'=1.37) from the UV experiment is not dehydronifedipine (Bay-b-4579) as predicted in Diagram 1.

4.4 Solvent Extraction

The three most polar solvents evaluated, ethyl acetate, chloroform and methanol, extracted too many co-eluting interferences from the blood. The three non-polar solvents, carbon tetrachloride, diethyl ether and dichloromethane also extracted co-eluting interferences but little evidence was seen of any drug material.

Therefore none of the solvents used gave acceptable results. The more polar solvents, in which nifedipine and its metabolites are more soluble, extracted so many interferences from the blood that it was impossible for peak identification. Since blood is such a complex mixture, it is difficult to use a simple solvent extraction without back extraction. No back extraction was tried here because the dihydropyridine ring of nifedipine is very sensitive to external conditions and trying a chemical step such as protonation of the secondary amine would cause oxidation to

dehydronifedipine, the first metabolite. Non -polar solvents will extract lipids and proteins, which along with the very poor solubility of nifedipine, is why hydrocarbon based solvents were not attempted. Slightly more polar solvents such as carbon tetrachloride, diethyl ether and dichloromethane are also poor for nifedipine.

4.5 Solid Phase Extraction

This was evaluated extensively. The first column tested was the least selective non-polar material, Bond Elut C18, with phosphate buffer at pH 5.2, 7.3 and 10.3 used to condition the columns instead of water. The results are shown in Table 5.

Table 5 : Bond-Elut C18 Column Elution with Dichloromethane : Effect of pH during conditioning

compound	% recovery		
	pH 5.2	pH 7.3	pH 10.3
Bay H 2228	/	/	/
Bay B 4759	66	57	49
nifedipine	37	34	46
nitrendipine	40	38	35
Bay O 4160	/	/	/

Examination of the results shows that the pH of the buffer used to condition the sorbent does not greatly affect the percentage recovery of the drug or its internal standard. However, only one of the metabolites was

eluted, prompting an evaluation of chloroform as an eluting solvent due to its greater polarity. This eluant was not used subsequently due to interference caused by co-extracted compounds. The eluting solvent was changed to 5% butan-1-ol in dichloromethane with Bond Elut C18 and C8 sorbents being used. The results are shown in Table 6.

Table 6: Elution with 5% Butan-1-ol in Dichloromethane

COMPOUND	% RECOVERY	
	C18	C8
BAY H 2228	/	8.6
BAY B 4759	102	62
NIFEDIPINE	81	61
NITRENDIPINE	53	47
BAY O 4160	/	30

The percentage recovery of nifedipine, nitrendipine and BAY B 4759 from the C18 column was improved, although the C8 material did extract all of the substances from the blood. Further evaluation was carried out of C8 and the more selective sorbents Bond Elut C2, CH and PH using 10% butan-1-ol in dichloromethane as eluant. The results are shown in Table 7.

Table 7 : Elution with 10% Butan-1-ol in Dichloromethane

COMPOUND	% RECOVERY			
	C8	C2	CH	PH
BAY H 2228	22	23	19	13
BAY B 4759	77	68	95	96
NIFEDIPINE	61	44	85	93
NITRENDIPINE	49	60	95	61
BAY O 4160	25	/	16	/

An increased recovery from the C8 sorbent was observed and high recoveries of nifedipine, nitrendipine and the main metabolite BAY B 4759 were obtained from the other sorbents. From these results either C8, C2, CH or PH sorbents combined with eluting solvent of 10 % butan-1-ol in dichloromethane are suitable for nifedipine extraction from whole blood. When the percentage of butan-1-ol was increased further (to 20% by volume) significant co-extracted interferences were noted. However, a major disadvantage of this method was that approximately 1 in 3 solid phase extraction columns became blocked by the blood.

The only polar sorbent tried in these studies was Extrelut® (diatomaceous earth). Elution with methanol was found to result in many interfering peaks while chloroform elution gave poor recoveries, as shown in Table 8.

Table 8 : Elution with Chloroform from Extrelut®

COMPOUND	% RECOVERY
BAY H 2228	/
BAY B 4759	34
NIFEDIPINE	15
NITRENDIPINE	14
BAY O 4160	/

Although recoveries from direct loading of blood on non-polar sorbents were good in some cases, the method had to be modified to allow the sample to flow through the column more easily without blockage.

4.6 Protein Precipitation and Solid Phase Extraction

To overcome the viscosity problem an initial step of either Extrelut® or protein precipitation was tried. With Extrelut®, 3 eluants were tried followed by a second stage of solid phase extraction on Bond Elut PH sorbent. The results are shown in Table 9.

Table 9 : Extrelut® and Bond Elut PH Solid Phase Extraction

COMPOUND	% RECOVERY		
% METHANOL IN CHLOROFORM	0	20	40
BAY H 2228	19	43	10
BAY B 4759	30	103	21
NIFEDIPINE	23	37	9
NITRENDIPINE	14	45	15
BAY O 4160	/	6	8

The results suggest that 20% methanol in chloroform is the optimum eluting solvent. Some decomposition of nifedipine to BAY B 4759 occurred here. This may have been due to the quantity of solvent required to be removed by evaporation (10 ml from Extrelut® + 5 ml from PH elution). This suggests the method is unsuitable as many methods in the literature caused no significant degradation.

A combination of protein precipitation in methanol followed by solid phase extraction using Bond-Elut C8 was tried with no degradation problems. The results are shown in Table 10.

Table 10 : Protein precipitation in methanol + C8 solid phase extraction

Compound	% recovery
BAY-H-2228	59
BAY-B-4759	71
Nifedipine	48
Nitrendipine	53
BAY-O-4160	25

4.7 Conclusions

This was the final method used in all subsequent nifedipine analysis. The method may not have matched SPE on its own in terms of percentage recovery, but did have the advantage of not blocking the SPE cartridges when whole blood is used.

Chapter 5 : Applications

5.1 Introduction

The final recommend method was protein precipitation in methanol followed by SPE on Bond-Elut C8 columns. This method was used to construct a calibration curve for nifedipine and also to analyse several authentic toxicology cases.

The method was also attempted with another common dihydropyridine, nicardipine, with a calibration curve and a toxicology case.

5.2 Nifedipine : method validation and toxicology cases

The calibration curve for nifedipine was linear over a range of concentrations from 10-1000 ng/ml and the method could be extended to concentrations as high as 10μg/ml. Six calibration curves were carried out over 3 days (2 curves each day) and the data combined to give the day to day variation (curve as shown in Diagram 6). The equation of the line is $y = 0.024x + 0.037$, the correlation coefficient was 0.999, the limit of detection is 5 ng/ml and the limit of quantitation is 15ng/ml. Raw data used for construction of the curve is shown in Table 11 and the statistical data shown in Table 12.

Diagram 6 : calibration curve for nifedipine

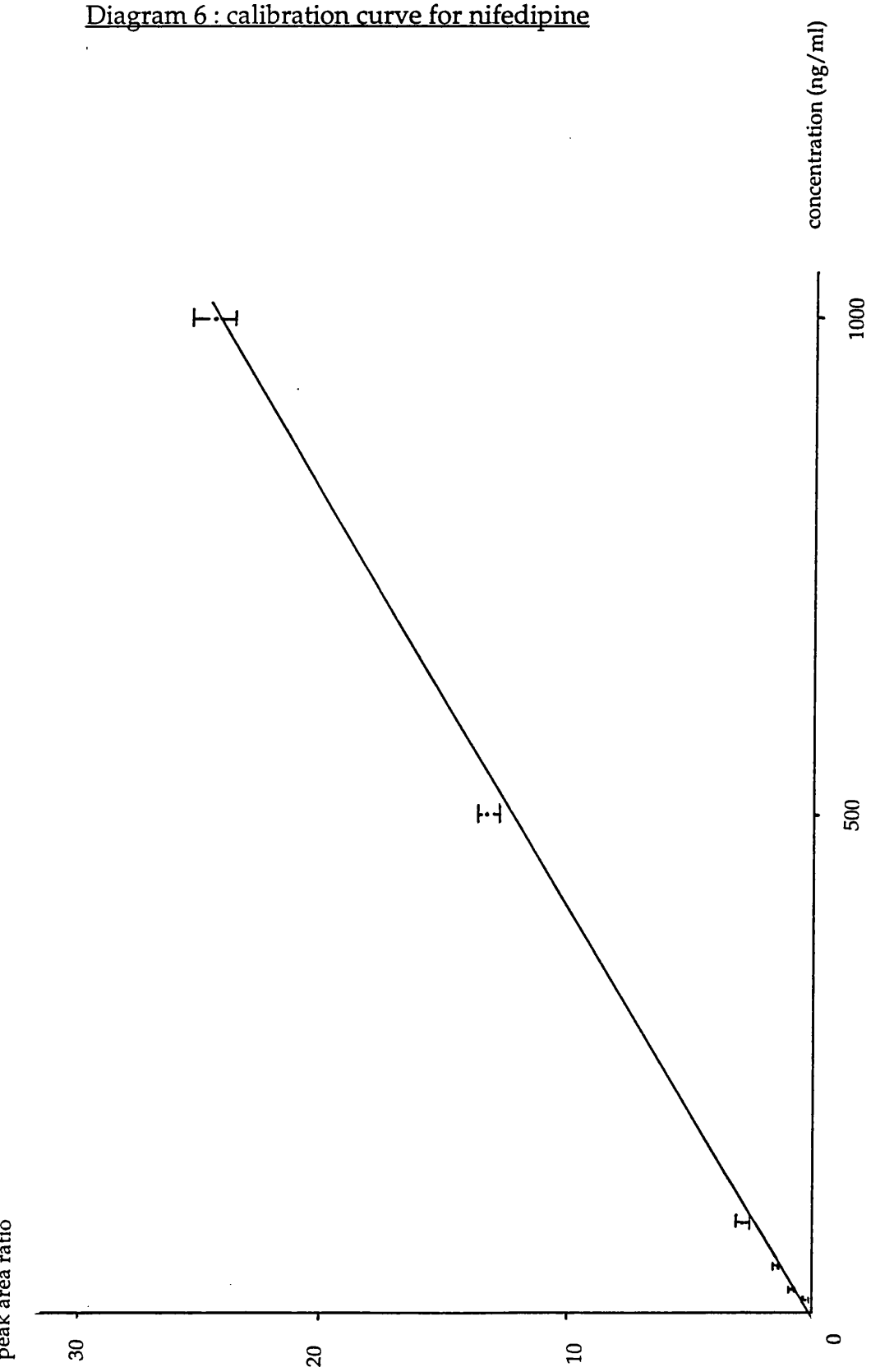


Table 11 : Raw data for construction of calibration curve

conc. ng/ml	calibration curve 1		calibration curve 2		calibration curve 3		calibration curve 4		calibration curve 5		calibration curve 6	
	A	B	A	B	A	B	A	B	A	B	A	B
10	0.516	0.598	0.545	0.566	0.495	0.525	0.566	0.543	0.588	0.564	0.601	0.581
25	0.824	0.774	0.811	0.824	0.783	0.821	0.833	0.801	0.793	0.841	0.888	0.864
50	1.504	1.521	1.498	1.513	1.522	1.454	1.602	1.401	1.488	1.510	1.519	1.539
100	2.814	2.868	2.994	2.822	2.735	2.804	2.846	2.855	2.848	2.855	2.827	2.001
500	13.02	13.41	13.10	13.05	12.82	14.05	12.70	13.08	13.03	13.01	13.04	13.00
1000	24.21	23.22	26.30	24.25	24.28	25.31	24.26	24.21	25.21	24.30	22.24	24.22

Table 12 : Statistical data

conc. ng/ml	arithmetic mean	standard deviation	coefficient of variance (%)
10	0.557	0.033	5.92
25	0.821	0.033	4.02
50	1.506	0.048	3.19
100	2.772	0.250	9.02
500	13.13	0.334	2.54
1000	24.33	1.01	4.16

To date in the Forensic medicine and Science Department 15 requests (13 toxicology, 2 road traffic) for nifedipine have been received. 4 of the cases were found to be positive by the routine technical staff. The results are shown in Table 11.

Table 13 : Post-mortem blood analysis positive for nifedipine and/or metabolites

case	compound	level (µg/ml)
1	nifedipine	8.3
	BAY-H-2228	0.5
2	nifedipine	6.9
	BAY-B-4759	2.7
	BAY-H-2228	0.4
3	nifedipine	0.7
	BAY-B-4759	3.1
	BAY-H-2228	1.8
4	nifedipine	0.75

The calibration curve and the toxicology results show that the method for the analysis of nifedipine is robust and sensitive.

Nicardipine : calibration curve

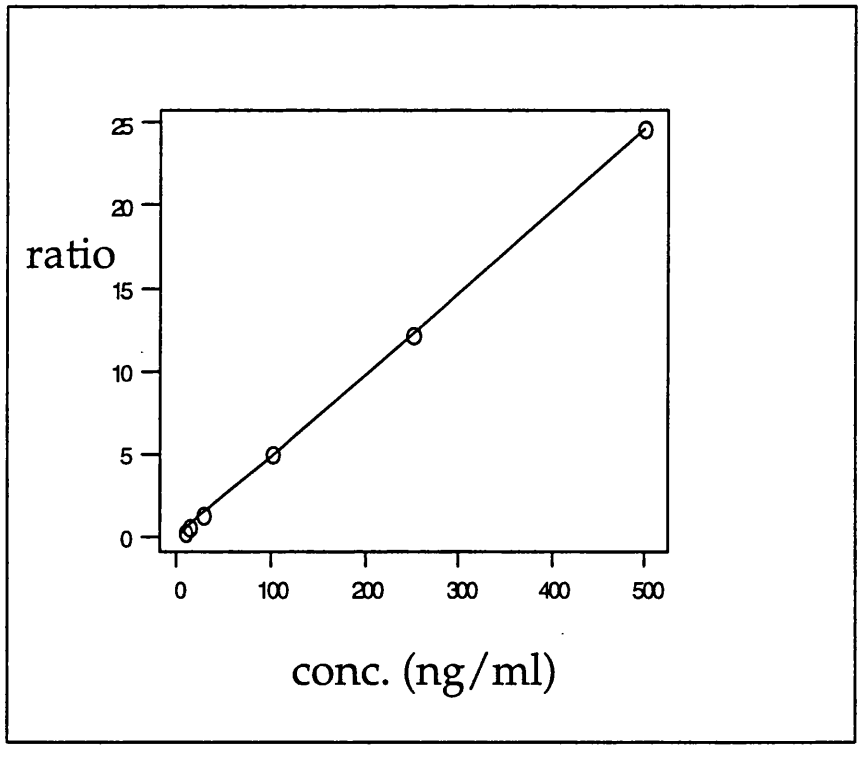
Nicardipine is the second most commonly prescribed dihydropyridine after nifedipine. For this reason the method was tried with nicardipine.

The extraction conditions and light exclusion precautions used here were the same as those applied in the nifedipine analysis. The percentage recovery of nicardipine was 59%, and using the chromatographic system in section the capacity factor for nicardipine is 4.93.

The calibration curve for nicardipine was linear over a range of concentrations from 10-500 ng/ml and, as is the case for nifedipine, can be extended to 10µg/ml.

The correlation coefficient was 0.987 and the calibration curve is shown in Diagram 7 . The limit of detection of the method is 6ng/ml and the limit of quantification is 18ng/ml.

Diagram 7 : calibration curve for nicardipine



One toxicology case was requested for nicardipine analysis. The technical staff, using the nifedipine analysis method, found this to be negative.

Section III : Chiral Analysis

Chapter 6 : Introduction to Stereochemistry

6.2 General

Stereochemistry is a very important aspect of chemistry and biochemistry because it is concerned with the three dimensional arrangement of molecules. Chirality is a fundamental concept which is predominant throughout nature. All living systems depend upon chirality and it is now being found to have an impact in other areas such as particle physics [38].

6.2 Historical overview

In the first half of the 19th century several French scientists studied diffraction, intereference and polarisation of light. The study of linear polarisation of light and rotation of the plane of polarisation in particular attracted attention because of the possible relationship between these phenomena and the structure of matter.

Optical activity was discovered in 1815 by Jean-Baptiste Biot and in 1848 Louis Pasteur made a set of observations on tartaric acid salts. The observations led him to make the following proposal : optical activity of organic solutions is determined by molecular asymmetry, which produces nonsuperimposable mirror image structures. An extension of this idea was made in 1874 when a theory of organic structure in three dimensions was advanced independently

and almost simultaneously by van't Hoff and LeBet. By this time it was known from Kekule's work in 1858 that carbon was tetravalent. Van't Hoff and LeBet proposed that the four valences of the carbon atom were not planar, but directed in a three dimensional arrangement with Van't Hoff specifically stating that the shape was tetrahedral.

A compound with four different groups, which Van't Hoff defined as an asymmetric carbon would therefore be capable of existing in two distinctly different nonsuperposable forms. The asymmetric carbon atom was therefore the cause of molecular asymmetry and thus optical activity [39].

6.3 Basic terms and concepts

6.3.1 Symmetry [40]

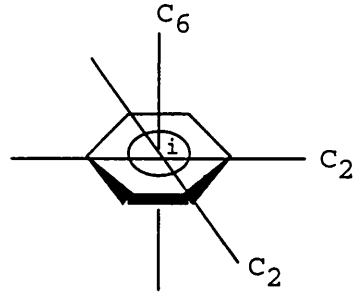
Symmetry or asymmetry are properties found in all molecules. At a mathematical level, symmetry elements and operations have been devised to display the geometrical properties of molecules. The elements and operations in symmetry are shown in Table 14.

Table 14 : Elements and operations of symmetry

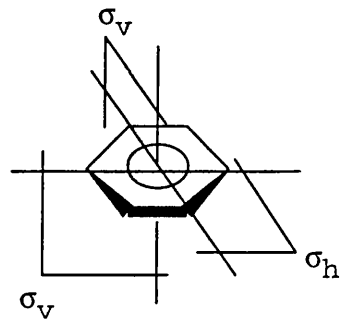
symmetry element	symbol	symmetry operations
proper (simple) axis of rotation	C_n	rotations
planes of symmetry	σ	reflections
centre of symmetry (of inversion)	i	inversion
rotation - reflection axes (mirror axes, improper axes, alternating axes)	S_n	rotation-reflections

To illustrate these elements the benzene molecule will be used as an example. Structures 9 and 10 in Diagram 8 show the symmetry elements of benzene.

Diagram 8 : symmetry elements of benzene



structure 9 : benzene - symmetry axes and centre of symmetry



structure 10 : benzene - planes of symmetry

A molecule is said to have a symmetry axis C_n if a rotation of 360°/n around this axis yields an arrangement indistinguishable from the original, i.e. benzene has C_6 and C_2 axes.

When a plane divides a molecule into 2 symmetrical halves, it is called a plane of symmetry. Benzene has 2 vertical planes (σ_v) and 1 horizontal plane (σ_h) of symmetry.

A centre of symmetry , (i), exists in a molecule where every atom has a symmetrical counterpart with respect to the centre of symmetry, and this is true of benzene.

In general molecules may possess none, one, or a number of elements of symmetry. Although the number of molecules in existence is huge, the possible combinations of symmetry operations are few and the combinations are called point groups.

The principal point groups for chiral molecules are shown in Table 15.

Table 15 : Point groups for chiral molecules

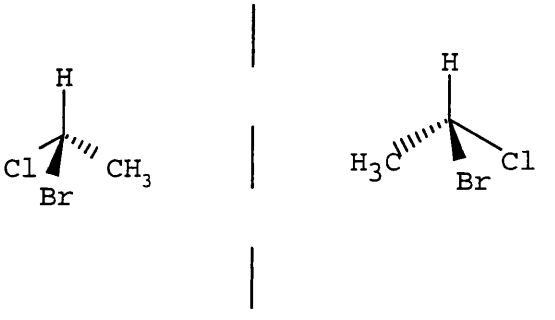
point group	elements
C_1	no symmetry elements (asymmetric)
C_n	C_n(n>1) dissymmetric (axial symmetry)
D_n	C_n, nD_n (dissymmetric) (dihedral symmetry)

6.3.2 Chirality

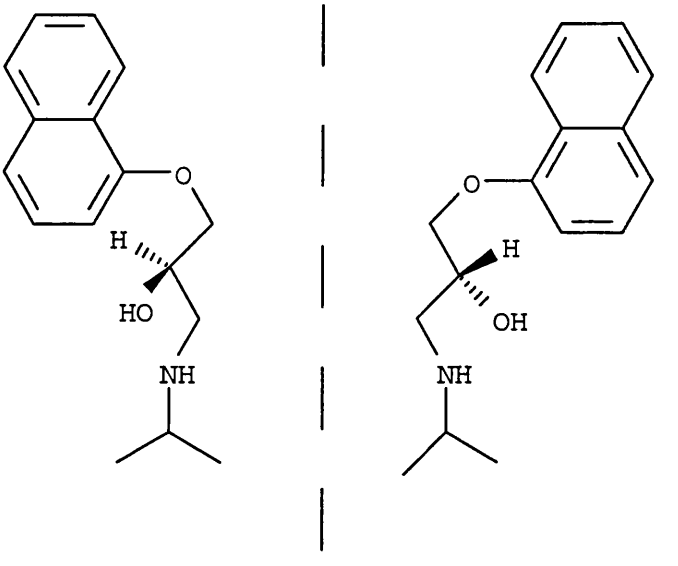
Chirality is defined as the property displayed by any object which is not superposable (superimposable) on its mirror image. Therefore a molecule cannot be chiral if it contains a plane of symmetry, such molecules being termed achiral. The word chiral comes from the Greek χειροσ (chiros) which means hand. This seems a sensible description for the property, since each hand is a mirror image of the other and they are non superposable.
Several structural features cause chirality including a chiral centre, alternating chiral axis, chiral plane and helical structures.

6.3.2.1 Chiral centre

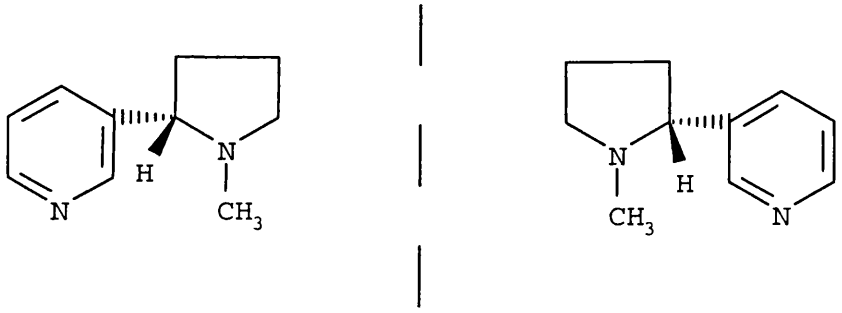
A chiral centre is described as a tetrahedral distribution of 4 different groups around an atom. Most commonly the atom is carbon and several examples are shown below (structures 11-13).



structure 11 : 1-bromo-1-chloroethane



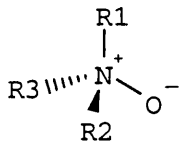
structure 12 : propranolol



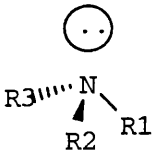
structure 13 : nicotine

Other elements in group IV such as silicon and tin can also have chiral centres due to their tetravalency.

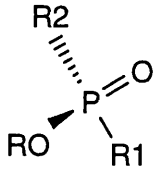
Atoms need not belong to group IV to possess a chiral centre, provided a stable tetrahedral arrangement can be reached. Examples are nitrogen (structures 14 and 15), phosphorus (structure 16) and sulphur (structure 17).

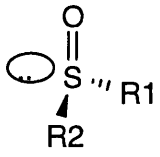


structure 14 : ammonium ion



structure 15 : trisubstituted ammonia

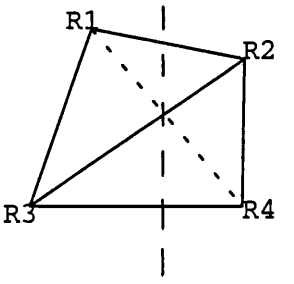




structure 16 : phosphinate

6.3.2.2 Alternating chiral axis [41]

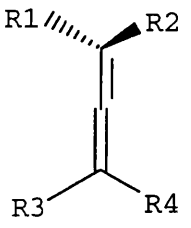
The alternating chiral axis really describes the chirality of 4 groups arranged out of plane in pairs about an axis. This is illustrated by the elongated tetrahedron in structure 18.



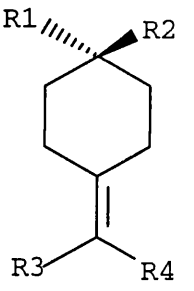
structure 18 : elongated tetrahedron

When no distinction exists between the 4 groups, R1-R4, the tetrahedron has a 4 fold axis of symmetry. Differences of any kind between the groups cause chirality. It should be noted that fewer differences in groups are required compared with a chiral centre, e.g. the pairs must be different, R1≠R2, R3≠R4 but this is the only constraint.

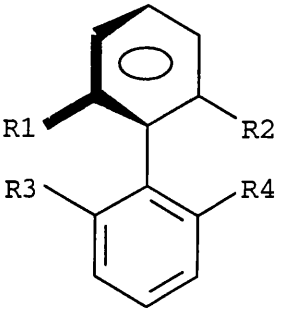
Many examples of this exist including allenes (structure 19), alkylidenecycloalkanes (structure 20) and biaryls such as biphenyl in structure 21.



structure 19 : allene



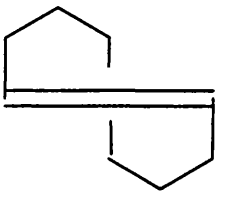
structure 20 : alkylidenecycloalkane



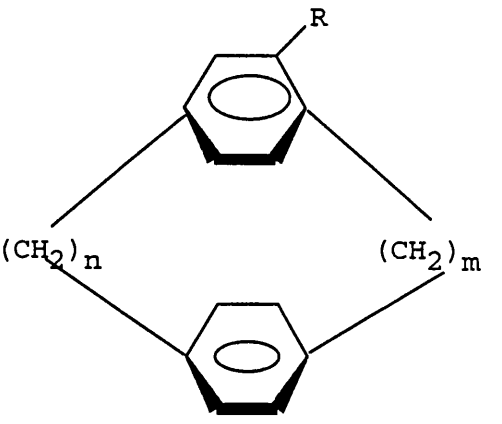
structure 21 : biphenyl

6.3.2.3 Chiral plane [42]

A plane of chirality occurs in molecules where the molecular plane is removed by the presence of a bridge. Examples of this include trans-cycloalkenes such as trans cyclooctene (structure 22) or substituted paracyclophanes (structure 23).



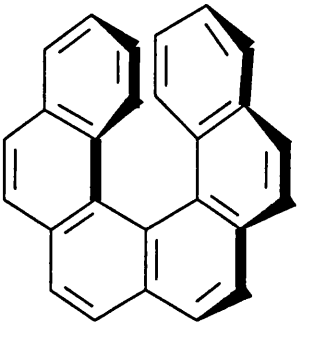
structure 22 : transcycloctene



structure 23 : substituted paracyclophane

6.3.2.4 Helical structures

Helices are chiral objects found in the everyday world (e.g. helical shells, left and right handed screws) as well as in chemistry. Examples are helicenes such as hexahelicene (structure 24) and biological macromolecules eg. DNA.

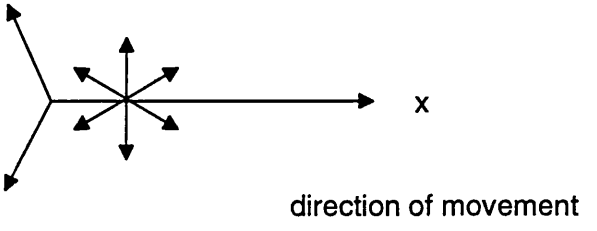


structure 24 : hexahelicene

6.3.3 Spectroscopic measurement of optical activity

Light [43], when considered as a wave , vibrates at right angles to the direction of travel. There are an infinite number of planes passing through the line of propagation, with the light vibrating in all of these planes. This phenomena is represented in Diagram 9.

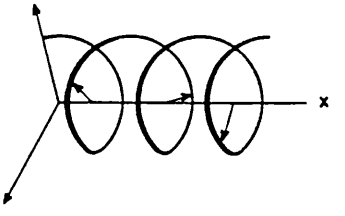
Diagram 9 : Natural light



6.3.3.1 Circularly polarised light

Circularly polarised light is light in which the electric field vector rotates around the direction of movement, either clockwise or anticlockwise. This effectively produces light which moves in a circular helical manner and a representation of this is shown in Diagram 10.

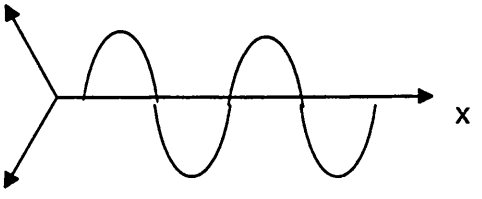
Diagram 10 : Circularly polarised light



6.3.3.2 Plane polarised light

In plane polarised light the electric field vector remains in a plane containing the propagation axis as shown in Diagram 11.

Diagram 11 : Plane polarised light



Plane polarised light can be considered as the resultant of the composition of two contra-rotating (circularly polarised) light beams of equal amplitude.

6.3.3.3 Elliptic light

Elliptic light is the composition of two unequal contra-rotating circularly polarised light beams giving light in which the electric vector describes an ellipse when observed in a plane perpendicular to the beam. The elliptic light is regarded as left or right circularly polarised depending upon which light (left or right) has the greatest amplitude.

6.3.3.4 Interaction of plane polarised light with chiral molecules [44]

Linearly polarised light is thought of as being two beams of circularly polarised light which propagate together in phase but in opposite rotational senses. When the linearly polarised light passes through an achiral medium, each of the two circularly polarised light beams pass through the material at the same velocity and thus have the same refractive indices. However, when the linearly polarised light passes through a chiral medium, phase differentiation occurs because the two circularly polarised light beams will have different refractive indices. This is because the chiral medium will absorb one of the components of circularly polarised light more than the other, and thus produce elliptic light.

It is the elliptic light which is measured by optical rotatory dispersion (ORD) and circular dichroism (CD).

6.3.3.5 Optical rotatory dispersion and circular dichroism

ORD and CD are both spectroscopic techniques which measure the interaction of UV light with a chiral medium over a full UV spectrum. In ORD the extent to which the beam of linearly polarised light is rotated (angular rotation) is the only parameter measured. However in CD the angular rotation is measured along with the different absorbances produced by the two circular components (of plane polarised light) as they travel through the chiral media.

6.3.3.6 Polarimetry and nuclear magnetic resonance (nmr)

The other two main spectroscopic techniques that can be used for analysis of chiral media are polarimetry and nmr. Polarimetry is very similar to ORD except that ORD measures the full UV spectrum , whereas polarimetry only measures selected wavelengths. One of the older naming systems used for optically active compounds was derived from the use of polarimeters. If the rotation of the light is clockwise, then the substance is dextrorotary, and if the rotation is anticlockwise, the substance is levorotarory. The symbols (+) or d are given for dextrorotary and (-) or l for levorotarory. The actual value of the (+) or (-) shift can be measured in degrees using a polarimeter.
NMR is a useful technique for analysis but one drawback is the requirement for a relatively large amount of sample with a minimum mass of a few milligrammes. Depending upon the geometry of the sample, a chiral additive may be required for nmr. The theory behind this technique can be found in most advanced chemistry textbooks, and a good review paper on its application to chiral phenomena was published in 1991 [45].

6.3.4 Labelling optically active compounds [46]

One method of naming optically active compounds has been mentioned above in section 6.3.3.6.

6.3.4.1 R/S notation

Another method used is the Cahn-Ingold-Prelog R/S rules. These rules are designed so that a particular stereochemistry of a molecule can be mentioned without drawing the structure.

Two steps are involved in this process :

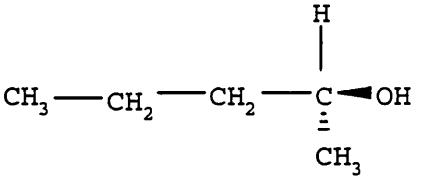
Step 1 : Following a set of sequence rules a priority is assigned to the four atoms or groups attached to the chiral centre.

The sequence rules are :

Sequence rule 1 : if the four atoms attached to the chiral centre are all different, priority depends on atomic number, with the atom of higher atomic number getting higher priority

e.g. Cl > C > H

Sequence rule 2 : if the priority cannot be decided by rule 1 then go to the next atom in the group (and so on, working outwards from the chiral centre). An example of this is shown in structure 25.

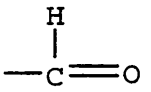


structure 25 : pentan-2-ol

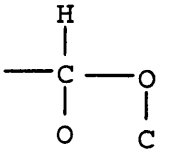
Priority here is :

OH > propyl > methyl > H

Sequence rule 3 : when a multiple bond is present, both atoms are also multiplied, as shown in structures 26 a,b.



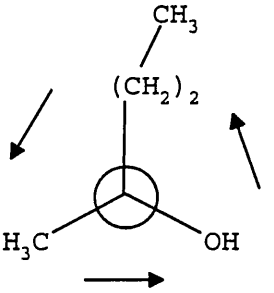
structure 26a : aldehyde



structure 26b : consequence of rule 3

Now that the sequence has been established, step 2 involves visualisation.

Step 2 : the molecule is orientated so that the ligand of lowest priority is directed away from the viewer. If we view pentan-2-ol (structure 25) in this manner the arrangement below is observed.



structure 27 : (S) - pentan-2-ol

When proceeding from the ligand of highest priority to the lowest, a clockwise rotation is specified as R and anticlockwise as S.

It should be noted that the R/S and (±) notations have no association. This is because (±) unlike R/S is a physical property of a substance. If both labels are known, then they should be used e.g. R-(-)- etc.

6.3.4.2 Helices

Helices have been classified as M or P where M denotes a left handed helix and P denotes a right handed helix.

6.3.5 Enantiomers and racemates

One of the requirements for a chiral molecule is that it cannot be superimposed on its mirror image. These mirror images are known as enantiomers, which have similar chemical and physical properties except for optical activity. A 50:50 mixture of enantiomers is known as a racemate (or a racemic mixture).

Often the products of a reaction, or a mixture of enantiomers, are not racemic and the ratio is defined as the enantiomeric excess i.e. % major - % minor.

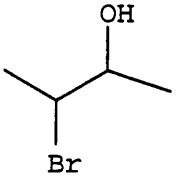
For example, a mixture with R=major, [R] donates concentration of R

$$\text{enantiomeric excess} = \frac{[R] - [S]}{[R] + [S]} \times 100$$

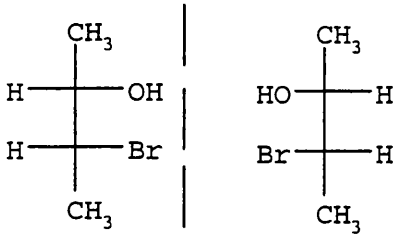
6.3.6 Diastereoisomers

So far the discussion has been based on molecules with 1 chiral centre. A molecule with 2 or more chiral centres is known as a diastereoisomer (sometimes shortened to diastereomer), has 2^n separate forms and 2^{n-1} pairs of enantiomers, where n=number of chiral centres.

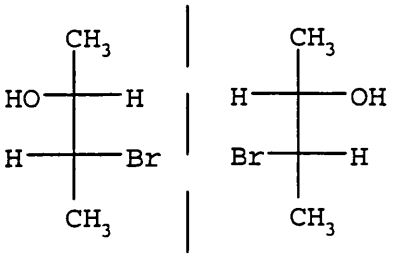
For example the compound 2-bromo-butan-3-ol (structure 28) has 2 chiral centres and thus 4 enantiomers (structures 28a,b,c,d).



structure 28 : 2-bromo-butan-3-ol



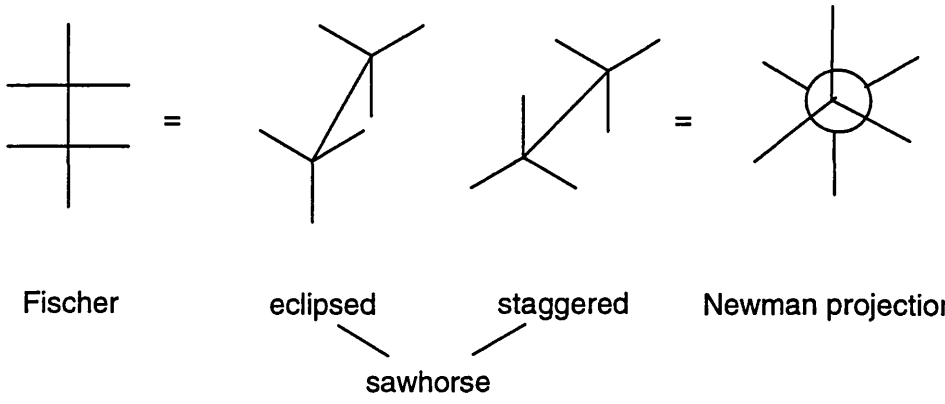
structure 28a,b : (±) erythro form



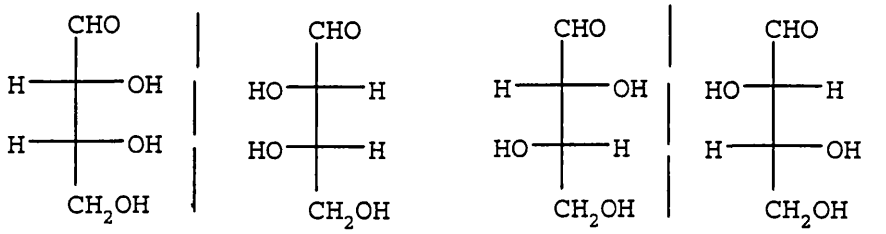
structure 28c,d : (±) threo form

The projection above is known as a Fischer projection and its relationship to other spatial representations is shown in Diagram 12.

Diagram 12 : Spatial representations



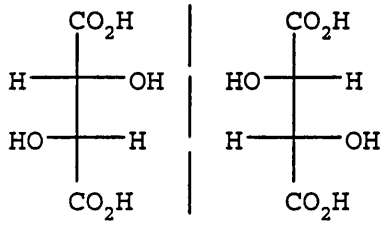
The naming system in 28a,b,c,d is based on the sugar erythrose (structure 29) and threose (structure 30) and the standard nomenclature for compounds with 2 chiral centres and different substituents at each centre. In the Fischer projection, when the highest priority groups are on the same side, the enantiomers are the erythro form, and on the opposite sides for the threo form.



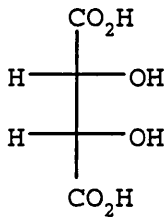
structure 29 : (±) erythrose

structure 30 : (±) threose

The 2^n rule is broken if each chiral centre has identical substituents. In this case there are 3 forms : (+), (-) and meso. (+) and (-) are related as enantiomers and to the meso form as diastereoisomers. This is illustrated by tartaric acid (structures 31 a,b,c).



structure 31a,b : (±)tartaric acid



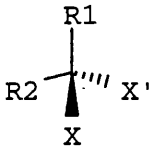
structure 31c : meso tartaric acid

6.3.7 Prochirality [47]

This describes non-chiral molecules which have the potential to become chiral by a chemical reaction. There are 2 types of prochirality : prochiral centre and prochiral faces.

6.3.7.1 Prochiral centre

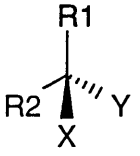
A prochiral centre usually involves 4 groups around a tetrahedral carbon, as shown in structure 32.

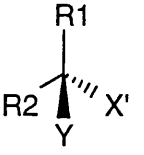


structure 32 : prochiral centre

Here R1≠R2, but X=X'. Replacement of X' with another group ,Y, gives the (R)-enantiomer (structure 33a) and replacement of X gives the (S)-enantiomer (structure 33b). X' is known as the pro-R group and X is known as pro-S. X and X' are collectively known as enantiotopic groups if R1 and R2 are non chiral. If

diastereoisomers are formed (i.e. R1 and/or R2 are chiral) they are known as diastereotopic groups.

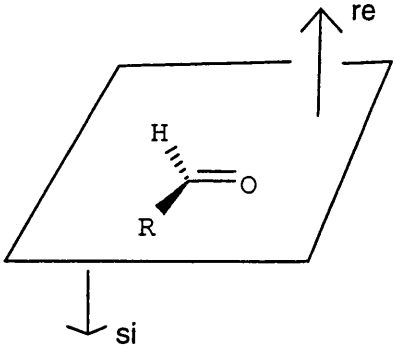


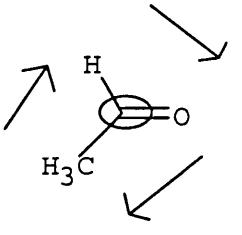


structure 33a : R-enantiomer strucutre 33b : S-enantiomer

6.3.7.2 Prochiral faces

Similarly, non chiral molecules containing carbonyl or alkene groups can be described. If a general carbonyl group is taken in a plane (structure 34) there are 2 possible faces available for attack in a reaction. When the molecule is viewed from above and the R/S sequence rules are used the Newman projection shown in structure 35 is obtained.





structure 34 structure 35

In this case the top face is a clockwise rotation of priority and is labelled re. An anticlockwise rotation is labelled si, as may be viewed from the bottom in this case. These faces are known as enantiotopic if R is achiral and diastereotopic faces if R is chiral.

6.3.8 Pharmacological definitions

Two description of enantiomers which often appears in the literature are eutomer and distomer. The eutomer describes the enantiomer most potent for the desired action and the enantiomer without such action is called the distomer.

The eudistic ratio is the ratio of the activities of eutomer and distomer and the eudistic proportion is the proportion of the concentrations of eutomer and distomer in the biological matrix (i.e. blood, plasma etc.).

Chapter 7 : Stereochemistry and Pharmacology

7.1 Introduction

Most naturally occuring medicinal agents exist in their optically active or single isomer form. Since these compounds are produced in nature, they exist in one isomeric form, due to the inherent chirality of the natural world. For example proteins are made up of L-amino acids, while carbohydrates are of the D-configuration [48].

However, many synthetic chemicals are produced as the optically inactive racemate. A recent survey [49] shows that in 1991 a greater percentage of synthetic drugs were being sold as single enantiomers as compared to 1982. This trend is mainly due to the improvement of chiral separation techniques and tighter regulatory controls.

Since human and animal systems are chiral environments, and many drugs have stereochemical properties, it is important to review stereochemical effects in pharmacology in some detail. The two main aspects are the stereochemical nature of drugs and the effects of stereoselectivity in drug pharmacology.

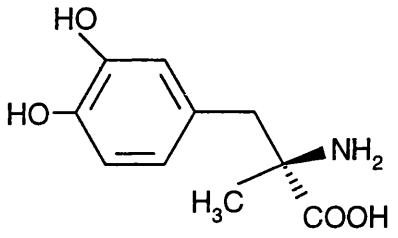
7.2 Stereochemical nature of drugs [50]

The stereochemical nature of drugs can result in their subdivision into two broad catagories with respect to pharmacology. In the first of these the pharmacological activity is due to one enantiomer only, while in the second, the activity differs both qualitatively and quantitavely in each enantiomer.

7.2.1 Pharmacological activity is, or appears to be, due to 1 enantiomer

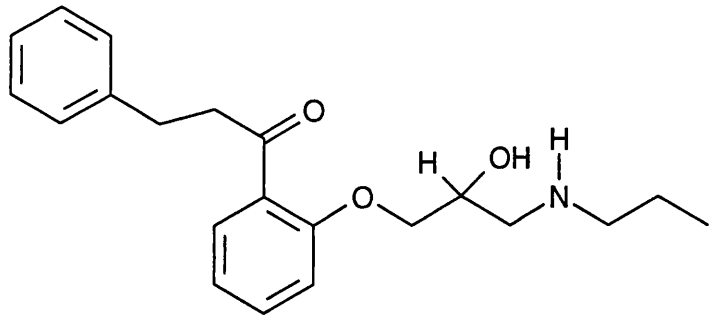
Three possible situations can result in pharmacological activity being associated with one enantiomer.

The first of these is where all activity does in fact reside in one enantiomer. An example of this is the antihypertensive agent α-methyl dopa (structure 36) [51].



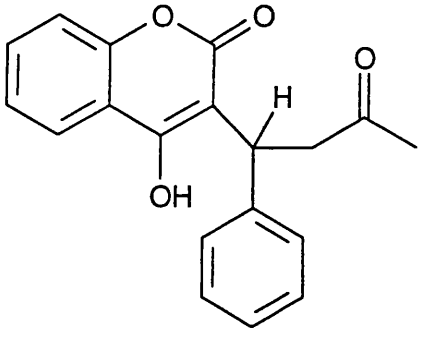
structure 36 : α-methyl dopa

The second case is when both enantiomers have similar activity and potency. This is a very rare situation since receptors and enzymes react to stereochemical differences. An example of this is propafenone (structure 37), where the 2 enantiomers have equal activity with regard to antiarrhythmic activity [52].

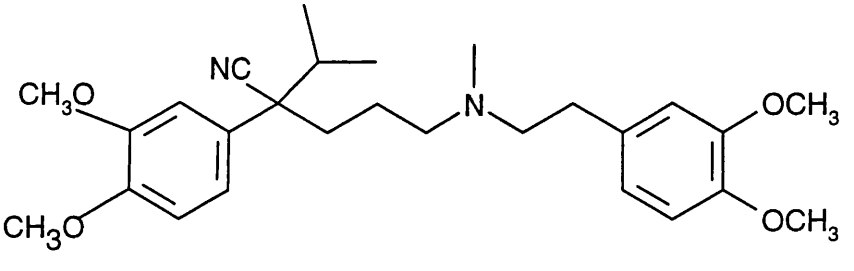


structure 37 : propafenone

The third situation is when the enantiomers have similar activities, but potencies differ. This situation does not present a therapeutic problem and examples include warfarin (structure 38) and verapamil (structure 39). The potency of S-(-)-warfarin, *in vivo*, is from 2 to 5-fold greater than R-(+)-warfarin [53]. Verapamil, a calcium channel blocker used as an antianginal agent and for the treatment of supraventricular tachyarrhythymias, shows different potencies between the two enantiomers. Both R(+)- and S(-)-verapamil equally increase cardiac blood flow through coronary vasodilation, but the (R) isomer is eight- to ten-fold less potent a cardiodepressant as compared with the S(-) isomer [54].



structure 38 : warfarin

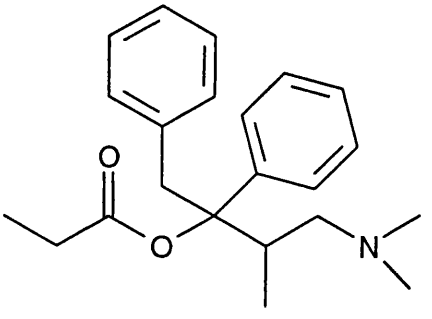


structure 39 : verapamil

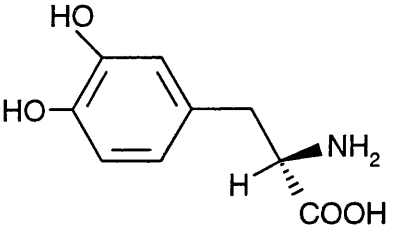
7.2.2 Pharmacological activity differs qualitatively and quantitatively in each enantiomer

Drugs of this type can again be divided into 3 different catagories.

The first of these is when single enantiomers are marketed because of the effects of the other enantiomer. Examples of this are L-dopa and α-dextropropoxyphene (structure 40).



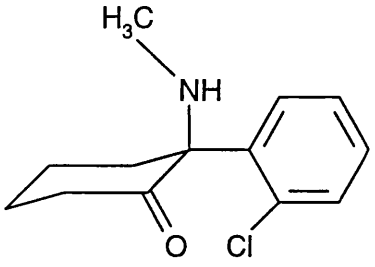
structure 40 : dextropropoxyphene



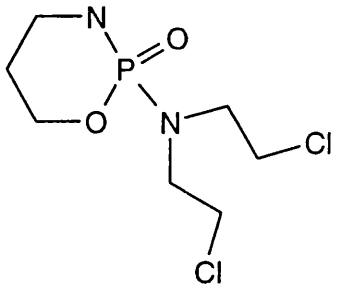
structure 41 : dopa

For dopa (structure 41) it was noticed that many of the serious side effects, such as granulocytopenia, were due to the D-isomer [55]. D-propoxyphene has analgesic properties, whereas its optical isomer L-propoxyphene has antitussive properties, but no analgesic properties [56]. The company Lilly gave them trade names which are also mirror images of each other, Darvon® and Novrad®.

The second situation arises when a racemic drug is developed despite unwanted activity in one isomer. Examples of this include ketamine (structure 42) and cyclophosphamide (structure 43).



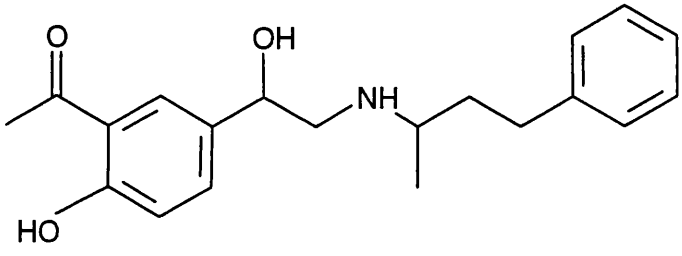
structure 42 : ketamine



structure 43 : cyclophosphamide

All of these are marketed as racemic mixtures with the enantiomers of each drug varying greatly in their pharmacological and toxicological profiles. Ketamine is a commonly used anesthetic and analgesic drug. Post anasthesia reactions to ketamine include hallucinations and agitation. When the potency ratio was investigated, the S-(+)-enantiomer had 3-4 times the potency of the R-(-)-enantiomer, with the side effects associated with the R-(-)- enantiomer [57]. The drug cyclophoshphamide (structure 43) owes its asymmetry to a chiral phosphorus atom and its (-)-enantiomer is twice as effective an antitumour agent but no more toxic than the (+)-enantiomer [58]. It can be seen in the two examples above that it would have been advantageous to market single enantiomers.

The third case of differing pharmacology is when the racemic product may have therapeutic advantages. A good example of this is labetalol (structure 44) for which the racemic product is useful for a percentage of the patient population.



structure 44 : labetalol

Labetalol is diastereomeric and is marketed as an equal mixture of all 4 stereoisomers. The pharmacological effects of each isomer [59] are summarised in Table 16.

Table 16 : Pharmacological effects of labetalol stereoisomers

isomer	effect
R,R	non specific β_1-, β_2- blocker
S,R	α_1-blocker
S,S	inactive
R,R	inactive

The combined product is useful because some patients require β-blockage (R,R) and also vasodilation (S,R). On the other hand the two inactive isomers might advantageously be excluded from the product. Because of this and the fact that some patients do not require vasodilation, the single R,R isomer is being developed (dilevalol).

7.3 Stereochemistry of drug pharmacology

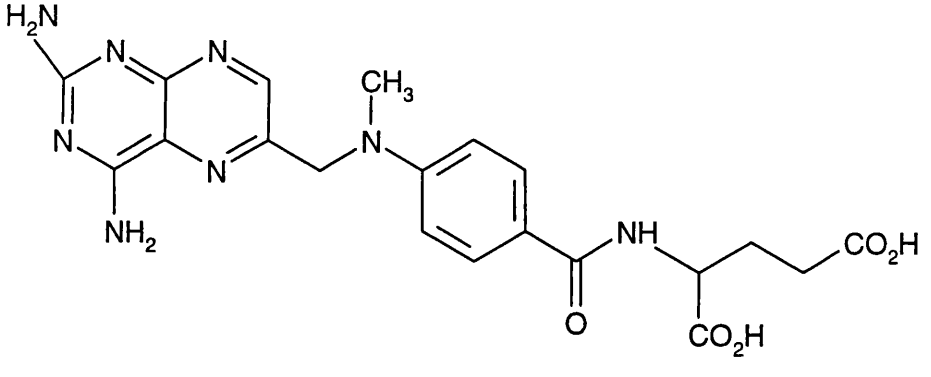
Once the stereopharmacological nature of the drug is known, the disposition and metabolism of the drug must be considered.

7.3.1 Drug disposition, binding and clearance [60]

Many of the events involved in drug disposition do not involve stereochemical interactions. However exceptions to this do occur if the drug molecule interacts specifically with biological macromolecules.

7.3.1.1 Drug absorption

Most drugs are absorbed from the intestine, airways or skin by passive diffusion of unionised drug across lipid membranes. The diffusion is controlled by two properties which do not show stereoselectivity, namely the degree of ionisation of the drug (dependent on pKa) and the solubility of the unionised form. Two drugs which do show stereoselectivity in absorption are methotrexate (structure 45) [61] and dopa (structure 41) [62], with the L-forms of each being actively absorbed and D-forms crossing the intestinal wall passively. Methotrexate is used for the treatment of solid tumours.



structure 45 : methotrexate

7.3.1.2 Drug distribution

In the drug distribution phase the majority of drugs cross the blood-brain barrier or penetrate organs by passive diffusion. However, one example of stereochemically selective distribution involves the R-isomers of 2-arylpropionic acids (e.g. ibuprofen) which are selectively incorporated into adipose tissue [63].

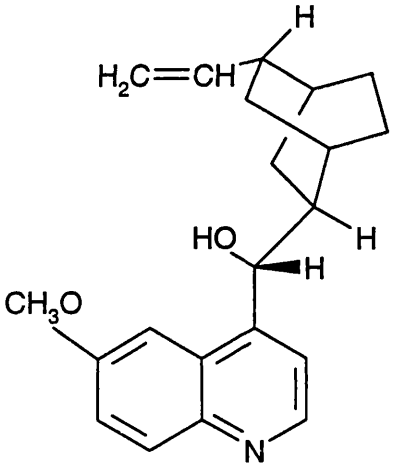
7.3.1.3 Plasma protein binding [64]

Unlike absorption and distribution, protein binding often depends upon stereochemical conformations, and most drugs undergo some degree of binding to plasma proteins. Two plasma proteins are responsible for the majority of drug binding : serum albumin which accounts for the bulk of drug binding, especially acidic solutes, and α_1-acid glycoprotein (AGP) to which weakly basic drugs bind preferentially. Although isomers may differentially bind to particular binding sites on plasma proteins, large conformational flexibility of the proteins may allow binding to other sites [65]. This area is still undergoing vigorous investigation.

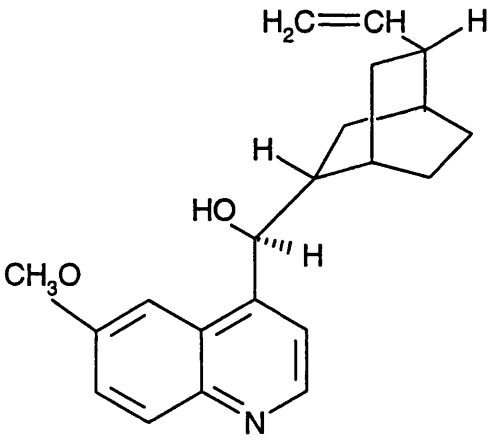
7.3.1.4 Renal excretion

The elimination of drug substances and/or metabolites from the kidney is a major elimination route. Glomerular filtration itself is not stereoselective and only unbound drug in plasma water is filtered. Therefore other factors such as stereoselective protein binding will result in isomers exhibiting differences in renal clearance. However drugs which are cleared

by active secretion in the renal tubules may show stereoselective elimination. An example of this is quinidine (structure 46) which has fourfold higher renal clearance than its diastereoisomer quinine (structure 47) [66].



structure 46 : quinidine



structure 47 : quinine

7.3.2 Metabolism [67]

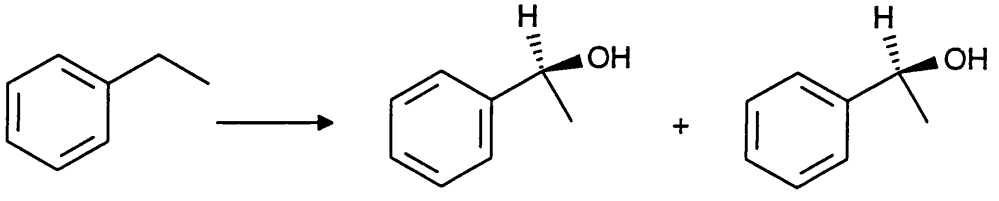
As mentioned earlier, absorption, distribution and excretion are generally passive processes which do not discriminate between enantiomers. However protein binding and enzymatic metabolism can show a high degree of stereoselectivity. This comes from the fact that interaction of the enantiomers of a chiral drug molecule with a chiral macromolecule, e.g. an enzyme, will give a pair of diastereoisomeric complexes. Similarly, the binding of a prochiral substrate to an enzyme, will orientate two enatiotopic groups differently, making them diastereotopic.

The metabolic transformations of compounds may be categorised in terms of five stereochemical courses. Their transformations are prochiral to chiral, chiral to chiral, chiral to diastereoisomeric, chiral to non-chiral and metabolic chiral inversion.

7.3.2.1 Prochiral to chiral transformations

This is a metabolic transformation which could occur at enantiotopic groups, either at a prochiral centre, or at a remote site from the chiral centre. An example of this is the transformation of ethylbenzene (structure 48) to 1-phenylethanol (structures 49 a,b) [68] as outlined in Diagram 13.

Diagram 13 : Transformation of ethyl benzene to 1-phenylethanol



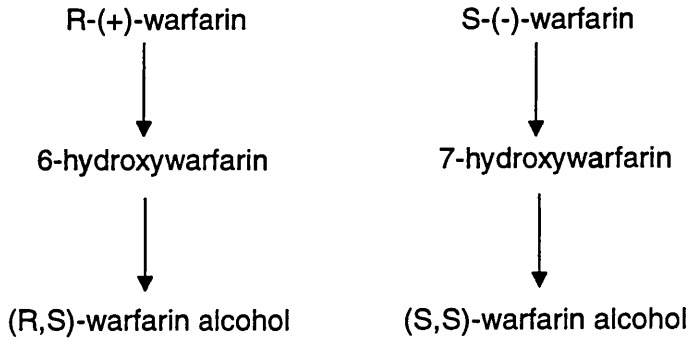
structure 48 : ethylbenzene structures 49 a,b : 1-phenylethanol

A general situation which can occur is the reduction non-symmetric ketones giving a chiral centre in the secondary alcohol product. A number of prochiral ketones have been examined as substrates giving the (S)-alcohols in 80% or greater [68]. These reactions are potentially difficult to predict or control because the binding orientation of the substrate towards the enzyme active site is not open to control. Therefore the chirality of the product is solely a function of the biological system responsible for the metabolism and cannot be influenced by the drug substrate administered, since stereochemical differences do not exist until metabolism has occurred.

7.3.2.2 **Chiral to chiral transformations**

Many examples exist of chiral compounds whose individual enantiomers are transformed at different rates and/or by different routes to metabolites with the original stereochemistry. An example of this is the stereoselective metabolism of warfarin enantiomers. The metabolism of warfarin administered as separate enantiomers in humans [69] is summarised in Diagram 14.

Diagram 14 : Metabolism of warfarin



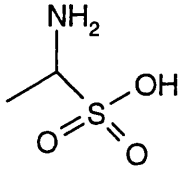
7.3.2.3 Chiral to diastereoisomeric metabolites

Diastereoisomeric metabolite formation is very common in drugs already containing a chiral centre. The way this can happen is by transformation of prochiral groups or by formation by reaction with a conjugate molecule from the body. An example of this could be one of the steps in the complex metabolism of warfarin . Two of the principal routes of metabolism are the aromatic hydroxylation of the 6- and 7-position of the coumarin ring and ketone reduction in the side chain [70]. This ketone

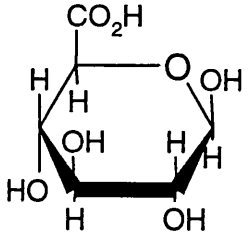
reduction results in the formation of diastereoisomers as outlined in Diagram 14.

Conjugation reactions in the body are very common and the endogenous conjugating agents are often called endocons. Two groups of endocons exist, achiral and chiral. Examples of achiral endocons are methyl and acetyl groups, sulphate and the amino acids glycine (structure 50) and taurine (structure 51). Common chiral endocons are glucuronic acid (structure 52), glucose (structure 53), glutathione (structure 54) and glutamine (structure 55). Conjugation of enantiomeric drugs with these chiral endocons will give enantiomers, and since the endocons are of fixed conformation, two will be possible. Many examples of this exist including 3-hydroxylated benzodiazepines (oxazepam, temazepam, lorazepam and lormetazepam) with glucuronic acid [71].

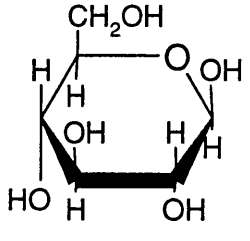
structure 50 : glycine



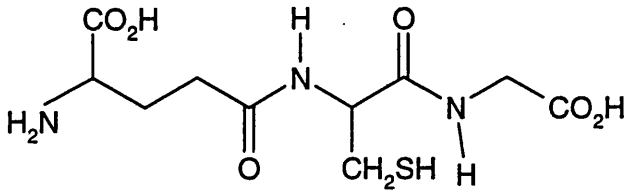
structure 51 : taurine



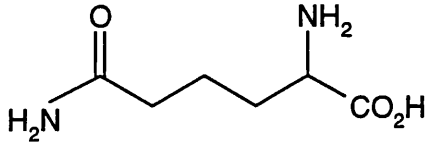
structure 52 : glucuronic acid



structure 53 : a-D-glucose



structure 54 : glutathione



structure 55 : glutamine

7.3.2.4 Chiral to non-chiral transformations

Examples of this transformation are rare, although it may occur when secondary alcohols yield ketones. A good example is of the deamination of amphetamine to yield phenylacetone [72].

7.3.2.5 Metabolic chiral inversion

Some drugs undergo quite extensive racemisation *in vivo* and data on the biological properties of a single enantiomer should be interpreted carefully. Many of the 2-arylpropionic acids (profens) undergo this conversion. These non steroidal anti-inflammatory drugs (NSAID's) have a chiral centre α to a carbonyl group, and the biological activity resides mainly in the (S)-enantiomer. The (R)-enantiomers are often weakly active or inactive. *In vitro* the metabolic inversion of the (R)-enantiomer to the (S)-enantiomer occurs [73, 74]. The initial observations were made with ibuprofen but since then the metabolic transformation has been observed for many other 2-aryl propionic acid drugs.

Chapter 8 : Temazepam and benzodiazepines

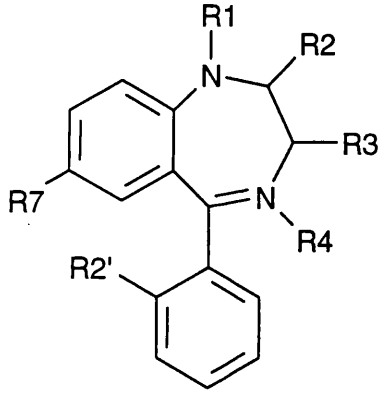
8.1 General

Temazepam is a member of the benzodiazepine class of drugs. Over 3000 benzodiazepine type drugs have been synthesised. About 35 are in clinical use throughout the world, with approximately 14 recommended for use in the UK. Temazepam in particular has been prescribed for sedation and insomnia relief, but more recently has been abused by drug users.

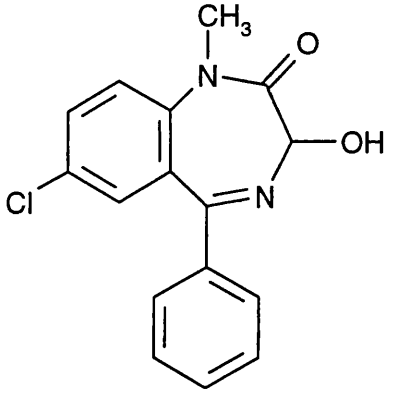
8.2 Chemistry

8.2.1 Planar structure

The term benzodiazepine refers to a molecule which contains a benzene ring fused to a seven membered diazepine ring. However all the important benzodiazepines contain a 5-aryl substituent and a 1,4-diazepine ring, and the term has come to mean 5-aryl-1,4-benzodiazepines [75]. The basic structure [76] and that of temazepam are illustrated below



structure 56 : general 5-aryl-1,4-benzodiazepine

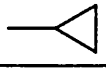


structure 57 : temazepam

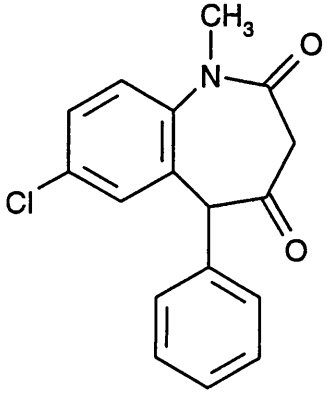
Most commonly in the 5-aryl-1,4-benzodiazepine structure, R_7=Cl, F or NO_2, R_2=carbonyl, R_2'=H or halide, R_1=H or alkyl.

Other common benzodiazepines are summarised in Table 17.

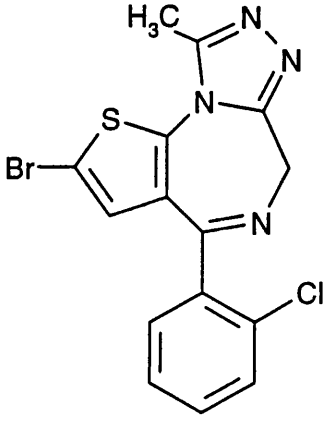
Table 17 : common benzodiazepines

benzodiazepine	R_1	R_2	R_3	R_7	R_2'
bromazepam*	H	=O	H	Br	H
chlordiazepoxide	-	$NHCH_3$	H	Cl	H
clobazam	CH_3	=O	H	Cl	H
clonazepam	H	=O	H	NO_2	Cl
clorazepate	H	=O	COO^-	Cl	H
demoxepam	H	=O	H	Cl	H
diazepam	CH_3	=O	H	Cl	H
flunitrazepam	CH_3	=O	H	NO_2	F
flurazepam	CH_2CH_2 $N(C_2H_5)_2$	=O	H	Cl	F
halazepam	CH_2CF_3	=O	H	Cl	H
lormetazepam	CH_3	=O	OH	Cl	Cl
lorazepam	H	=O	OH	Cl	Cl
nitrazepam	H	=O	H	NO_2	H
nordazepam	H	=O	H	Cl	H
oxazepam	H	=O	OH	Cl	H
prazepam		=O	H	Cl	H
quazepam	CH_2CF_3	=S	H	Cl	F
temazepam	CH_3	=O	OH	Cl	H

Modifications of this basic structure have yielded compounds with similar activities, including 1,5-benzodiazepines (e.g.clobazam, structure 58) and those in which the benzene ring has been replaced with heteroaromatic rings such as thieno (e.g. brotazolam, structure 59).



structure 58 : clobazam

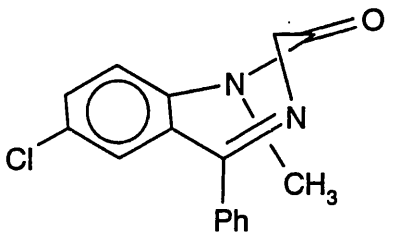


structure 59 : brotazolam

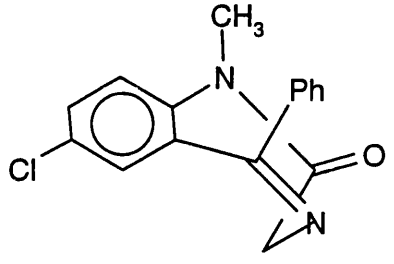
8.2.2 Three-Dimensional structure and conformations of 1,4-benzodiazepines

Diazepam

Diazepam is considered to be achiral because it has no chiral centres. However it does have a chiral plane [77] and the 2 conformations are shown below :



structure 60a : M - diazepam



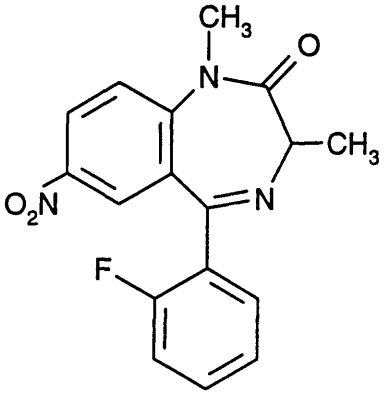
structure 60b : P - diazepam

It can be seen that the 7-membered heterocyclic ring forms a boat shape with the N1 methyl and C5 phenyl groups pointing away from the C3 point. However, the barrier for inversion of the heterocyclic ring is not high enough to prevent ring inversion, and the 2 forms are in thermal equilibrium at room temperature.

3-substituted 1,4-benzodiazepines

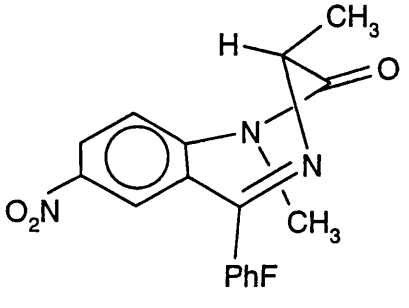
Monosubstitution of the C3 position stabilises one of the conformations, depending on the absolute conformation of position C3. For the (R)-enantiomer the P conformation is more stable, whereas for the (S)-enantiomer the M conformation is more stable. This is because the larger

substituent at C3 cannot point into the ring for steric reasons. The crystal structure for temazepam [78, 79] agrees with this and suggests this conformational argument is true for 3-hydroxyl derivatives. X-ray crystal analysis has also been carried out on (R)- and (S)- 1,3-dimethyl-5-(2-fluorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one [80]. The molecule is shown below (no stereochemical features shown) in structure 61.

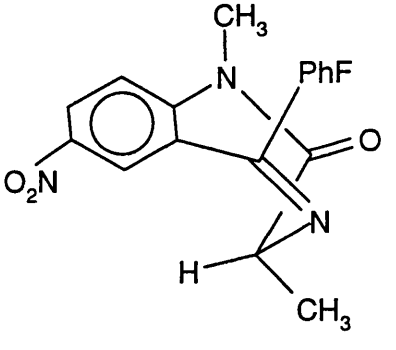


structure 61 : 1,3-dimethyl-5-(2-fluorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one

From the crystal data the conformations preferred for each enantiomer are shown below :



structure 62a : R-conformer



structure 62b : S-conformer

These two conformations can be generalised for the (S)- and (R)- enantiomers of 3-substituted 1,4 benzodiazepine.

8.2.4 Circular dichroism (CD) of 1,4-benzodiazepin-2-ones

Several investigations have taken place on the properties of 1,4-benzodiazepin-2-ones [81, 82, 83, 84, 85]. The studies which investigated 3-substituted-analogues did not include either oxazepam or free (non-complexed) temazepam, but looked at various 3-alkyl substituents [81, 83], 3-ester substituents and lormetazepam. Other investigations obtained data on diazepam [85], and complexes of several 1,4-benzodiazepin-2-ones (including temazepam) with β-cyclodextrin [84].
It was noted in all of these studies involving free or non-complexed chiral 1,4-benzodiazepin-2-ones that there was a positive CD below 300 nm and a negative CD between 300-350 nm for the (S)-enantiomers, with the opposite being true of the (R)-enantiomers.

8.2.5 Chemical and Physical properties

Temazepam is a white crystalline powder with m.p. 156-159°C. It is practically insoluble in water, but soluble 1 in 10 in both ethanol and chloroform. Its pKa is 1.6.

8.3 Pharmacology

8.3.1 Effects

The effects of the benzodiazepines all result from actions of these drugs on the central nervous system (CNS). These include sedation, hypnosis,

decreased anxiety, muscle relaxation, anterograde amnesia and anticonvulsant activity. Only two effects on peripheral tissues have been noted, these being coronary vasodilation, seen after intravenous administration of therapeutic doses of certain benzodiazepines, and neuromuscular blockage, seen only in very high doses.

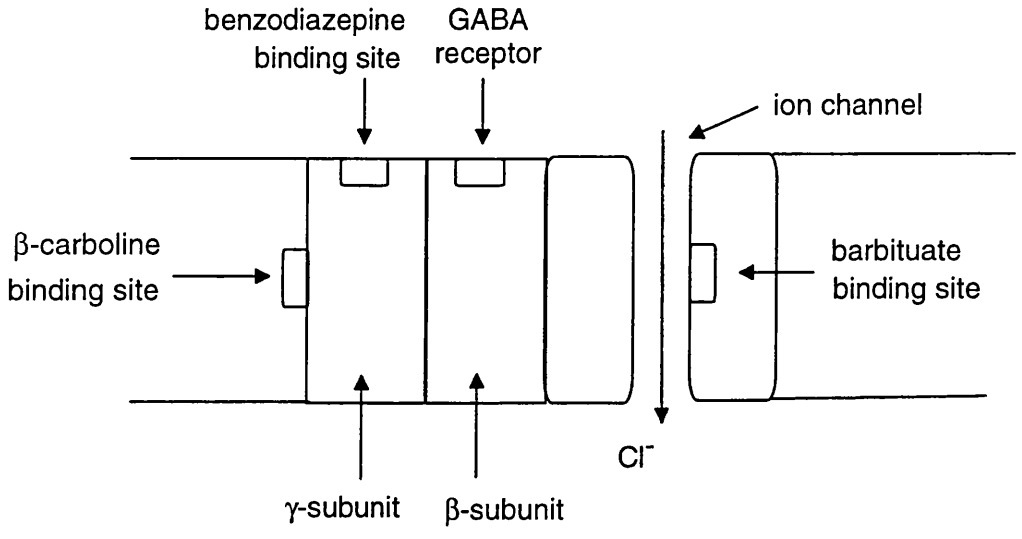
8.3.2 Benzodiazepine binding sites and GABA receptors

The main mechanism through which benzodiazepines are believed to exert all their actions, including anxiolytic, sedative, anticonvulsant and muscle relaxation, is by enhancing the inhibitory activity of GABA (gamma aminobutyric acid) at $GABA_A$ inhibitory synapses.

GABA is the principal inhibitory neurotransmitter in the CNS, and there are at least two types of GABA receptor in the brain, namely $GABA_A$ and $GABA_B$. The $GABA_A$ receptor controls chloride ions at the anion channel and $GABA_B$ receptor controls potassium ions at the cation channel. Benzodiazepines exert their action by binding to $GABA_A$ causing an increase in neuronal inhibition. Since the $GABA_B$ binding site is not influenced by benzodiazepines it will not be discussed.

$GABA_A$ consists of several subunits named α, β, γ, δ, with benzodiazepines binding strongly to γ and GABA to the β subunit. The important receptors and binding sites are summarised below in Diagram 15 [86].

Diagram 15 : binding sites at $GABA_A$ receptors



Other classes which interact with benzodiazepine receptors include inverse agonists and specific antagonists. Inverse agonists such as betacarbolines have opposite effects on the receptors resulting in reduced affinity of the GABA receptor to cause anxiologenic and preconvulsant effects. Specific antagonists such as flumazenil bind to the benzodiazepine receptor and block both types of modulatory agonists [87, 88].

8.3.3 Classification

The benzodiazepines may be classed as long-, intermediate- or short-acting compounds [89]. The long acting drugs are either N1-desalkyl derivatives, e.g. delorazepam and nordazepam, or are oxidised in the liver to these compounds, e g. chlordiazepoxide, diazepam, flurazepam and prazepam. These long-acting benzodiazepines are characterised by formation of desalkyl (nor) derivatives. Further biotransformation of the nor derivatives is slower than the initial desmethylation of the parent compounds and

accumulation can occur after a few days treatment. The rate limiting step of their metabolism (with the exception of 1,5- derivatives) is C3 hydroxylation to oxazepam or lorazepam (2' halogenated derivative).

The 7-nitro derivatives such as clonazepam, flunitrazepam and nitrazepam are metabolised by nitroreduction with no important known active metabolites and are classed as intermediate acting.

The short acting benzodiazepines include C3-hydroxylated compounds such as lorazepam, lormetazepam, oxazepam and temazepam which undergo rapid conjugation with glucuronic acid to water-soluble inactive metabolites that are excreted in the urine [90]. Temazepam is also converted to oxazepam in small amounts [91].

The half-lives of several of these compounds are shown in Table 18 along with their therapeutic doses.

Table 18 : Half-lives and therapeutic doses of commonly prescribed benzodiazepines

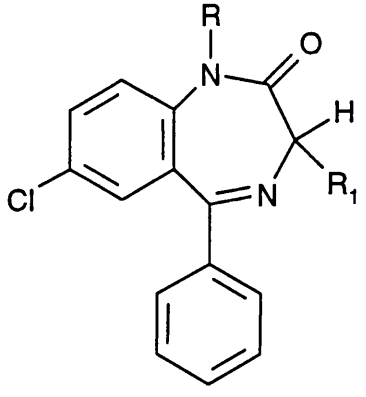
benzodiazepine	use	half-life (hrs) (plasma)	daily dose (mg)
flurazepam	hypnotic	2-3 *	15-30
loprazolam	hypnotic	4-11 (mean 7)	1-2
lormetazepam	hypnotic	10	0.5-1
nitrazepam	hypnotic	18-38 (mean 28)	5-10
temazepam	hypnotic	3-38 (mean 10)	10-30
flunitrazepam	hypnotic	10-70 (mean 25)	0.5-1
lorazepam	anxiolytic	9-24 (mean 14)	1-4
chlordiazepoxide	anxiolytic	5-30 (mean 15)	40-100
diazepam	anxiolytic	20-100 (mean 48)	6-30
clobazam	anxiolytic	10-58 (mean 25)	20-60
bromazepam	anxiolytic	8-19 (mean 12)	3-18
oxazepam	anxiolytic	4-25 (mean 8)	180
clorazepate K	anxiolytic	2 *	7.5-22.5
alprazolam	anxiolytic	6-20 (mean 12)	0.25-3

8.4 Stereoselective chiral binding

Several studies have been completed which suggest differential binding of the enantiomers of chiral 1,4-benzodiazepines to human plasma proteins and animal brain tissue.

8.4.1 Human serum albumin

One study [92] involved the use of circular dichroism (CD) to investigate the binding of four 1,4-benzodiazepines to human serum albumin (HSA). The main structure and ring substitutions are shown below in structure 63 and the table below :



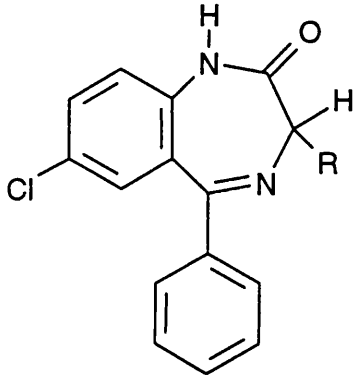
structure 63 : 5-aryl-1,4-benzodiazepin-2-one

R	R_1	name
H	H	nordazepam
CH_3	H	diazepam
H	CH_3	3-methyl-nordazepam
H	$OCO(CH_2)_2CO_2H$	

Three other studies [93, 94, 95] have used a chiral stationary phase, based on HSA bound to silica, for investigation of structural requirements and binding sites. Two of these studies [93, 94] suggest that there is more than one binding site on HSA for benzodiazepines. This conclusion does not agree with the CD study [92]. However the studies [93, 94, 95] should be treated more carefully as it is difficult to say how well a chromatographic system will mimic HSA in the human body.

8.4.2 Bovine brain tissues

Another study [96] focuses on the binding of three chiral 1,4-benzodiazepines (3-substituted nordazepams) to bovine cerebral cortex. The structures of these are shown below in structure 64a :



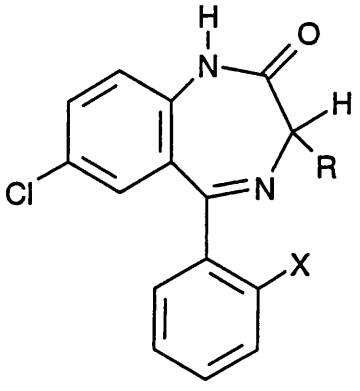
$R = CH_3$, $OCOCH_3$ or $OCOC(CH_3)_3$

structure 64a : 5-aryl-1,4-benzodiazepin-2-one

It was found in this study that the (S)-enantiomer was up to one hundred fold more potent in displacing ^{3}H-flunitrazepam from cerebral receptors than the (R)-enantiomer.

The results have been confirmed by a similar study on bovine brain membranes [82]. Once again, tritiated flunitrazepam was displaced, the (S)-

enantiomers showing greater affinity for the brain membranes. The compounds were 3-substituted nordazepam derivatives and are summarised below :



structure 64b : 5-aryl-1,4-benzodiazepin-2-one

R	X
OCOMe	H
OCOMe	Cl
$OCOBu^t$	H
$OCO(CH_2)_2CO_2^-$	H
Me	H

8.4.3 Rat brain tissues

Several studies have been carried out on rat brain tissues. One of these [97] suggests that the (+)-enantiomer of oxazepam sodium hemisuccinate displaces ^{3}H-diazepam binding 10X as effectively as the (-)-enantiomer in rat cortex tissue.

Another study focused on ^{3}H-diazepam displacement from rat cerebral cortex [98] and noted differential enantiomer binding.

8.5 Medical problems with benzodiazepines

8.5.1 Dependence

Benzodiazepines, although very useful, can be addictive. Dependence is frequently a problem when prescriptions of duration 3-4 weeks and longer are given out and is thought to occur in about one third of all patients receiving benzodiazepines. Research has shown that approximately 35% of patients prescribed benzodiazepines for longer than 4 weeks are likely to develop a significant degree of dependence [99]. The meaning of dependence here is that a person is unable to stop the drug after the appropriate period without withdrawal problems. Major symptoms of withdrawal occur in less than 20% of patients and include acute brain syndrome, delirium, confusion and psychosis. The majority of patients develop more minor conditions such as increased anxiety, insomnia, irritability, dysphoria, headache, nausea, tremors and difficulty in concentration and/or sensory disturbances [100]. It is quite ironic that some of these conditions may have been the reason for administration in the first place.

8.5.2 Toxicity

The benzodiazepines are thought to be less toxic than many other drugs in overdose. However a recent study [101] has shown that certain benzodiazepines have a higher fatal toxicity index (measured in deaths per million prescriptions) than some of the tricyclic antidepressants. Flunitrazepam, temazepam and diazepam have the highest indices. Another study [102] shows there is a large difference in sedation levels obtained after

overdose for several benzodiazepines. However temazepam is one of the most likely to cause death, possibly also due to the habit of injecting this drug [101]. It may also be taken orally in conjuction with alcohol, especially in suicide cases.

8.5.3 Injection of temazepam

Temazepam has a high fatality index which was thought to stem in part from its widespread popularity with drug abusers. However injecting temazepam tends to be habitual in young people and only 45% of temazepam deaths in England and Wales occurred in young people, the same as triazolam [101]. Triazolam is the other widely prescribed short acting hypnotic, which is not commonly employed by drug users [103]. Several studies have been published suggesting a growing number of drug users injecting temazepam only [103, 104, 105], or as a cocktail with other drug substances such as buprenorphine [106, 107, 108]. One of the problems with injection of temazepam was the use of gel filled capsules as a source of the drug. When the contents of these capsules are injected intraarterially instead of intravenously, complications such as limb ischaemia can occur [109].

8.6 Extraction

Many methods have been published in the literature concerning benzodiazepine extraction from biological fluids. Liquid-liquid extraction is commonly used, but problems here include possible hydrolysis of the ring system from acids/bases used in the back extraction. A recent review [110] focused on all extraction techniques as well as biopharmacological data and high performamce liquid chromatography. Another recent paper [111]

reviewed solid phase extraction of 1,4-benzodiazepines from biological fluids. For urinary benzodiazepine conjugates, enzymatic and acid hydrolysis has also been reported [112].

8.7 Chromatography

8.7.1 Achiral

Nonchiral chromatographic assays for benzodiazepines are numerous in the literature. Methods include HPLC and GC with a review from 1990 [113] summarising many chromatographic methods.

HPLC is a commonly used technique for benzodiazepine analysis with non polar phases such as octadecyl (C18) being popular. An example of this is the gradient analysis of 15 benzodiazepines and selected metabolites from post-mortem blood [114].

GC has been used with various detectors including flame ionisation detection [115], nitrogen phosphorus detection [116, 117] and dual analysis with electron capture and nitrogen-phosphorus detection [118]. Gas chromatography with mass spectral detection is commonly employed especially for urinary metabolites [119, 120], selected ion monitoring for "natural" benzodiazepines in human serum [121] and negative chemical ionisation in urine samples [122].

8.7.2 Chiral

Chromatographic separation of chiral benzodiazepines has been extensively carried out by high performace liquid chromatography. Four phases have been investigated, namely Pirkle type phases and those based on ovomucoid protein, human serum albumin and β-cyclodextrin.

Pirkle phases have been used to separate lorazepam and several esters (found on the 3-position) [123]. In this study 6 closely related Pirkle phases were used. Another study [124] used the Pirkle phase (S)-N-(3,5-dinitrobenzoyl)phenylalanine as a chiral selector for the separation of oxazepam, temazepam, lorazepam and lormetazepam. All four drugs had a resolution, R>1 on one of the three mobile phases evaluated. The two studies involve a chromatographic analysis of pure standards and not of biological samples.

The protein phase, ovomucoid bound to silica, was used for the separation of the chiral metabolites of medazepam [125]. Medazepam is not chiral itself, but the 3-hydroxy metabolites have a chiral centre. Again this application did not involve biological samples.

Human serum albumin, as well as being a transport protein, is used as a selector for chiral stationary phases. Three studies [93, 94, 95] have used this stationary phase to examine binding sites and structural requirements for various benzodiazepines.

Several studies have involved β-cyclodextrin bound to silica as a chiral selector for benzodiazepines. Two of these [126, 127] analysed separation factors for several racemic benzodiazepines. Another two studies [128, 129] involved the analysis of benzodiazepine glucuronides in human urine samples. In both cases some separation was obtained on octadecyl (C18)

bonded silica with full separation of diastereoisomers being obtained on β-cyclodextrin.

Chapter 9 : Chromatographic enantiomeric separation

9.1 Introduction

Over the past 25-30 years there has been an increase in the popularity of chromatography for separating enantiomers. These methods require formation of a diastereoisomeric complex, with the main two methods being direct and indirect resolution. Direct resolution involves injecting the enantiomers into the chromatographic system without chemical alteration, which has a stationary or mobile phase with some kind of chiral recognition capability. Indirect resolution involves reaction of the enantiomer(s) with a stereochemically pure reagent to form diastereoisomeric complexes, prior to achiral chromatography.

9.2 Direct resolution

Several advantages of this method exist over indirect resolution. The main one is that no sample pre-derivatisation is required, leading to direct on-column separation. Several chiral phases have been developed and applied to techniques such as HPLC and GC.

9.2.1 High performance liquid chromatography

All chiral HPLC stationary phases are based on a chiral selector molecule bonded to the silica. There are 5 basic types of selector, based on the recognition and mechanisms of formation and these are summarised as follows [130]

Type I : solute/stationary phase complexes are formed by attractive interactions, hydrogen bonding, π-π interactions, dipole stacking etc.;

Type II : the primary mechanism for the diastereoisomeric complex formation is attractive interaction, but inclusion can also be important;

Type III : the solute enters a cavity in the stationary phase, forming an inclusion complex;

Type IV : the solute is part of a diastereoisomeric metal complex;

Type V : the stationary phase is a protein and the complexes are formed though a combination of hydrophobic and polar interactions;

New stationary phases : antibiotics

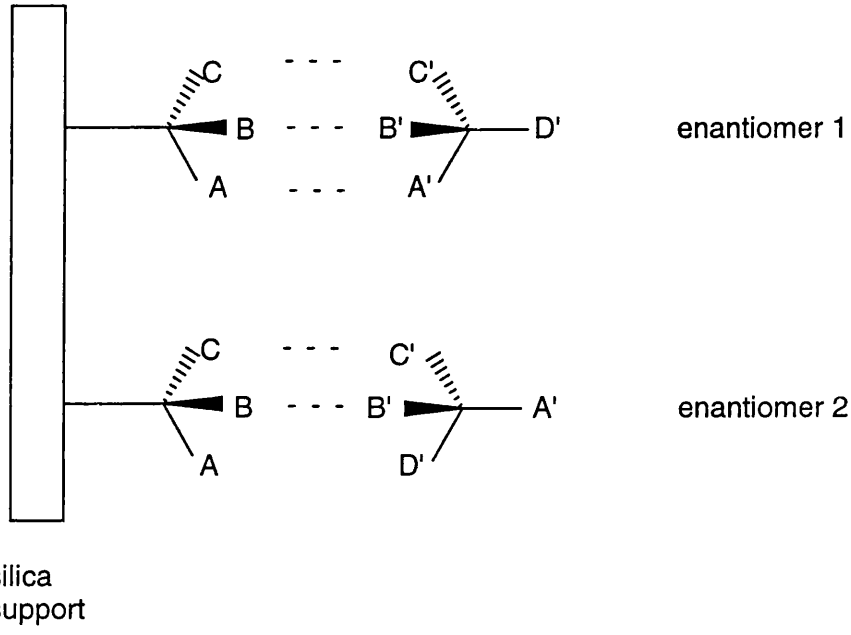
9.2.1.1 Type I stationary phases : Pirkle type phases

The type I phases may also be known as synthetic multiple-interaction phases, but are more commonly referred to as Pirkle phases. This is after W.H. Pirkle who has done much work in this area from its beginnings in 1979 to the present day. A review has recently been published covering the history of the Pirkle laboratory and many of the phases designed [131].

The Pirkle stationary phases depend on the three-point rule [132], which states chiral recognition requires a minimum of three simultaneous interactions between the stationary phase and at least one of the

enantiomers. At least one of the interactions must be stereochemically dependent. A pictorial representation is shown below in Diagram 16.

Diagram 16 : three point rule

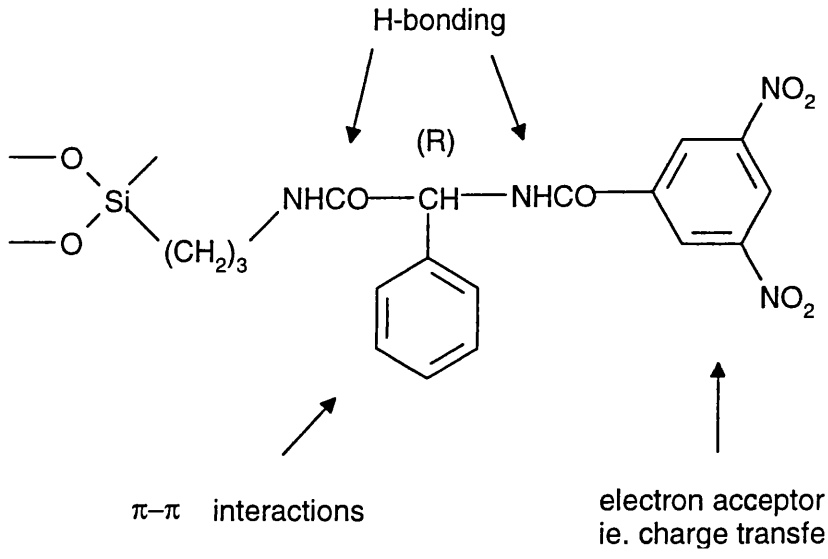


From Diagram 16 it can be seen enantiomer 1 interacts at three sites : A-A', B-B', C-C', whereas mirror image 2 does not interact at A-A'. Therefore differences in binding energy are possible and each enantiomer will spend a different amount of time on the phase. The stationary phases are designed so that each interaction is different.

Commonly, the 3 functional groups are :

1. π-acidic or π-basic aromatic groups, i.e. capable of donor-acceptor interactions ;
2. polar hydrogen bond and/or dipole stacking sites ;
3. bulky non-polar groups, providing steric repulsion, van der waals interactions, and/or conformational control.

An example of a commercially available phase is (R)-phenylglycine-3,5-dinitrobenzoyl, shown in the following structure :



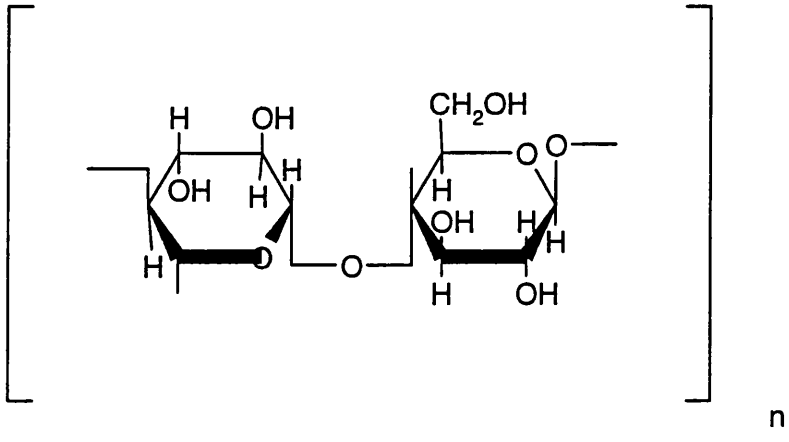
structure 65 : (R)-phenylglycine-3,5-dinitrobenzoyl

In the above structure, the three interactions for this particular phase are shown, and many other phases are similar with minor structural differences. For example the π-π interactions could be increased by changing the benzene ring to a napthalene ring, or an electron donor group could replace the dinitrobenzoyl group.

Many classes of compound have been separated on Pirkle phases with several drug classes of interest being antihypertensives, antianginals and antiinflammatories [133].

9.2.1.2 Type II stationary phases : cellulose [134]

Cellulose is a good choice of material for chiral stationary phases due to its inherent optical activity and abundance in the biosphere. It is a highly ordered crystalline polymer consisting of D-(+)-glucose units joined by α-1,4 linkages as shown in structure 66

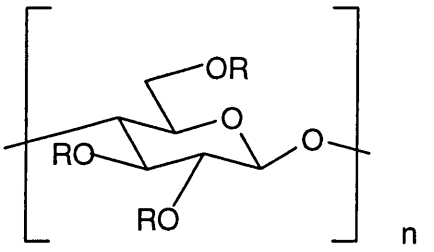


structure 66 : cellulose

X-ray analysis has shown that these chains lie side by side in bundles, held together by hydrogen bonds. These bundles become twisted together forming rope-like helical structures. Since cellulose consists of chiral subunits (D-(+)-glucose), chiral recognition may occur either through attractive interactions between the solute and D-(+)-glucose or by inclusion of the solute into the cavities/channels between the chains.

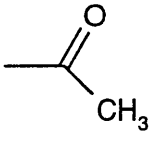
However it was found that natural cellulose did not have enough mechanical strength for HPLC, which prompted manufacture of several derivatives. These are shown below in Diagram 17.

Diagram 17 : derivatives of cellulose

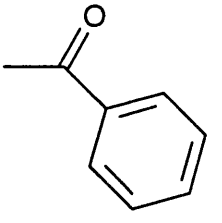


basic cellulose structure

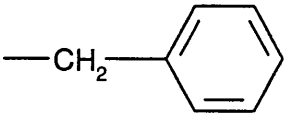
some possible structures for R



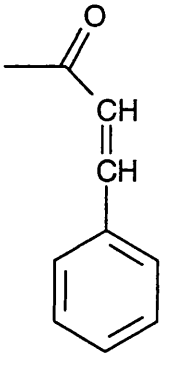
acetate



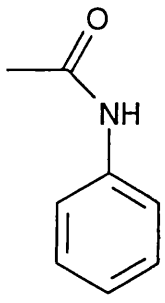
benzoate



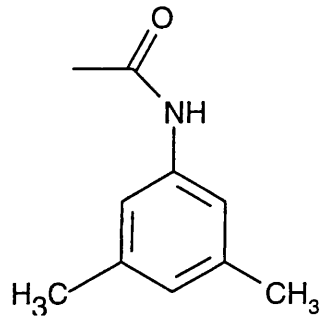
methyl ether



cinnamate



phenyl carbamate



3,5-dimethyl-
phenylcarbamate

One important practical consideration for these stationary phases is that chlorinated solvents will remove the derivatised cellulose support. This is also true of acetone, DMSO and methyl ethyl ketone. However, solvents such as hexane, propan-2-ol, ethanol, methanol or water can be used safely.

The celluloses have been used for separation of many drug compounds including mephobarbital and mephenytoin on cellulose tris(4-methylbenzoate) [135] and bevantolol on cellulose tris(3,5-dimethylphenylcarbamate) [136].

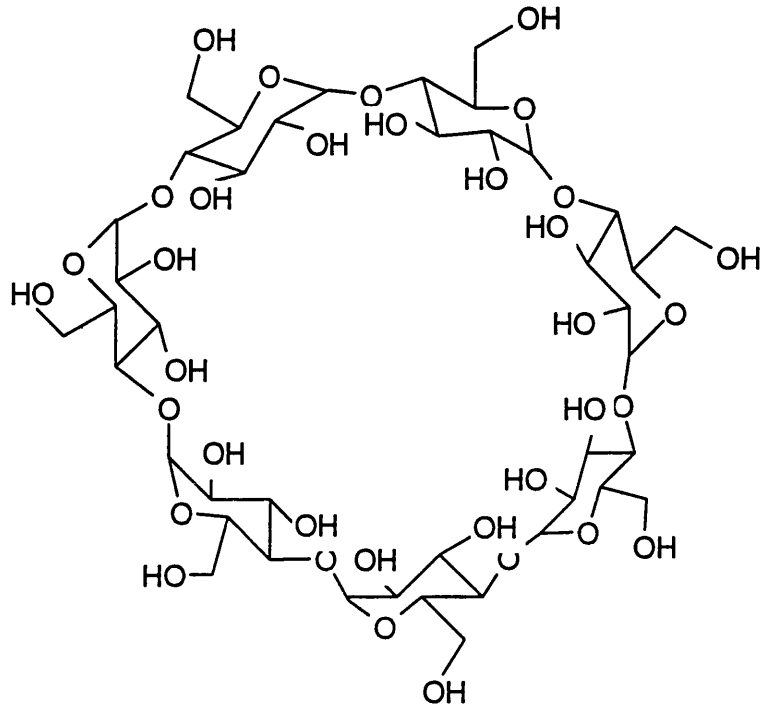
9.2.1.3 Type III stationary phases : inclusion complexes

These phases require an inclusion complex to form between the solute and stationary phase. Three types of molecule are bound to silica, namely cyclodextrins, polymethacrylates and crown ethers.

9.2.1.3.1 Cyclodextrin bonded phases [137]

Cyclodextrin molecules are cyclic oligosaccharides comprising six to twelve glucose molecules bonded in α-1,4 linkages. The molecules are toroidally shaped and the most commonly used in chromatography are α, β

and γ-cyclodextrin, containing 6, 7, 8 glucose units respectively. β-cyclodextrin is shown in structure 67.



structure 67 : β-cyclodextrin

One or two of the primary hydroxyl groups are used to link the cyclodextrin to the support, with the secondary hydroxyl groups lining the mouth of the cavity. The mouth of the cyclodextrin is therefore more hydrophilic with the lining being relatively hydrophobic due to the glycosidic oxygen bridges.

The mechanism of separation relies on the fact that part of the solute molecule should fit into the cyclodextrin cavity (inclusion) so that the centre of asymmetry of the analyte is associated with the polar hydroxyl groups at the edge of the cavity. Therefore the asymmetric centre may be up to 4 bonds away from the aromatic moiety. The size of the cyclodextrin cavity is very important in determining the type of compound separated. As a general rule substituted phenyl, naphthyl and biphenyl rings can be

separated on β-cyclodextrin while smaller molecules may separate on α-cyclodextrin. Larger molecules such as 4 or 5 ring steroids can be separated on γ-cyclodextrin.

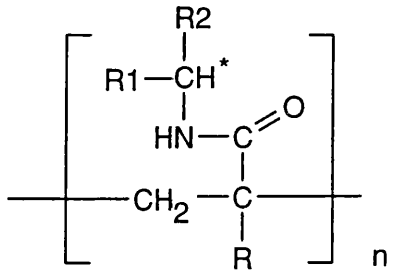
Many derivatives of cyclodextrins have been produced with β-cyclodextrin being the most commonly altered. The molecules are bonded to the secondary hydroxyl and examples include (R)- and (S)-naphthylethylcarbamate [138], carboxymethyl- and carboxyethyl-[139], all bonded to β-cyclodextrin.

Commercially available derivatives [140] include acetyl (α, β and γ-cyclodextrin), (R)- and (S)- napthyl ethyl carbamate (β-cyclodextrin) and 3,5-dimethyl phenyl carbamate (β-cyclodextrin).

A number of drugs have been separated on β-cyclodextrin including ibuprofen [141], terfenadine [142] and several amphetamines [143].

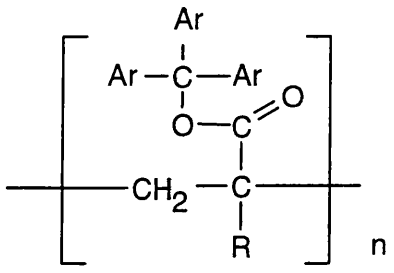
9.2.1.3.2 Polymethacrylate phases

These phases have been developed from polymerisation reactions forming optically active synthetic networks or helices. The polymerisation must be completed carefully with the use of a chiral reagent or chiral catalyst. If a chiral monomer is used, then crosslinking polymerisation occurs which results in a chiral polymer network. Examples of this are polyacrylamides and polymethylacrylamides of which the general structure is shown in structure 68.



structure 68 : polyacrylamide

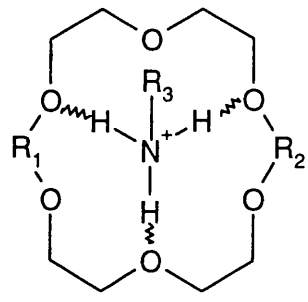
If a chiral catalyst is used on an achiral monomer then isotactic (stereoregular) single handed helical structures are produced. An example of the basic structure is shown below in structure 69.



structure 69 :

9.2.1.3.3 Chiral crown ethers-

Crown ethers are synthetic macrocyclic polyethers that can form selective complexes with various cations. An 16-crown-6-ether is shown in structure 70 , where the 16 indicates the total number of atoms in the polyether ring and 6 the number of oxygen atoms.



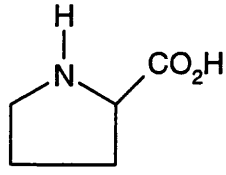
structure 70 : 16-crown-6-ether

In structure R_1 and R_2 are the chiral elements of the crown ether. R_1 and R_2 are chosen and added to hinder rotation of the crown ether about its axis of disymmetry.

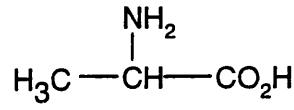
The 16-crown-6-ether is particularly useful in forming stable complexes with potassium and ammonium ions because the cavity size and electrostatic properties of the oxygen atoms suit these molecules. Thus a structural requirement in the analyte is a primary amine moiety, such as in amino acids and small peptides.

9.2.1.4 Type IV - Ligand exchange chromatography [144, 145]

Chiral ligand exchange chromatography works on a principle involving metal atoms for diastereoisomeric formation. The separation occurs on a square planar transition metal atom such as copper(II) or nickel(II) with four possible binding sites for ligands. Two of the binding sites on the atom are taken by one enantiomer of a chiral bidentate amino acid such as L-proline (structure 71) or L-alanine (structure 72).



structure 71 : proline



structure 72 : alanine

This complex takes on a "chiral" character and enantiomeric mixtures of suitable compounds, such as amino acids or other bidentate molecules, are separated due to interaction with the complex.

In most cases a nonpolar stationary phase such as C18 is used with a mobile phase containing the chiral selector (amino acid and copper(II) salt). However several stationary phases have been manufactured. These have an amino acid such as proline or valine bonded directly to the silica and the copper(II) salt is added to the mobile phase.

9.2.1.5 Type V : Protein based stationary phases [146]

Proteins are high molecular weight biological polymers and are composed of L-amino acids. One function of proteins in biological systems is to bind xenobiotic compounds. Because of this and the fact they are inherently chiral, proteins have been bonded to silica as chiral stationary phases.

Proteins which have been bound include α_1-acid glycoprotein (AGP), albumin (SA) as either human serum albumin (HSA) or bovine serum albumin (BSA) and the enzyme ovomucoid (OVM) obtained from chicken egg whites.

The most common conditions for type V phases are similar to a biological environment i.e. aqueous mobile phase containing buffer at pH 7.

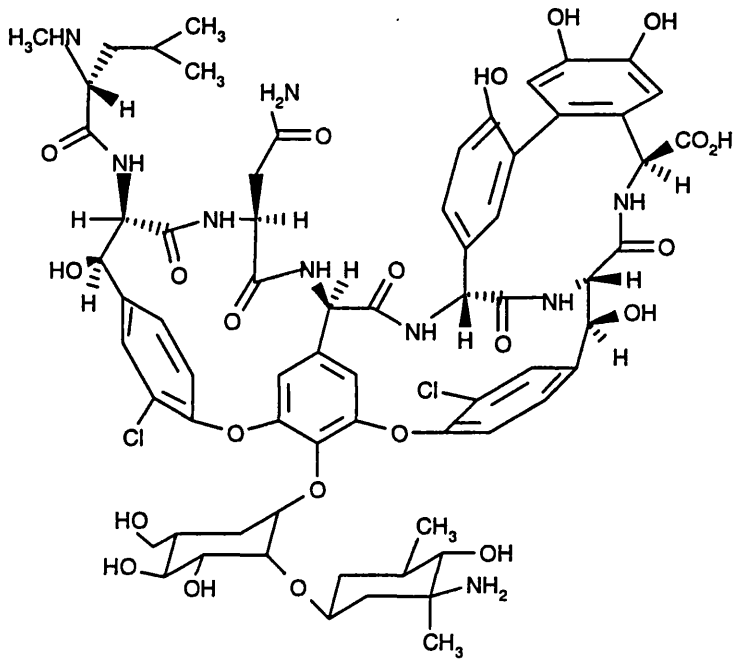
Under these conditions many interactions occur including hydrogen bonding, hydrophobic and electrostatic interactions.

Many chiral separations of drugs have been established on protein phases including warfarin on OVM [147], β-blockers on AGP [148] and omeprazole (gastric acid inhibitor) on AGP, OVM and BSA [149].

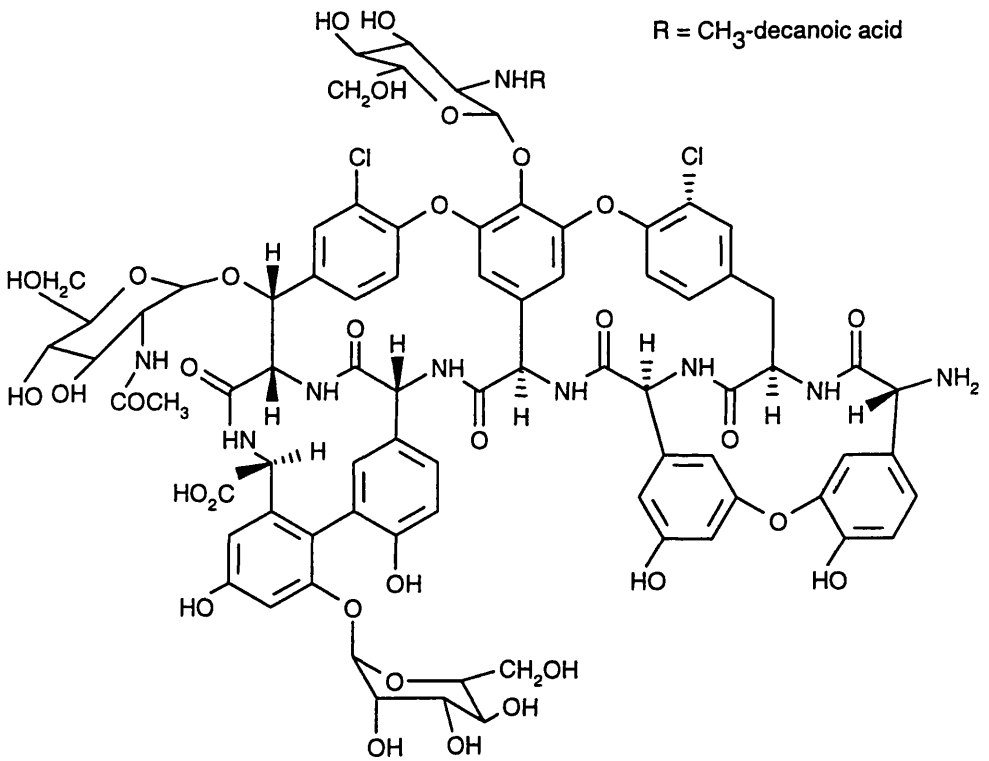
9.2.1.6 New stationary phases : antibiotics [150]

A new class of chiral selectors has been developed, namely macrocyclic antibiotics bonded to silica. The two commercially available at present are vancomycin (structure 73) and teicoplanin (structure 74) [151].

Both of these could be described as multimodal phases because the complex chiral environments they contain. For example they can mimic Pirkle phases in certain respects due to the abundance of amine, amide, carboxylic acid and substituted benzyl groups. Also, these phases contain cavities with possibilities for inclusion complexation.



structure 73 : vancomycin



structure 74 : teicoplanin

9.2.2 Gas chromatography [152]

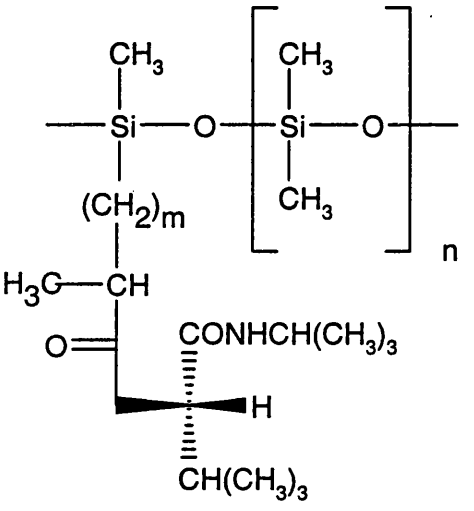
Direct separations by gas chromatography have not made the same advances as in liquid chromatography. One of the main reasons is the difficulty in preparing a stationary phase which will withstand the high temperatures often encountered in gas chromatography.

The three main types of stationary phase used are hydrogen bonding phases, chiral metal coordination compounds and cyclodextrin type phases.

9.2.2.1 Hydrogen bonding stationary phases

These phases require the formation of hydrogen bonds between the analyte and phase. The analytes often require derivatisation to increase volatility and/or introduce suitable groups for hydrogen bonding. Reagents used for this include isocyanates and phosgene.

Several commercial phases are based on the following structure :



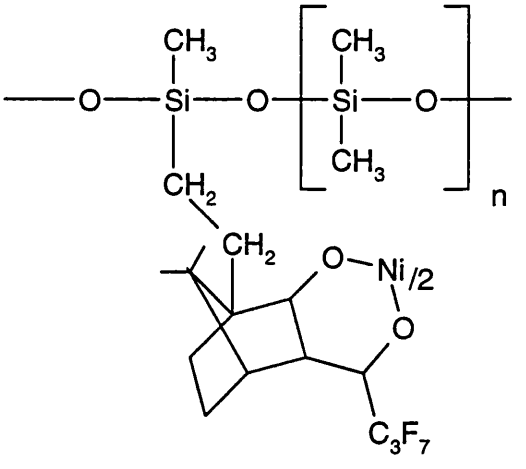
where m=1-3

structure 75 : Chirasil-Val

9.2.2.2 Metal coordination compounds

Another type of stationary phase is that resulting from the complex resulting from divalent transition metal ions and terpene ketones.

The most successful is nickel(II)-[3-(heptafluorobutanoyl)-(1R)-camphonate]. This was bonded to dimethylsiloxane with the resulting structure shown below.



Chiral recognition takes place when the analyte donates electrons to the nickel atom forming a diastereoisomer. Compounds which have been separated include cyclic ethers, esters, acetals, aldehydes, ketones and alcohols from classes such as pheromones and essential oils. The major disadvantage is the low temperature range of operation which is 25-100°C. The 2 in structure 75 means that another heptafluorobutanoyl-(1R)-camphonate molecule is bound to the nickel atom.

9.2.2.3 Cyclodextrin type phases [153]

Cyclodextrin based polysiloxane derivatives have been the most successful stationary phases for GC having operating temperatures from 25-250°C. Above this temperature the phase is still mechanically stable, but the cyclodextrin has lost its enantioselectivity. It should be noted that differences between phases from different manufacturers may result in an upper limit between 200-250°C.

Native β- and γ-cyclodextrin are available as stationary phases, but the vast majority are per-O-alkyl derivatives such as heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin. These structures are very similar to the cyclodextrin structure shown in section 9.2.1.3.1 except alkyl groups usually replace the alcoholic hydrogen atoms of the glucose molecules.

Chapter 10 : Experimental

10.1 Materials

10.1.1 Chemicals

Methanol, ethanol, propan-1-ol, propan-2-ol, acetonitrile were HPLC grade from BDH, Merck Ltd, Poole, UK. Hexane was purchased as HPLC grade from Aldrich Chemical Company, Gillingham, UK.
Potassium dihydrogen orthophosphate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate and orthophosphoric acid were GPR™ grade, all being obtained from BDH.
The Bond-Elute solid phase extraction columns C8 (octylsilica) were supplied by Varian, Harbour City, CA, USA.

10.1.2 Drug standards and biological specimens

The reference drug standards of benzodiazepines (temazepam, oxazepam, lormetazepam, lorazepam and diazepam) were obtained from Sigma-Aldrich Chemical Company Ltd, Poole, UK.

10.2 Chromatography

The HPLC system consisted of a solvent pump, LKB 2150 (LKB-Produkter AB, Box 305, S-161 26 Bromma, Sweden), external injection valve with a 20µl loop, column 250 x 4.0 mm i.d., LiChroCART® 250-4 ChiraDex®

β-cyclodextrin (E. Merck, Darmstadt, Germany), Waters 490-MS UV detector (Millipore UK Ltd/Waters Chromatography division, Harrow, Middlesex) and a pen recorder (Linseis NRE-68). The reversed phase column used was a 250 x 4.6 mm i.d. ODS HL5 (capital HPLC, Broxburn, West Lothian, UK).

10.3 Extraction

10.3.1 Extrelut® extraction

The Extrelut® extraction method for benzodiazepines used routinely in the Department of Forensic Medicine and Science was used. The method involved the addition of 0.25 ml of 5% ammonia solution and 1 ml phosphate buffer (0.05M, pH 7.4) to 1 ml blood. The blood was then mixed for several minutes. While mixing occurred 0.25 ml 5% ammonia solution is added to the columns containing Extrelut® (approximately 8 grammes per column). The mixture was then added to the columns and left for 5 minutes for absorption. The drugs were then eluted with the addition of 8 ml diethyl ether. The ether was then evaporated and the sample was reconstituted with mobile phase prior to chromatography.

10.3.2 Solid phase extraction on octylsilica (C8)

The solid phase extraction method was designed such that no steps involved acid or base, because of possible racemisation of temazepam under these conditions.

The sample pre-treatment and C8 methods were as follows :

pre-treatment : add 1ml (or 2ml) 0.05 M phosphate buffer (pH 6.8) to 1ml (or 2ml) blood or serum and mix well.

SPE

1. conditioning : 1 column volume of methanol
   1 column volume of 0.05 M phosphate buffer (pH 6.8)
2. sample : add slowly over 2-3 minutes
3. wash : 2 column volumes of water
   1 column volume of hexane
4. elution : 1 column volume of 80% methanol in water

The samples were then evaporated to dryness and reconstituted in mobile phase.

This solid phase extraction method was used on plasma samples for chiral and non-chiral analysis.

10.3.3 Liquid - liquid extraction for semi-preparative samples

Individual enantiomers were collected as fractions following HPLC using the mobile phase 10% acetonitrile in water. The excess acetonitrile was evaporated and a liquid - liquid extraction carried out. Several solvents were evaluated for this purpose, including diethyl ether, ethyl acetate, dichloromethane and chloroform. Ether was selected. Following the extraction, the ether was evaporated and the two enantiomers were dissolved in methanol, which was then evaporated. This extra step was to remove any traces of diethyl ether from the enantiomers.

Prior to this extraction of the enantiomers, several solvents were evaluated in the liquid-liquid extraction. The solvents were ethyl acetate, diethyl ether, dichloromethane and chloroform.

10.4 Circular dichroism

Circular dichroism spectra of temazepam enantiomers were recorded on a Jasco J-600 Spectropolarimeter with a 0.1 cm cell.

10.5 Oral administration of temazepam and collection of the samples

Two separate experiments were carried out on a healthy male volunteer. In the first study one 10mg tablet of temazepam was administered (swallowed with a mouthful of water) with collection times up to 24 hours. In the second study, two 10mg tablets were administered with collection times up to 60 hours.

The sample collection and storage were as follows : 10ml blood was collected in a heparinised vial and mixed gently. Serum was separated immediately by centrifugation at 3000 rpm for 10 minutes, with the separated serum frozen until analysis.

Chapter 11 : Results and discussion I

11.1 Introduction

High performance liquid chromatographic chiral separations using cyclodextrin bonded silica can be carried out in reversed, normal or polar organic mobile phase conditions. Because the β-cyclodextrin stationary phase contains a nonpolar inclusion cavity and polar hydroxyl groups at the mouth of the cavity, either of these can be supressed to give reversed phase or polar organic conditions.

When cyclodextrin stationary phases are used with aqueous mobile phases, the basic retention mechanism is inclusion complexation. This mechanism involves the attraction of the apolar molecule or molecular segment to the apolar cavity. The cavity of the cyclodextrin becomes very selective when an aromatic group is present as there is electron sharing between the aromatic methylene groups and the glucoside oxygens. It is therefore essential that if a chiral separation is attempted in reversed phase mode then the analyte must have at least one aromatic ring.

In the normal phase mode for cyclodextrins the mobile phase is very similar in theory to that used with achiral chromatographic columns. In this particular case inclusion complexation and polar interactions such as hydrogen bonding occur between the stationary phase and analyte.

The polar organic mode derives its name from having polar mobile phase constituents such as anhydrous triethylamine and glacial acetic acid and does not include any water. Usually a mixture of solvents is used containing small amounts of both triethylamine and glacial acetic acid. In

the polar organic phase, inclusion complexation is supressed and analytes interact with the secondary hydroxyl groups across the larger opening of the cyclodextrin. Since three points are required for chiral recognition, the solvent pool in the cyclodextrin cavity is seen as one. Therefore at least two functional groups capable of surface interaction must be present in the analyte. This mobile phase should be a good alternative to reversed phase systems because different requirements on the analyte are demanded, and separations may occur where inclusion was not possible.

In this study the reversed phase mode was evaluated for separation of 1,4-benzodiazepines. The chiral resolution factors were investigated for oxazepam, temazepam, lormetazepam and lorazepam, with particular reference to temazepam.

11.2 Chromatography

11.2.1 Mobile phase effects

Several mobile phases were tested and resolution factors obtained. These included water : alcohol mixtures, water : acetonitrile mixtures, buffer : alcohol mixtures and buffer : acetonitrile mixtures.

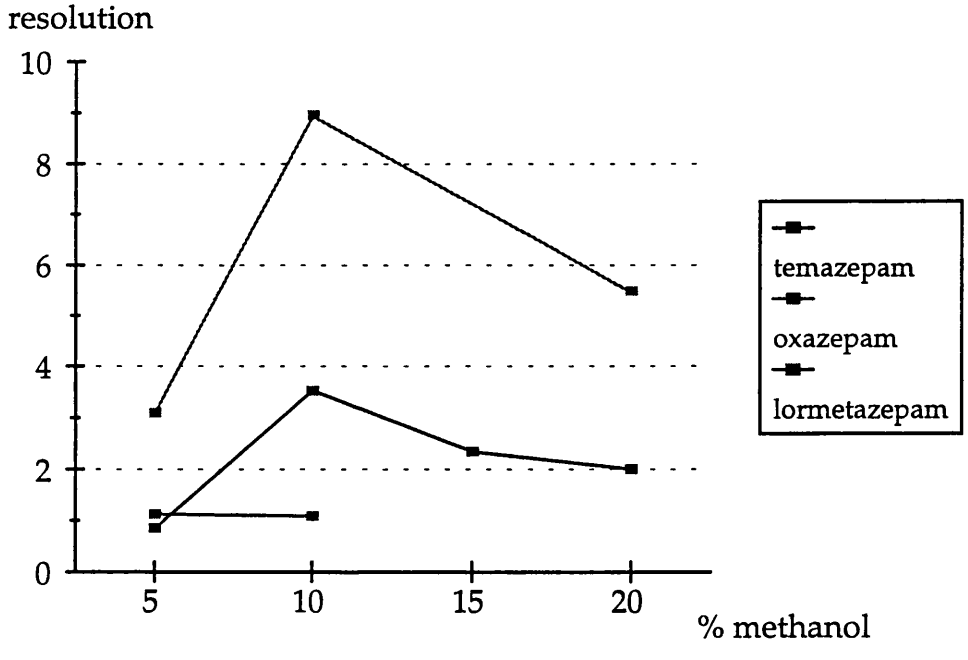
11.2.1.1 Water : alcohol/acetonitrile mixtures

In reversed phase systems using cyclodextrins it is known that the type and ratio of organic modifier to aqueous part in the mobile phase affects stereoselectivity. Several solvents were mixed in varying degrees with water, the solvents being methanol, ethanol, propan-1-ol and acetonitrile.

Methanol water : mixtures

For methanol : water mixtures 5 ,10 ,20 ,and 40% methanol mixtures were investigated. The results are shown in Diagram 18.

Diagram 18 : Enantiomeric resolution of chiral benzodiazepines versus percentage methanol



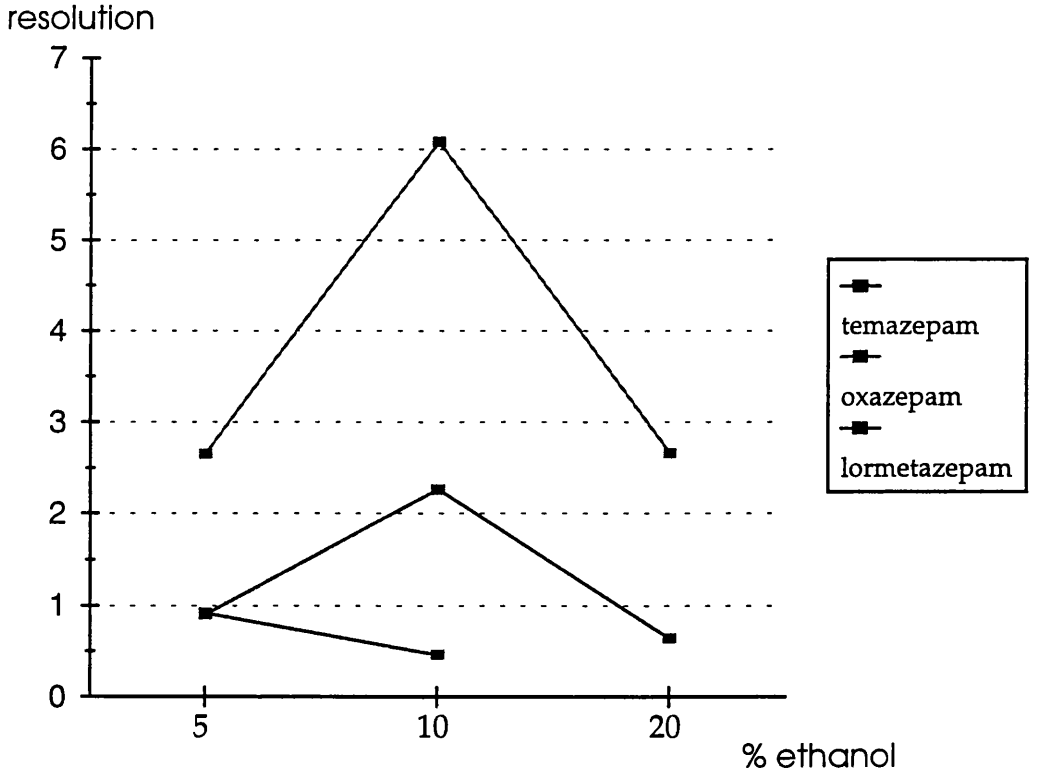
From the results it can be seen that 10% methanol gave the best separation of enantiomers for temazepam and oxazepam. Lormetazepam showed no real change when the percentage of methanol was changed from

5 to 10% methanol, and above this no resolution of enantiomers was noted with one peak only being observed. With 40% methanol all drugs elute with the solvent front.

Ethanol : water mixtures

The next solvent tried was ethanol. The percentages of ethanol used were 5, 10, 20 and 30% with the results shown in Diagram 19.

Diagram 19 : Enantiomeric resolution of chiral benzodiazepines versus percentage ethanol



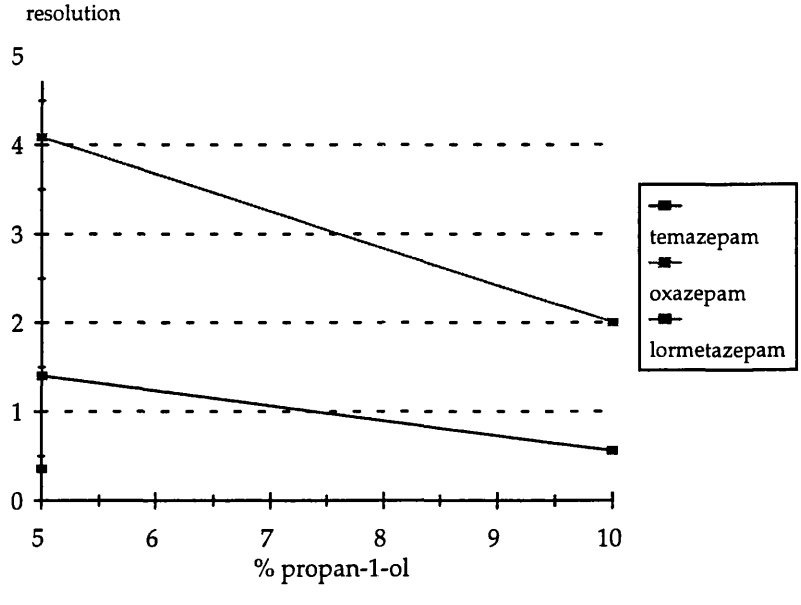
Again from the results it can be seen that 10% solvent gives the best separation of enantiomers for temazepam and oxazepam. The enantiomers

of lormetazepam were again poorly resolved with one peak only at 20% ethanol. At 30% ethanol all drugs eluted with the solvent front.

Propan-1-ol : water mixtures

Propan-1-ol : water mixtures attempted here were 5, 10 and 20% organic solvent with the results shown in Diagram 20.

Diagram 20 : Enantiomeric resolution of chiral benzodiazepines versus percentage propan-1-ol

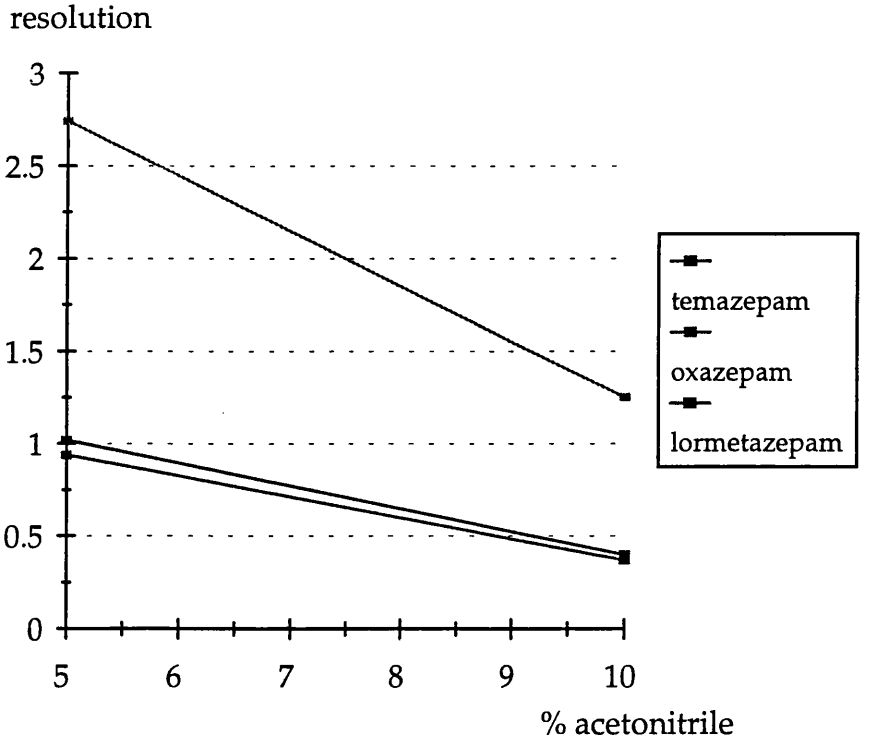


Here it can be seen that 5% propan-1-ol is the optimum amount of solvent required for temazepam and oxazepam, with lormetazepam giving a poor enantiomer separation at 5% propan-1-ol and one peak at 10% propan-1-ol. It should be noted that as the percentage of propan-1-ol is decreased chromatographic peaks broadened and capacity factors increase to a level unrealistic for analysis (k' > 16).

Acetonitrile : water mixtures

As with propan-1-ol, the percentages of acetonitrile tried were 5, 10 and 20%. The results are shown in table and Diagram 21.

Diagram 21 : Enantiomeric resolution of chiral benzodiazepines versus percentage acetonitrile



The results here are similar to propan-1-ol in that 5% solvent gives the optimum resolution of the drugs in the range evaluated.

The data outlined above can be plotted per drug, so that solvent effects are more apparent. Lorazepam was not plotted as no enantiomeric separations were observed on any of the mobile phases attempted. The combined results are shown in Diagrams 22-24.

Diagram 22 : Enantiomeric resolution of temazepam versus percentage solvent

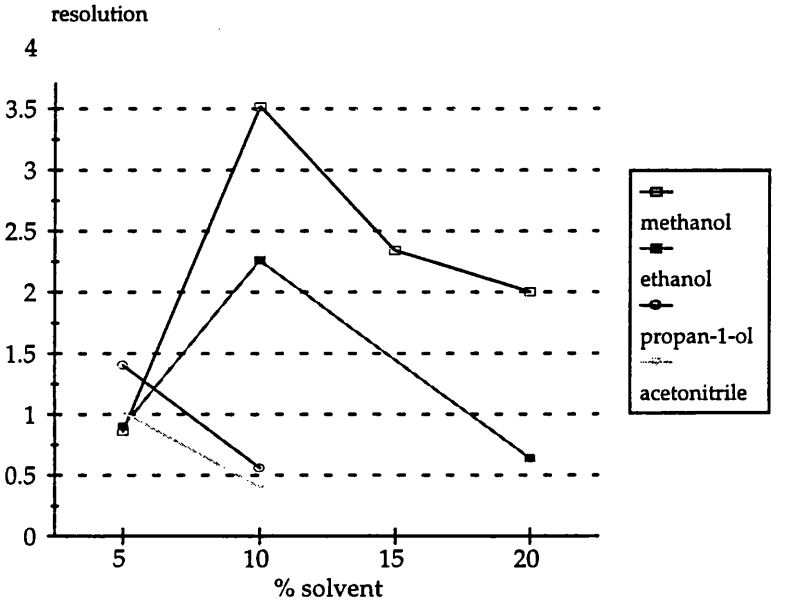


Diagram 23 : Enantiomeric resolution of oxazepam versus percentage solvent

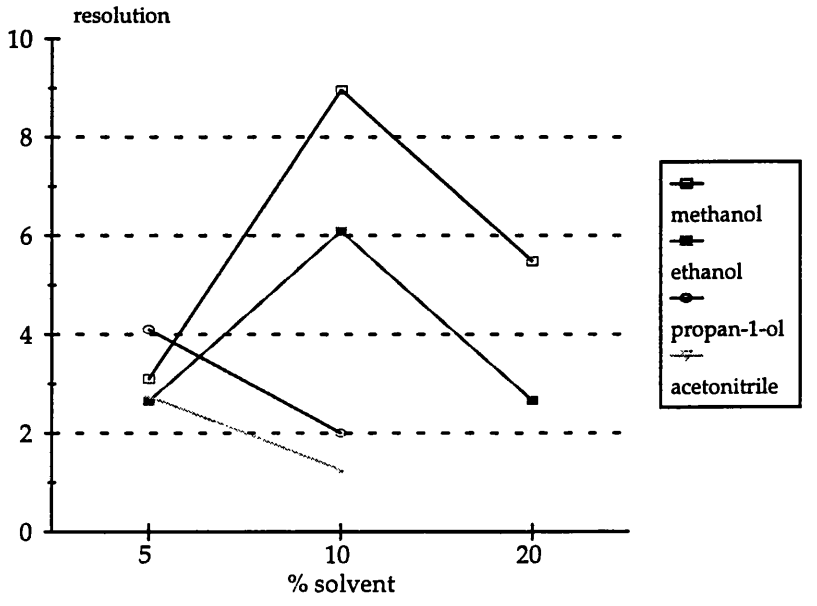
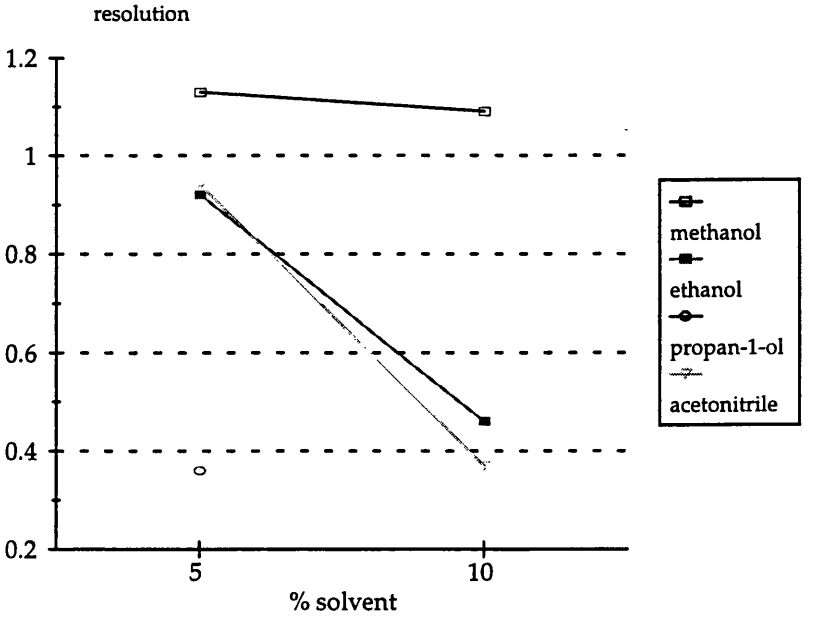


Diagram 24 : Enantiomeric resolution of lormetazepam versus percentage solvent



As can be seen in diagrams 22-24, the resolution of each drug was at an optimum value at a certain solvent concentration. For temazepam and oxazepam, the optimum amount of solvent was 10% for methanol and ethanol and 5% for acetonitrile and propan-1-ol. However any one of the mobile phases tried for oxazepam gave an acceptable enantiomeric resolution.

Lormetazepam and lorazepam chromatographed poorly on the aqueous : solvent systems. One explanation is that because these two drug molecules each have two chlorinated benzene rings which have a high affinity for the cyclodextrin, cavity and both may form a complex. If this is the case then the drug molecules will not form a complex in one particular way and so total resolution cannot occur. It may, however, be possible to separate the enantiomers of these 2 in the polar organic mode where hydrogen bonding would replace inclusion complexation.

One of the main problems which was noticed on all mobile phases was peak tailing and it was decided to substitute for water with phosphate buffer.

11.2.1.6 Effects of phosphate buffer

As suggested above, phosphate buffer replaced water in the mobile phase system. Also the mobile phase optimisation studies concentrated on temazepam and oxazepam, with no further work being done on lormetazepam or lorazepam.

A mobile phase tried was 10% methanol in 0.05M phosphate buffer (pH 6.8). The results are shown below in Table 19.

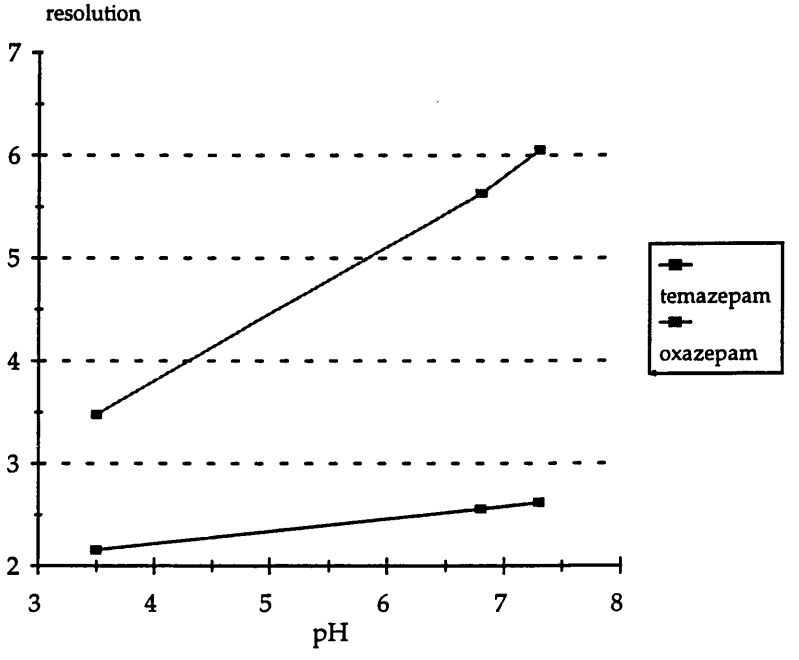
Table 19 : Comparison of enantiomeric resolution of temazepam and oxazepam between 10% methanol in water and buffer

mobile phase	resolution temazepam	resolution oxazepam
MeOH : buffer 10 : 90	2.28	6.07
MeOH : water 10 : 90	3.52	8.96

When the results are compared to 10% methanol in water it was found that peak shapes improved with less tailing noticed. However the resolution dropped from 3.52 to 2.28 and 8.96 to 6.07 for temazepam and oxazepam respectively and these results were more comparable to 20% methanol in water.

If 10% methanol in buffer (pH 6.8) gave similar enantiomeric resolutions to 20% methanol in water, then it may follow that 2.5% acetonitrile in buffer (pH 6.8) may be similar to 5% acetonitrile in water (since 5% was the optimum acetonitrile concentration in water). Improved enantiomeric resolutions were noted in the 2.5% acetonitrile in buffer (pH 6.8). However long retention times and broad peaks were noted and so 5% acetonitrile in buffer was tried. This mobile phase was tried with 3 different pH's and the results shown below in Diagram 25 .

Diagram 25 : Enantiomeric resolution of temazepam and oxazepam versus pH



From this 5% acetonitrile in buffer (pH 6.8) was selected as a possible mobile phase for two reasons. The first was that when biological extracts (originating from spiked whole blood) were used less interferences were noted from the biological matrix. The second reason is that 5% acetonitrile in buffer has acceptable capacity factor values for the analyst (k' 2.89 for temazepam enantiomer 1 and 4.37 for temazepam enantiomer 2)
As can be seen from Diagram 24 a near linear relationship exists between enantiomeric resolution, which increases with pH.

A pH of 7.35 was selected for the buffer. This pH gave a good enantiomeric resolution of both analytes and also fell within the pH range of the column (pH 3.0-7.5).

Chapter 12 : Results and discussion II

12.1 Introduction

The enantiomers of 3-substituted-1,4-benzodiazepin-2-ones have different binding constants to human and animal proteins and tissues and the binding to human serum albumin and bovine and rat cerebral cortex was discussed in section 8.4.

After evaluating the β-cyclodextrin stationary phase for separations of 3-hydroxy-1,4-benzodiazepin-2-ones from solution, it was evaluated as a separation technique for toxicological purposes. A pharmacokinetic study of temazepam enantiomers in serum samples from a human volunteer was designated as a suitable evaluation as a preliminary to the analysis of post-mortem blood samples.

After the evaluation for toxicological purposes a semi-preparative chiral chromatographic separation of temazepam enantiomers was carried out and the isomers were individually analysed by circular dichroism analysis.

12.2 Extraction

Whole blood was spiked with temazepam, oxazepam, and diazepam (internal standard) and two solid phase extraction methods were tested. The first method was based on the routine extraction procedure used in the Department of Forensic Medicine and Science using Extrelut® and the second was solid phase extraction solid phase extraction using octylsilica

stationary phase. In addition a liquid-liquid extraction technique was carried out on samples obtained from semi-preparative chiral chromatography.

12.2.1 Extrelut® extraction

The Extrelut® extraction will not be discussed further as extracts were extremely messy with quantification of the drugs impossible on the β-cyclodextrin stationary phase.

12.2.2 Solid phase extraction on octylsilica

Blank whole blood and blank serum were extracted by a non-polar solid phase extraction method on octylsilica. The recovery of each benzodiazepine is summarised in Table 20.

Table 20 : SPE extraction of three benzodiazepines from blood and serum

compound	% recovery from blood	% recovery from serum
oxazepam	65	68
temazepam	62	63
diazepam	59	62

As Table 18 shows, the % recovery of all three drugs is greater from serum samples than whole blood. This seems reasonable because whole blood is a more complex mixture than serum, and it is generally more difficult to quantify drugs from this matrix. The recoveries are relatively low compared to many solid phase extraction methods and the method could be improved for achiral chromatographic methods. As the method in section

10. shows, acidic or basic conditions are avoided in the extraction to exclude the possibility of racemisation.

12.2.3 Liquid-liquid extraction for semi-preparative samples

Four solvents were evaluated for the liquid-liquid extraction of racemic temazepam in water. The results are shown below in Table 21.

Table 21 : Liquid-liquid extraction of temazepam from water

solvent	% recovery
ethyl acetate	65
diethyl ether	61
dichloromethane	55
chloroform	60

As the above table shows, the four solvents extract a similar amount of temazepam from the water. Diethyl ether was chosen as the solvent of choice due to its low volatility and thus ease of removal.

12.3 Chromatography

12.3.1 Chiral

The chiral chromatographic system consisted of a β-cyclodextrin column with a mobile phase of 5% acetonitrile in 0.05M phosphate buffer

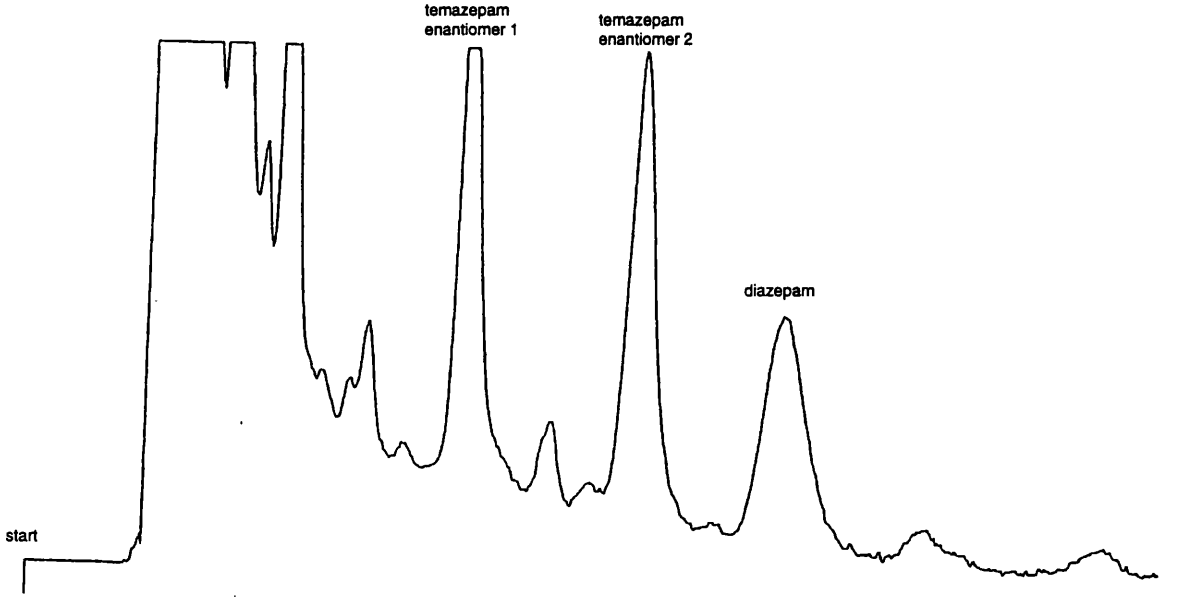
(pH 7.35). UV detection was at 254nm and the mobile phase flow rate was 0.8ml/min. The capacity factors are shown in Table 22.

Table 22 : Capacity factors of temazepam enantiomers and diazepam

benzodiazepine	k'
temazepam enantiomer 1	2.89
temazepam enantiomer 2	4.37
diazepam	5.63

A typical chromatogram is shown in Diagram 26.

Diagram 26 : Chromatogram of temazepam enantiomers and diazepam



12.3.2 Achiral

The method used for the non-chiral analysis is outlined in section 10., and is from the UN benzodiazepine manual [154]. The methods is recommended for dissolved tablet samples, but worked well here with samples derived from serum.

The capacity factors for the three benzodiazepines analysed are shown below in Table 23.

Table 23 : Capacity factors of three benzodiazepines on C18 stationary phase

benzodiazepine	k'
oxazepam	4.75
temazepam	5.75
diazepam	9.55

12.3.3 Semi-preparative chiral separation

A semi-preparative chiral separation of racemic temazepam was carried out so that larger quantities of each enantiomer could be collected. Twenty separate injections of racemic temazepam were performed with 24 µg loaded on column each time.

UV spectrometry was then employed to quantitate each enantiomer. The quantity of enantiomer 1 (first eluting enantiomer) was 54 µg and the quantity of enantiomer 2 was 50 µg, with both being quantified at 232nm. The UV spectra of each along with that of racemic temazepam standards is shown below in Diagrams 27-29.

Diagram 27 : UV spectrum of temazepam enantiomer 1

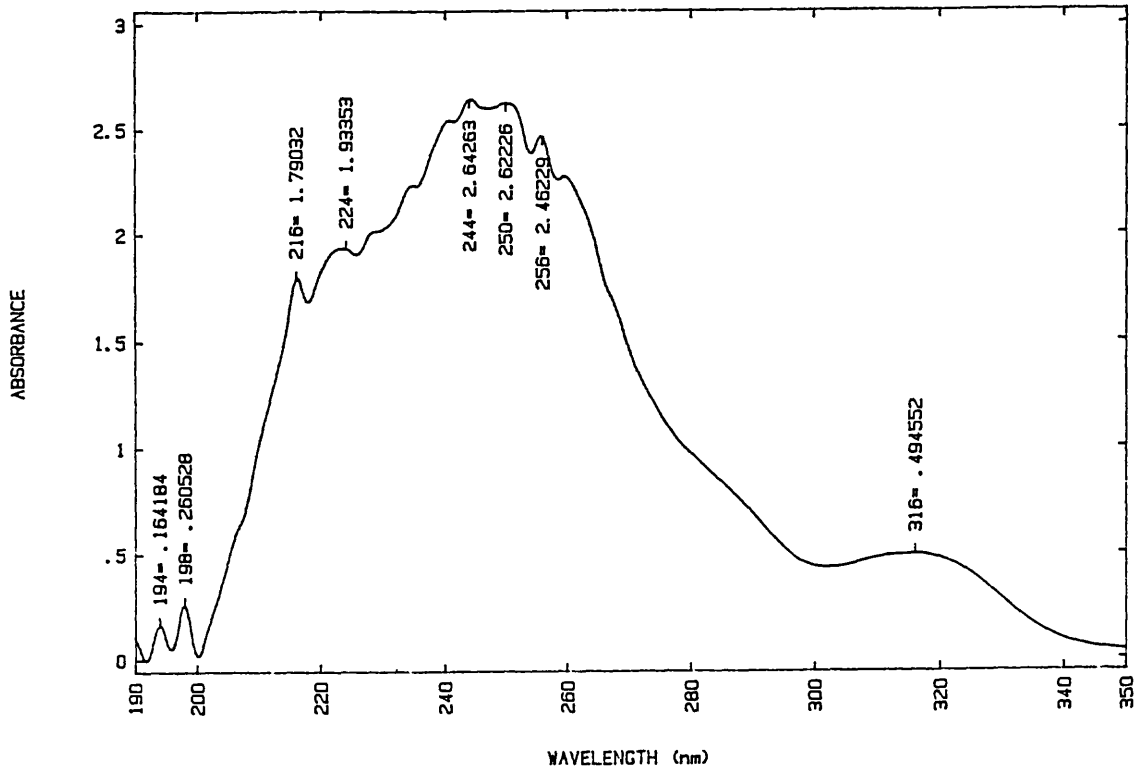


Diagram 28 : UV spectrum of temazepam enantiomer 2

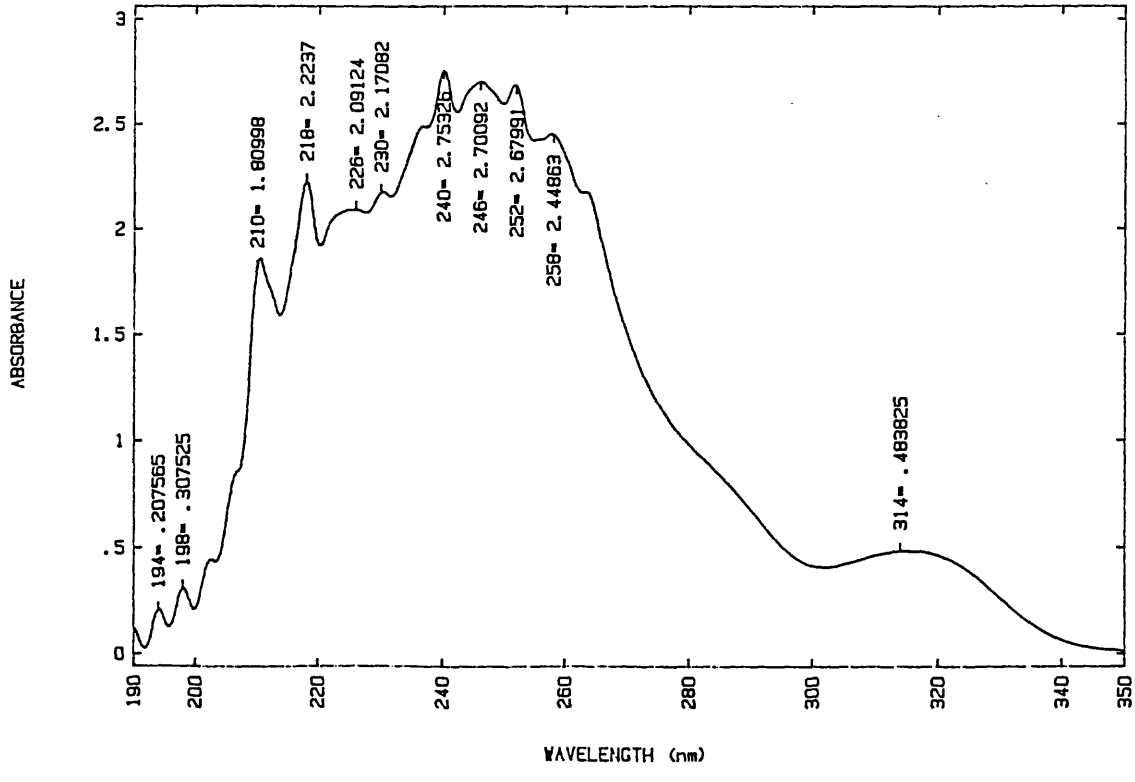
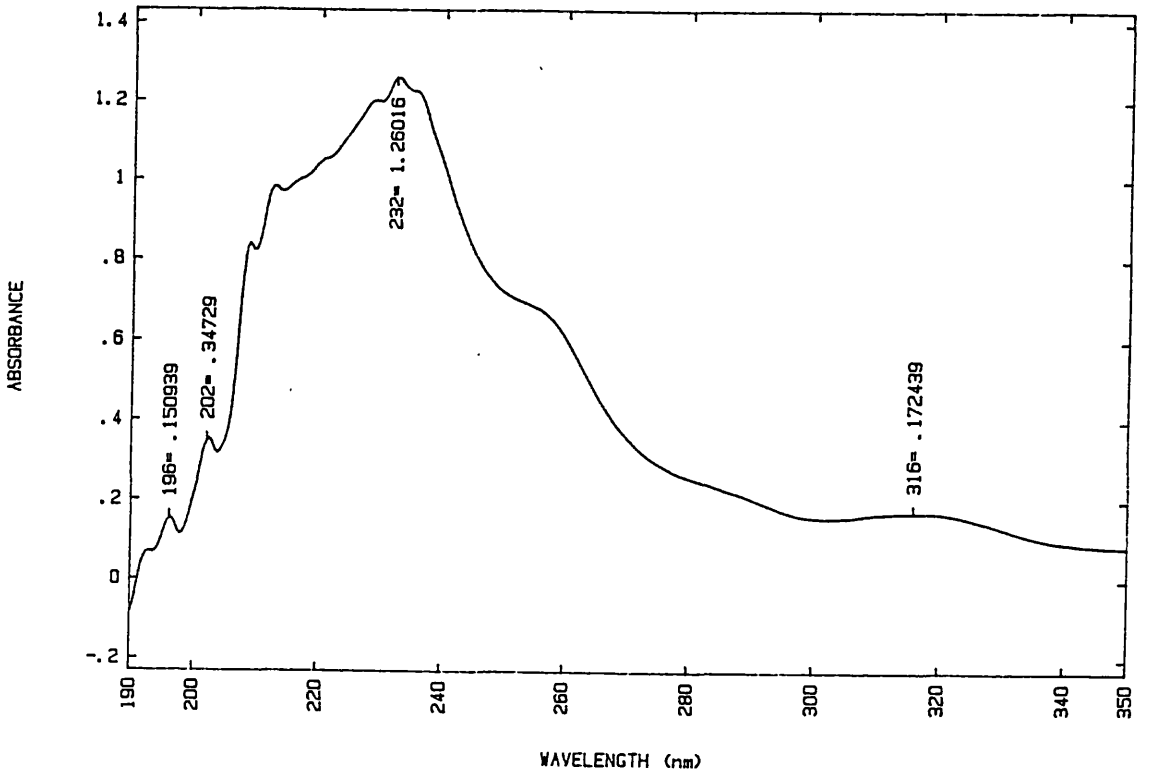


Diagram 29 : UV spectrum of racemic temazepam

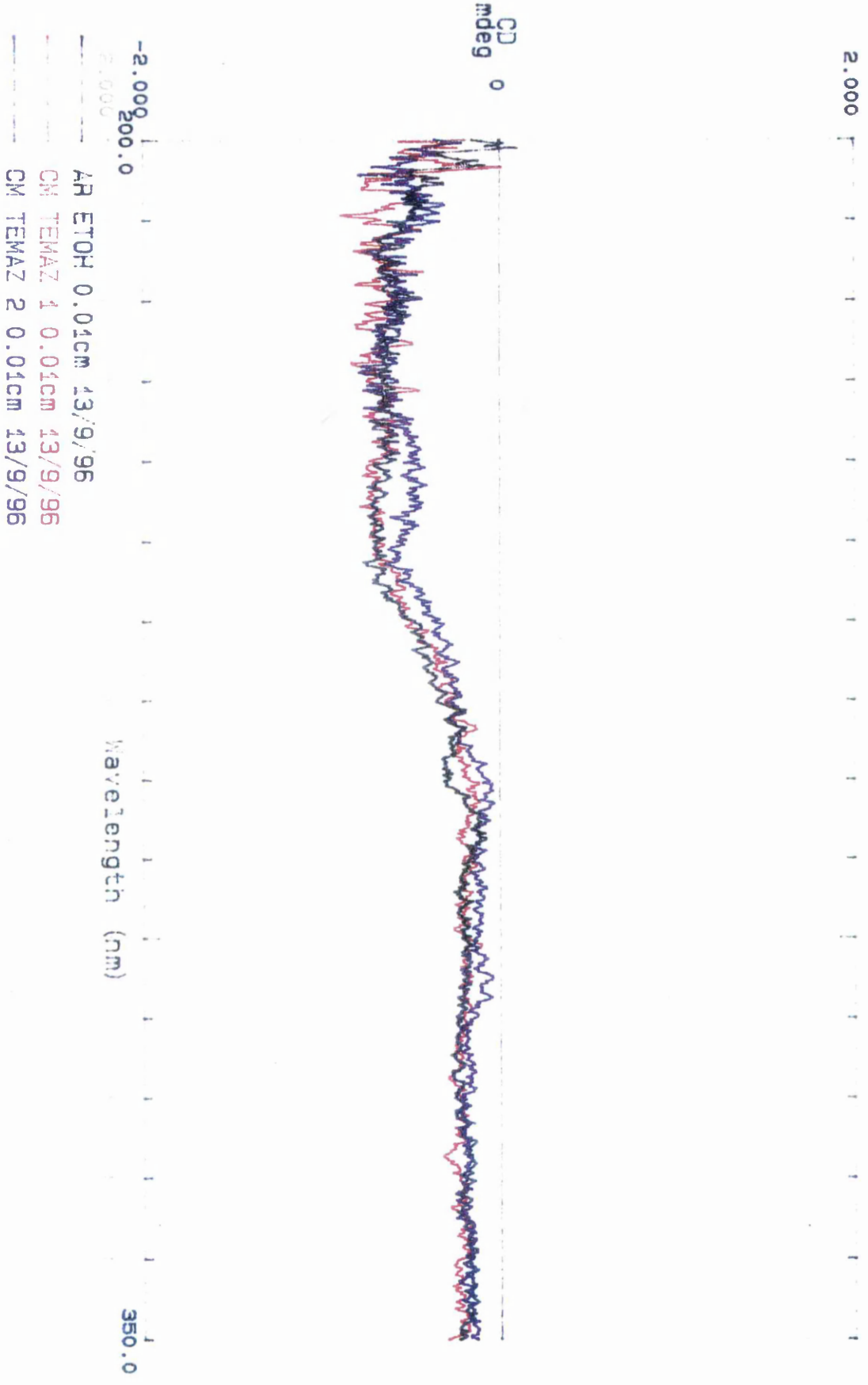


Diagrams 27-29 shown that there is a slight difference in the three UV spectra. The UV spectra of the two enantiomers are more similar to each other than the racemic temazepam spectra. However the UV spectra of the enantiomers have several shoulder peaks on the main absorbance at λmax. of 244nm for enantiomer 1 and 240nm for enantiomer 2. When the two UV spectra of each enantiomer are compared to that of the racemate in this way it suggests there are impurities present.

12.4 Circular dichroism spectrum

The circular dichroism spectrum of each enantiomer and that of the solvent, ethanol, is shown in Diagram 30. As the diagram shows both enantiomers and the solvent blank have the same spectral characteristics of no CD absorbance. This may be due to the samples being impure (as suggested by the UV spectra), racemisation of the enantiomers has occurred or the quantity of each enantiomer (approximately 50 μg) is too small.

Diagram 30 : Circular dichroism spectra of temazepam enantiomers



12.5 Pharmacokinetic studies

As mentioned in the introduction to this chapter, a healthy male volunteer completed two separate pharmacokinetic studies with temazepam, involving oral doses of 10 and 20 milligrammes racemic temazepam. The results from both pharmacokinetic studies are shown below in Diagrams 31 and 32

Diagram 31 : Plasma concentration curves of temazepam enantiomers following a 10 mg oral dose

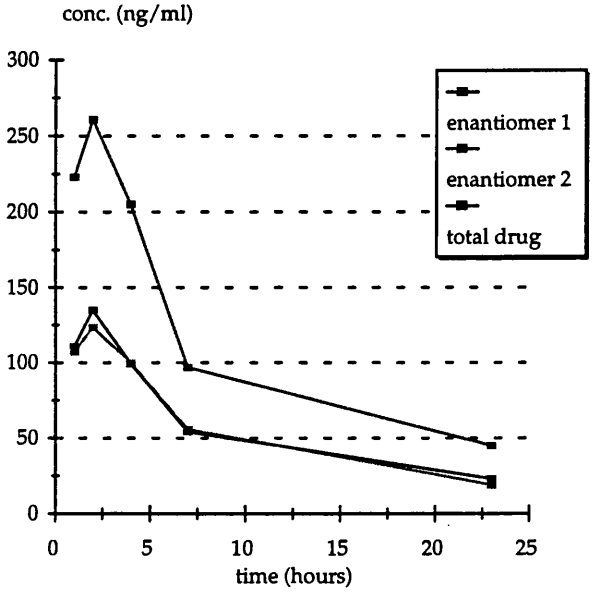
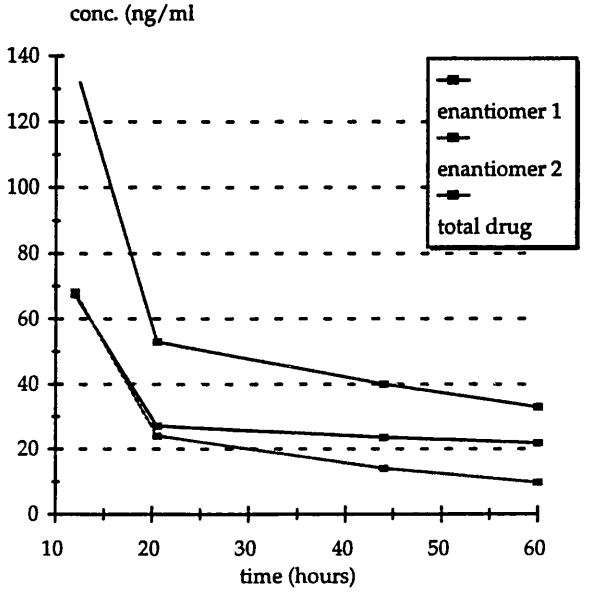


Diagram 32 : Plasma concentration curves of temazepam enantiomers following a 20 mg oral dose



It can be seen from Diagram 31 that the ratio of enantiomers is approximately one until 12-13 hours. After this the ratio changed slowly until 23 hours as the concentration of the second enantiomer decreased more quickly than the first enantiomer. A similar situation was noted with the 20 milligramme oral dose (shown in Diagram 32) and continued up to 60 hours. Oxazepam, the main metabolite of temazepam (ignoring glucuronides) was not seen in any of the samples. This suggests that the differences in concentration between the two temazepam enantiomers is due to stereoselective binding rather than stereoselective metabolism, although it is certainly possible that both could occur. It is still unclear from the study the fate of the temazepam after entering the body. One partial solution to this

problem is to extend the method to measure temazepam and oxazepam glucuronides in plasma, and also collect samples of urine for similar analysis.

Chapter 13 : Conclusions and suggestions for further work

13.1 Nifedipine analysis

This study involved finding a suitable and robust method for the analysis of nifedipine and its metabolites from whole blood. The extraction methods evaluated were liquid-liquid extraction, solid phase extraction and protein precipitation plus solid phase extraction.

It was concluded that the liquid-liquid extraction was unsuitable for the purposes because of many co-extracted interferences. Solid phase extraction was better because recoveries of drug material were high. However problems persisted because whole blood blocked one-third of all columns used. The method of choice was protein precipitation plus solid phase extraction because blood extracts were free of significant interferences. The method had a relatively low recovery of 48% for nifedipine but blockage of solid phase extraction columns did not occur.

13.2 Evaluation of β-cyclodextrin stationary phase for chiral chromatography of four common benzodiazepines

Evaluation of four benzodiazepines (temazepam, oxazepam, lorazepam, lormetazepam) was carried out using a β-cyclodextrin column, with several solvent combinations.

When using a β-cyclodextrin column under reversed phase conditions it could be concluded that the enantiomers of lorazepam and lormetazepam will not be resolved on this stationary phase, whereas the enantiomers of

temazepam and oxazepam would separate well under these conditions. Other conclusions are that a decrease in organic modifier concentration (in water or buffer) increased the enantiomeric resolution but also increased the retention, and that increasing the pH increases the enantiomeric resolution for temazepam and oxazepam.

13.3 Pharmacokinetic study

Two pharmacokinetic studies were carried out on temazepam enantiomers. Two separate doses of temazepam, 10 and 20 mg, were given to a healthy male volunteer and samples taken over 23 and 60 hours respectively.

It was concluded that the enantiomers were treated similarly in binding and/or metabolism until 12 or 13 hours as the enantiomeric ratio is 1:1 up to this point. After this time the concentration of the first eluting enantiomer fell more slowly than the second which suggests a process such as stereoselective binding or metabolism.

In the studies no trace of the temazepam metabolite, oxazepam, was noted which suggested this metabolite is not formed in blood. However the method is not sensitive for glucuronides and thus no conclusions about these metabolites could be made.

It could also be concluded that direct chiral chromatographic analysis is possible for toxicology but difficulties still exist due to the fragility of the β-cyclodextrin stationary phase toward extracted samples of biological origin.

13.4 Further work

Further work could (a) include a investigation of β-cyclodextrin columns from a different manufacturer to check if all β-cyclodextrin phases are fragile towards extracted biological samples, (b) applying the method to post-mortem blood samples, and (c) extending the study to analyse temazepam and oxazepam glucuronides in blood or urine. Since the glucuronides are diastereoisomers they could also be analysed by achiral chromatography.

Also, other methods of chiral analysis such as chiral derivatisation (to form diastereoisomers) for gas chromatography or high performance liquid chromatography could be evaluated as these may be more robust towards extracted biological samples. Mobile phase additives for capillary electrophoresis may be considered, as low sample volumes and small quantities of reagents are required. All of these analytical techniques could be applied to other optically active drugs of forensic interest such as the ring substituted amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA).

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Appendices

A. Analysis of Nifedipine in Whole Blood Samples

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Abstract

Nifedipine is an anti-anginal/anti-hypertensive drug which acts as a slow calcium channel blocker. It is widely used in the UK and occurs regularly in medicolegal cases requiring toxicological analysis. Careful sample handling and analysis is required, particularly the exclusion of light. Isolation involves solid phase extraction on octylsilica following deproteination with methanol. Other non-polar phases were evaluated (octadecylsilica, ethylsilica, phenylsilica and cyclohexylsilica). Analysis by HPLC utilised a ODS column and UV detection at 238nm. The limit of detection was 5ng/ml blood, and the method was evaluated using case samples of autopsy blood.

Keywords: Nifedipine, whole blood, SPE/HPLC, overdose

Introduction

Nifedipine is the prototype drug of the group of dihydropyridine calcium-channel blocking agents, also known as calcium antagonists, calcium entry blockers or slow calcium blockers. The drug inhibits the influx of

calcium ions into muscle cells and also binds to the intracellular calcium binding proteins. It has a selective effect as a dilator of arterial vessels. This causes dilation of the pulmonary, coronary and peripheral arteries resulting in a fall in blood pressure. The drug is used for the treatment of hypertension and angina pectoris.

The aim of this study was to find the best commercially-available non-polar sorbent for solid phase extraction (SPE) of nifedipine from blood. The only polar material tested was Extrelut®. The majority of papers published for the analysis of nifedipine from blood, plasma, serum or urine involve liquid-liquid extraction. A wide range of solvents has been used for nifedipine extractions and these are highlighted in a recent review paper [1]. Approximately five methods using solid phase extraction have been published to date. Two of these methods involve on-line pre-column extraction [2,3] while another combines SPE on octadecylsilica (ODS) and basic solvent extraction [4]. Extractions from fetal calf serum using octylsilica [5] and from human serum using ODS [6] have also been published.

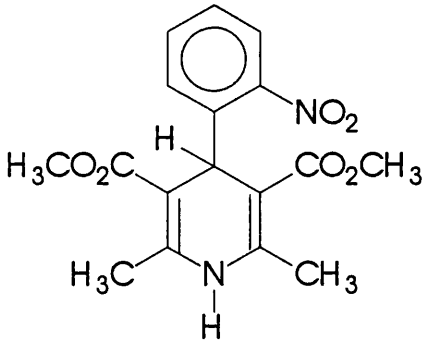
Absorption of nifedipine from the gastro-intestinal tract is rapid and almost complete, although bioavaliability after oral administration is between 45 and 75% [7] . Following administration by mouth, peak blood concentrations are reported to occur 30-120 minutes after consumption, with a plasma half life of 2-5 hours[8].

The metabolism of nifedipine takes place almost exclusively in the liver, where it is converted into three pharmacologically inactive metabolites which are excreted in the urine (80%) and faeces (20%). A scheme for this metabolism is outlined in Figure 1 and involves oxidative dehydrogenation to dehydronifedipine (BAY B 4759, structure 2), followed by demethylation to the pyridine mono-carboxylic acid metabolite (BAY O 2820, structure 3), then oxidation of the 2-methyl group to the primary alcohol (BAY H 2228,

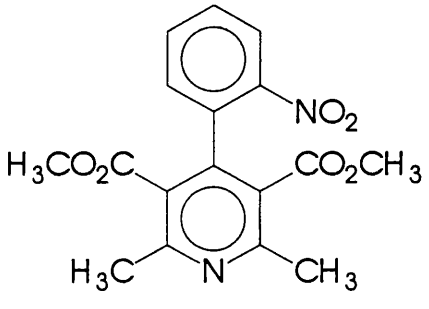
structure 4). This metabolite undergoes pH-dependent cyclisation to the lactone (BAY O 4160, structure 5) [9].

Overdose levels of nifedipine have not been reported in the literature, as far as we know, but several publications on nifedipine poisoning from a medical angle have been published [10-15].

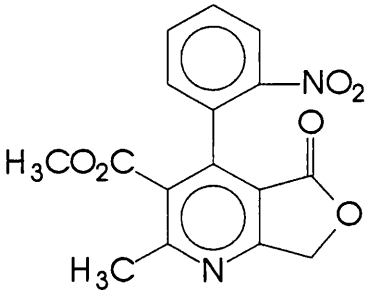
DIAGRAM 1 - METABOLISM OF NIFEDIPINE



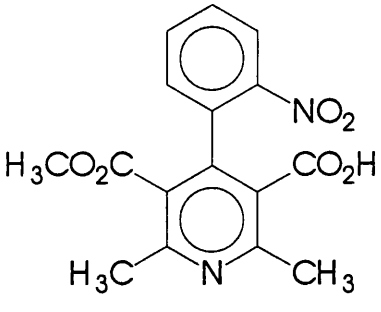
Structure 1 - Nifedipine



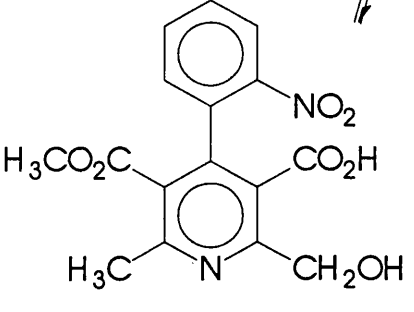
Structure 2 - BAY B 4759



Structure 5 - BAY O 4160



Structure 3 - BAY O 2820



Structure 4 - BAY H 2228

Experimental

Materials:

All sample handling and extraction steps were carried out with glassware wrapped in aluminium foil to prevent degradation of nifedipine by light. Dichloromethane, methanol, butan-1-ol, chloroform, acetonitrile and propan-2-ol were of HPLC grade and supplied by BDH. Hexane and pentane were purchased from Aldrich (UK). Cetrimide (hexadecyl-trimethylammonium bromide) was obtained from Sigma UK Ltd. C18 (octadecyl), C8 (octyl), C2 (ethyl), CH (cyclohexyl) and PH (phenyl) Bond-Elut solid phase extraction columns were supplied by Varian, and Extrelut® granules were obtained from Merck.

Reference standards of nifedipine, nitrendipine and metabolites were kindly donated by Bayer (Germany) Ltd. Specimens of autopsy blood were obtained at routine post-mortem examinations in the Glasgow area.

Standard solutions were prepared in bottles covered with aluminium foil and dissolved in methanol. Two stock solutions were prepared and had the following composition-
Stock solution 1: nifedipine 6.1 µg/ml, bay-b-4759 3.6 µg/ml, bay-o-4160 5.8µg/ml and bay-h-2228 7.1 µg/ml. Stock solution 2: nitrendipine 7.9 µg/ml.

Instruments:

The HPLC system consisted of a solvent pump (LDC/Milton Roy Constametric®3000), external injection valve with a 20µl loop, column

(250x4.6 mm i.d., ODS HL5), UV detector (Waters 490-MS) at 238nm and a recorder (Linseis NRE-68). The mobile phase consisted of water:acetonitrile 50:50 with 20mM cetrimide and was used at a flow rate of 1.0 ml/min. The pH of the mobile phase was 7.9.

Extraction procedures:

Several methods for the extraction of nifedipine from blood were evaluated. These included solvent extraction (A), direct loading of blood on a solid phase material (B) and protein precipitation or Extralut® (diatomaceous earth) extraction as an initial stage before solid phase extraction using modified silicas (C).

A. Solvent extraction with methanol, chloroform, ethyl acetate or diethyl ether:

0.9ml of spiked blood containing 100µl of stock solution 1 and 100µl of stock solution 2 was added to 10ml of the solvent in a dropwise manner. After centrifugation (2400rpm, 15 minutes), separation and evaporation of the organic layer the extract was reconstituted in methanol and analysed by HPLC.

B. Direct loading of blood on a solid phase material:

The materials tested were Extrelut® and Bond-Elut C18, C8, C2, CH and PH.

For Extrelut® extraction a 5ml syringe was filled to the 3ml level (approx. 0.7-0.75g) with Extrelut® granules. 1ml of blood containing 100µl of stock solution 1 and 100µl of stock solution 2 was then added and allowed to absorb for 5 minutes. The elution step involved passing of 10 ml of methanol

or chloroform through the cartridge followed by evaporation of the solvent and reconstitution in methanol.

For the Bond-Elut C18, C8, C2, CH and PH solid phases the following procedure was used-

1. Conditioning- 1 column volume of methanol
   - 1 column volume of water
2. Application of sample-the sample was allowed to flow through the column very slowly, over 3-5 minutes
3. Rinsing - 1 column volume of water
   - 1 column volume of hexane
   - 1 column volume of pentane
4. Elution - 2 column volumes of eluting solvent.

The solvent was evaporated and the extract reconstituted in methanol. Eluting solvents tried were dichloromethane, chloroform, 5% v/v butan-1-ol in dichloromethane and 10% v/v butan-1-ol in dichloromethane.

C. Combination of protein precipitation/Extrelut® with solid phase extraction

Protein precipitation: 1ml of blood containing 100µl of stock solution 1 and 100µl of stock solution 2 was added dropwise to 5ml of methanol and mixed for 15 minutes. After centrifugation (2400rpm, 15 minutes), separation and evaporation of the methanol, the extract was reconstituted in 1ml water and applied to a Bond-Elut PH solid phase extraction column.

Extrelut®: 1ml of spiked blood was added to Extrelut® as in (B). Elution was tried with 10 ml of chloroform, 20% methanol in chloroform and 40% methanol in chloroform. After the solvent had been evaporated the

extract was reconstituted in 1ml water and applied to a Bond-Elut C8 solid phase extraction column.

Procedure for Bond-Elut PH and C8 extraction :

1. Conditioning - 1 column volume of Methanol
   - 1 column volume of water
2. Application of sample - the sample was allowed to flow through the column very slowly over 3-5 minutes
3. Rinsing - 1 column volume of water
   - 1 column volume of hexane
   - 1 column volume of pentane
4. Elution - 2 column volumes of 10% butan-1-ol in dichloromethane.

The solvent was evaporated and the extract was reconstituted in methanol.

Results and discussion

Under the HPLC conditions described the following retention times were noted : BAY H 2228-4.4 minutes, BAY B 4759-5.4 minutes, Nifedipine-6.5 minutes, Nitrendipine-10.0 minutes and BAY O 4160-16.5 minutes.

Each of the extraction procedures which were evaluated worked to a certain extent except for solvent extraction.

A. Solvent extraction:

Regardless of the solvent used (4 were tried) too many interferences were co-extracted from the blood to allow drug or metabolite peak identification and quantification. Back-extraction was not attempted as Nifedipine and internal standard Nitrendipine have no ionisable groups

which could be used without structural alteration of the dihydropyridine ring.

B. Direct loading of blood on substituted silica sorbents :

This was evaluated extensively. The first column tested was the least selective non-polar material Bond Elut C18, with phosphate buffer at pH 5.2, 7.3 and 10.3 used to condition the columns instead of water. The results are shown in Table 1.

TABLE 1. Bond-Elut C18 Column Elution with Dichloromethane : Effect of pH during conditioning

COMPOUND	% RECOVERY		
	pH 5.2	pH 7.3	pH 10.3
BAY H 2228	/	/	/
BAY B 4759	66	57	49
NIFEDIPINE	37	34	46
NITRENDIPINE	40	38	35
BAY O 4160	/	/	/

Examination of the results shows that the pH of the buffer used to condition the sorbent does not affect the percentage recovery of the drug or its internal standard. However, only one of the metabolites was eluted, prompting an evaluation of chloroform as an eluting solvent due to its greater polarity. This eluant was not used subsequently due to interference caused by co-extracted compounds. The eluting solvent was changed to 5%

butan-1-ol in dichloromethane with Bond Elut C18 and C8 sorbents being used. The results are shown in Table 2.

TABLE 2: Elution with 5% Butan-1-ol in Dichloromethane

COMPOUND	% RECOVERY	
	C18	C8
BAY H 2228	/	8.6
BAY B 4759	102	62
NIFEDIPINE	81	61
NITRENDIPINE	53	47
BAY O 4160	/	30

The percentage recovery of Nifedipine, Nitrendipine and BAY B 4759 from the C18 column was improved, although the C8 material did extract all of the substances from the blood. Further evaluation was carried out of C8 and the more selective sorbents Bond Elut C2, CH and PH using 10% butan-1-ol in dichloromethane as eluant. The results are shown in Table 3.

TABLE 3: Elution with 10% Butan-1-ol in Dichloromethane

COMPOUND	% RECOVERY			
	C8	C2	CH	PH
BAY H 2228	22	23	19	13
BAY B 4759	77	68	95	96
NIFEDIPINE	61	44	85	93
NITRENDIPINE	49	60	95	61
BAY O 4160	25	/	16	/

An increased recovery from the C8 sorbent was observed and high recoveries of nifedipine, nitrendipine and the main metabolite BAY B 4759 were obtained from the other sorbents. From these results either C8, C2, CH or PH sorbents combined with eluting solvent of 10 % butan-1-ol in dichloromethane are suitable for nifedipine extraction from whole blood. When the percentage of butan-1-ol was increased further (to 20% by volume) significant co-extracted interferences were noted. However, a major disadvantage of this method was that approximately 1 in 3 solid phase extraction columns became blocked by the blood.

The only polar sorbent tried in these studies was Extrelut® (diatomaceous earth). Elution with methanol was found to result in many interfering peaks while chloroform elution gave poor recoveries, as shown in Table 4.

TABLE 4: Elution with Chloroform from Extrelut®

COMPOUND	% RECOVERY
BAY H 2228	/
BAY B 4759	34
NIFEDIPINE	15
NITRENDIPINE	14
BAY O 4160	/

Although recoveries from direct loading of blood on non-polar sorbents were good in some cases, the method had to be modified to allow the sample to flow through the column more easily without blockage.

C. Combination of protein precipitation/Extrelut® with solid phase extraction:

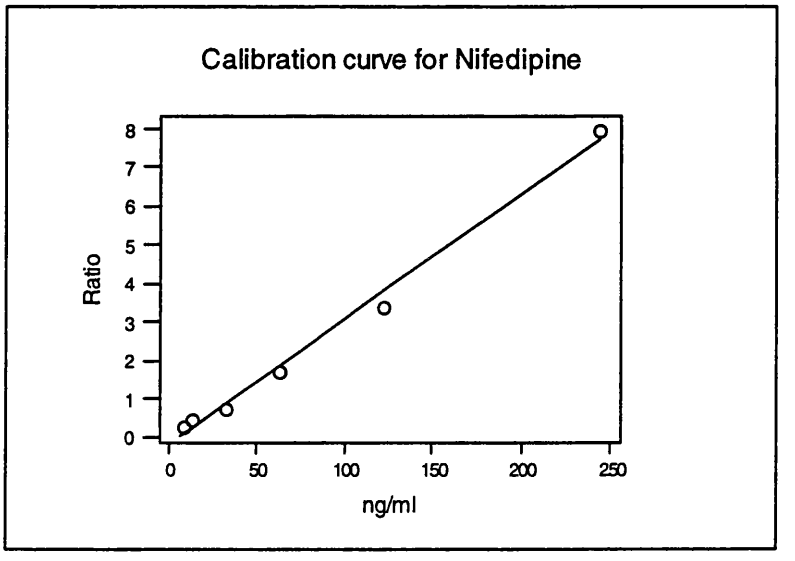
To overcome the viscosity problem an initial step of either Extrelut® or protein precipitation was tried. With Extrelut®, 3 eluants were tried followed by a second stage of solid phase extraction on Bond Elut PH sorbent. The results are shown in Table 5.

TABLE 5: Extrelut® and Bond Elut PH Solid Phase Extraction

COMPOUND	% RECOVERY		
% METHANOL IN CHLOROFORM	0	20	40
BAY H 2228	19	43	10
BAY B 4759	30	103	21
NIFEDIPINE	23	37	9
NITRENDIPINE	14	45	15
BAY O 4160	/	6	8

The results suggest that 20% methanol in chloroform is the optimum eluting solvent. A calibration curve was prepared using this method, although some decomposition of Nifedipine to BAY B 4759 occurred. This may have been due to the quantity of solvent required for removal by evaporation (10 ml from Extrelut® + 5 ml from PH elution). This suggests the method is unsuitable as many methods in the literature caused no significant degradation.

A combination of protein precipitation in methanol combined with solid phase extraction using Bond Elut C8 was subsequently used to construct a calibration curve.



The calibration curve for Nifedipine was linear over a range of concentrations from 6.1-244ng/ml. The correlation coefficient was 0.991.

Toxicology Cases

In 1994 our Department received 3 fatal toxicology cases in which analysis of nifedipine was requested. Autopsy blood was analysed using the method described for the preparation of the calibration curve. Two of the cases were found to be positive.

The first case was found to be positive for nifedipine (8.3 μg/ml) and metabolite Bay-H-2228 (0.5 μg/ml). The second case was found to be

positive for nifedipine (6.9 μg/ml), plus metabolites Bay-H-2228 (0.43 μg/ml) and Bay-B-4579 (2.7 μg/ml).

Conclusion

The work described above suggests that most non-polar methods of solid phase extraction are suitable for the extraction of nifedipine. When whole blood is used an appropriate protein precipitation step is recommended. Although nifedipine is photodegradable, the analysis can be carried out using conditions described in this paper quite easily and reliably providing a suitable light exclusion method is implemented.

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Pharmacokinetics of Temazepam Enantiomers in Human Serum

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Abstract

Organisations such as the EC and FDA have prepared draft guidelines for separate patents on enantiomers of chiral drugs. A recently published paper reviews current draft guidelines for Canada, EC, Japan and the United States [1].

In this study, a chiral chromatographic method has been developed for separation of the enantiomers of temazepam, a widely abused prescription drug important to the forensic toxicologist. A brief review of chiral pharmacodynamics and chromatography is also presented.

Extraction from human serum involved the use of solid phase extraction on octylsilica (C8), with direct chromatographic enantiomer separation on a β-cyclodextrin stationary phase. Total drug concentrations were measured on an ODS stationary phase.

A pilot metabolism study was carried out which consisted of oral administration of 2 separate therapeutic doses to a healthy male volunteer. The doses were 10 and 20 mg with blood samples collected up to 23 and 60 hours respectively.

Introduction

Chirality is defined as the property displayed by any object which is not superposable (superimposable) on its mirror image. It is an important property which many drug molecules possess. Most naturally occurring medicinal agents exist in their optically active or single isomer form. However, many synthetic chemicals are produced as the optically inactive racemate. A survey in 1993 showed that a greater percentage of synthetic drugs were being sold as single enantiomers as compared to 1982 [2]. This trend may be due to improvements in chiral separations or chiral synthesis and tighter regulatory controls.

Since human and animal systems are chiral environments, drug enantiomers may have different effects/actions in these systems. Three main situations exist when describing the pharmacodynamic effects of enantiomers.

The first of these is when each enantiomer has the same pharmacodynamic effect. This is true of temazepam and other 3-substituted 1,4-benzodiazepin-2-ones. In this case different potencies exist, with the (S)-enantiomers up to 100x more potent than the (R). The case of equal potencies is very rare an example being the antihistamine promethazine.

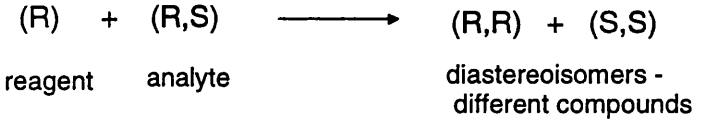
The second situation is where all the activity resides in 1 enantiomer and the other enantiomer is biologically inactive. An example of this is the antihypertensive agent α-methyldopa.

The third situation is when a useful/harmful pairing exists. Examples of this are numerous, perhaps because the harmful effects have attracted attention. These include the anaesthetic ketamine where most post operative side effects, such as nausea and hallucinations, are associated with the (R)-enantiomer. Other examples include the ring substituted amphetamines such as MDMA and MDA. In these drugs the S-(+) enantiomers undergo demethylation faster than the R-(-) enantiomers. The S-(+) enantiomers are more neurotoxic (from metabolite) and pharmacologically more active [3].

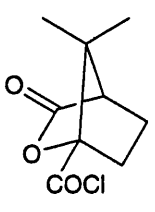
Terminology sometimes used in the literature should be noted here. Eutomer describes the isomer with the desired activity while the distomer is the enantiomer which is inactive or has the unwanted activity.

For enantiomeric separations by chromatography, diastereoisomeric complexes must be formed. This is because enantiomers are chemically and physically identical, differing only in optical rotation, whereas diastereoisomers are different compounds. Diastereoisomeric complexes can be formed in three main ways, namely by chiral derivatisation, using chiral mobile phase additives or using chiral stationary phases.

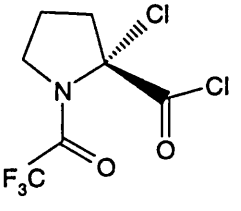
Chiral derivatisation involves the use of enantiomerically pure derivatising reagents to react with the analytes (mixture of enantiomers) to form diastereoisomeric complexes. These diastereoisomers, being different compounds, can be separated by achiral chromatographic methods, most commonly GC but also HPLC and CE.



typical reagents :



(-)-camphanic acid chloride



(S)-(-)-N-(trifluoroacetyl)-prolyl chloride

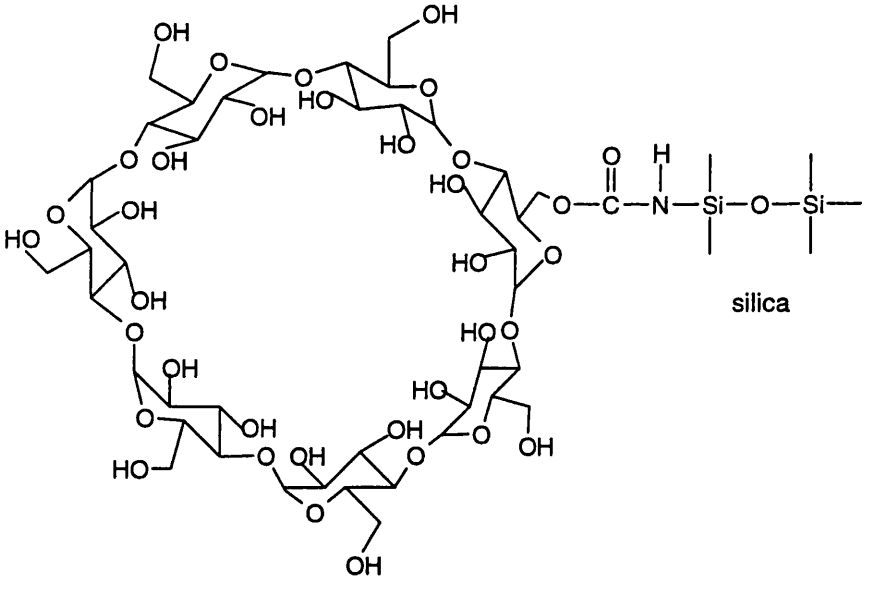
The second method of diastereoisomeric formation is the use of chiral mobile phase additives. Here optically pure molecules such as cyclodextrins or transition metal amino acid complexes are added to the mobile phase with an achiral stationary phase being used. This method is used in HPLC and CE analysis.

The third method of chiral analysis involves the use of chiral stationary phases. Many phases have been developed and are available commercially, and are grouped according to their interactions. The classes for HPLC are listed below :

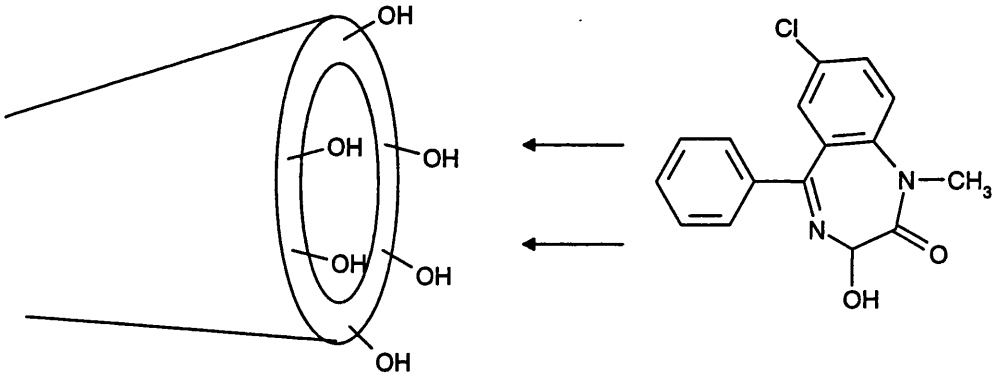
Type I : Pirkle phase
Type II : Celluloses
Type III : Inclusion complexes - cyclodextrins, crown ethers
Type IV : Ligand exchange phases
Type V : Bound proteins - HSA, BSA, AGP, OVM
New phase : macrocyclic antibiotics

The phases for GC are mainly based on cyclodextrins, but a few others include hydrogen bonding phases and metal coordination compounds.

The HPLC stationary phase used in this study was a native β-cyclodextrin stationary phase as show below :



The separation is based on the formation of inclusion complexes, as represented below :



Experimental

Materials :

Acetonitrile and methanol were of HPLC grade and were supplied by BDH. Hexane was supplied by Aldrich (UK). Potassium dihydrogen orthophosphate was of AnalaR® grade and supplied by BDH. Sodium hydroxide was GPR grade and supplied by BDH. C8 (octyl) Bond-Elut solid phase extraction columns were supplied by Varian, Harbour City, CA, USA.

Chromatography :

The HPLC columns were as follows :

Chiral analysis - 250 x 4 mm i.d. Chiradex® β-cyclodextrin + guard column (Merck) ;

Achiral analysis - 250 x 4.6 mm i.d. ODS HL5 + guard column (Capital HPLC, Broxburn, West Lothian, UK).

UV detector : Waters 490-MS (Millipore UK Ltd/Waters Chromatography Division, Harrow, Middlesex).

HPLC pump : LKB 2150 (LKB-Produkter AB, Box 305, S-161 26 Bromma, Sweden)

Integrator : model HP 3396A

Conditions : UV detection was at 254nm

Chiral : mobile phase 5% acetonitrile in 0.05M phosphate buffer (pH 7.3), flow rate 0.8 ml/min.

Achiral : mobile phase MeOH:H_2O:0.05M phosphate buffer (pH 7.25) flow rate 1.5 ml/min.

Sample collection and storage :

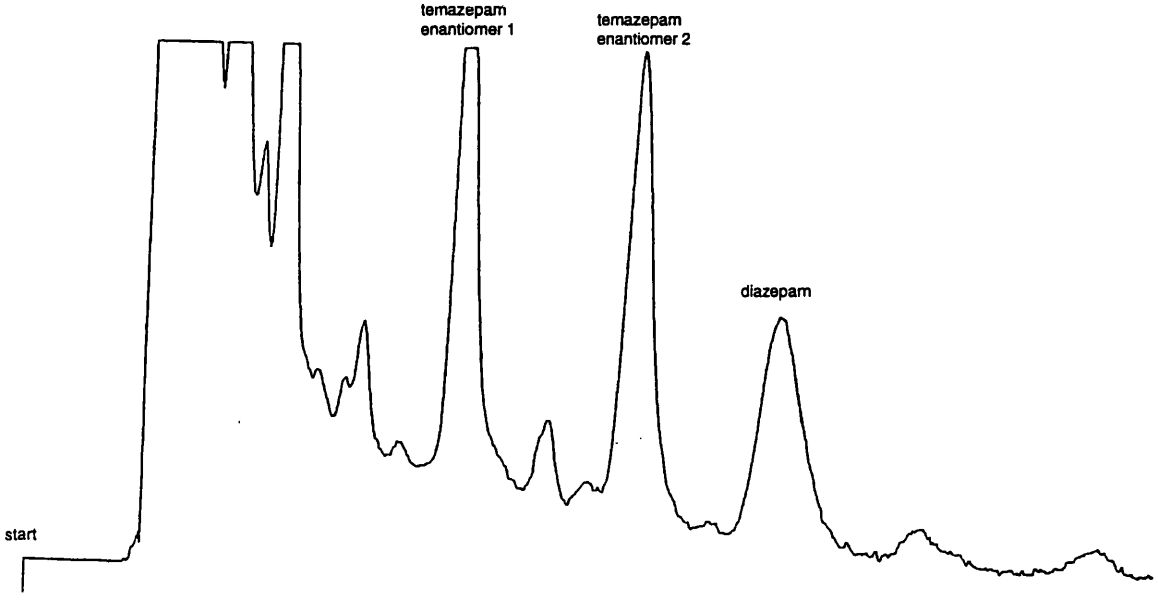
Blood (10ml) was collected in a heparinised vial and mixed gently. Plasma was separated immediately by centrifugation at 3000rpm for 10 minutes and was frozen until analysed.

Extraction scheme :

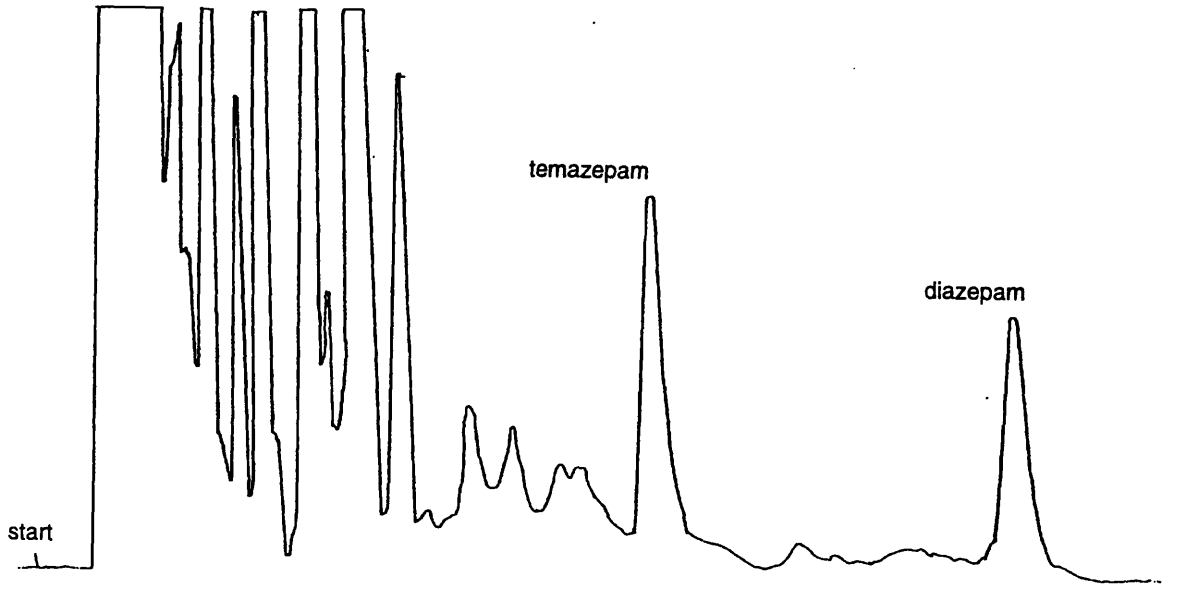
1. defrost samples
2. add 1 ml 0.05M phosphate buffer (pH 6.8) and internal standard (diazepam)
3. mix samples for 10 min.
4. solid phase extraction on C8

conditioning : 1 column vol. MeOH
1 column vol. buffer (as above)
add sample slowly over 2-3 min.
column wash : 1 column vol. H_2O
2 column vols hexane
elution : 1 column vol. 80% MeOH in H_2O

Specimen chromatograms are shown below :



Chromatogram of 2 hour plasma sample run on β-cyclodextrin column



Chromatogram of 2 hour plasma sample run on C18 column

Results and discussion

The results from both pharmacokinetic studies are shown below in figures 1 and 2.

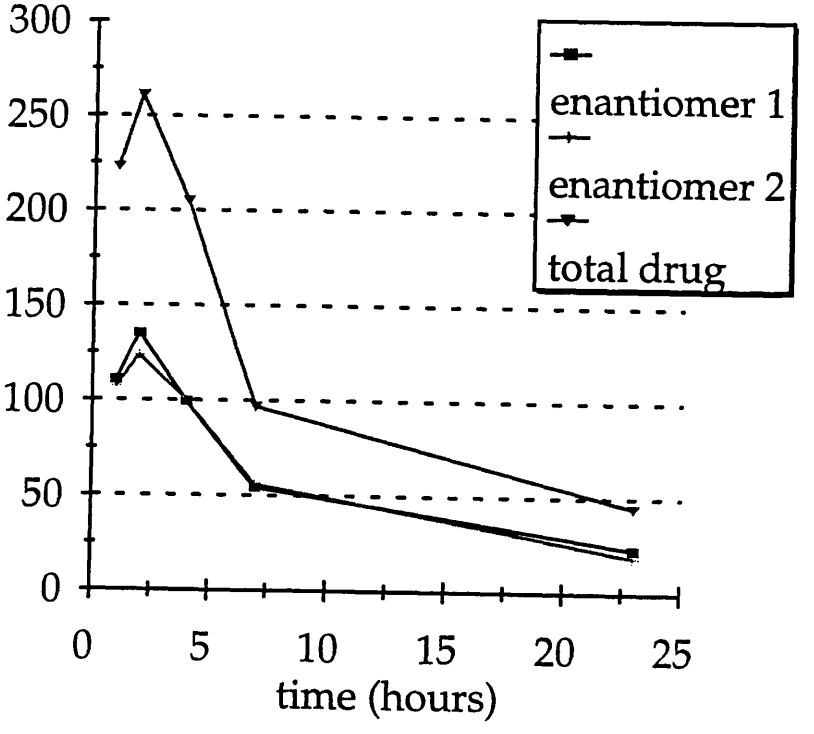


Figure 1 : Plasma concentration curves of temazepam enantiomers in following a 10 mg oral dose

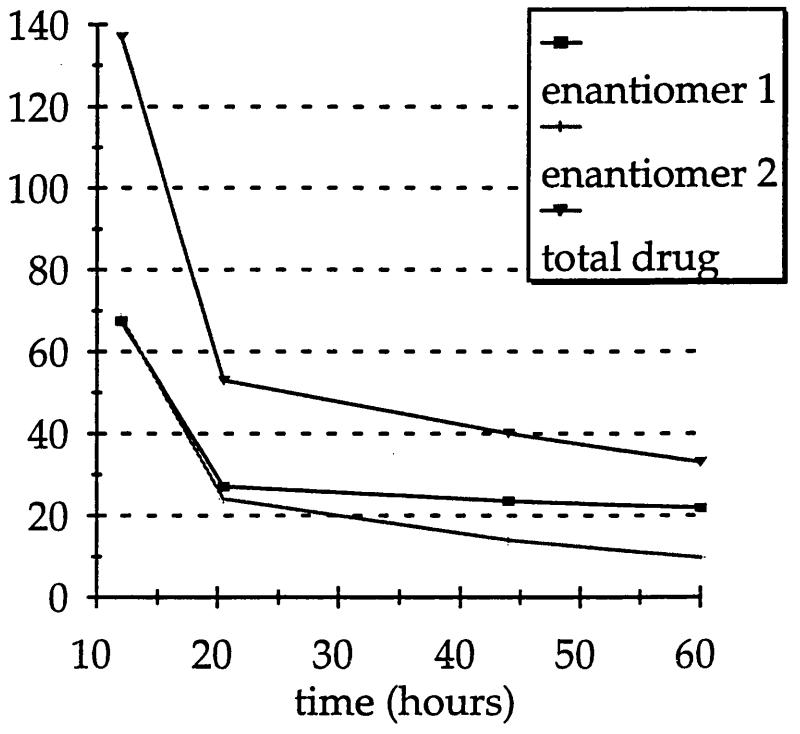


Figure 2 : Plasma concentration curves of temazepam enantiomers in following a 20 mg oral dose

It can be seen from figure 1 that the ratio of enantiomers is approx. 1 until 12-13 hours. After this the ratio begins to change. This trend is also noted in the 20 mg oral dose and continues up to 60 hours. In both cases it is the second enantiomer which has the lower concentration.

Oxazepam, the main metabolite of temazepam (ignoring glucuronides) was not seen in any of the samples.

Future applications of this method include using the enantiomeric ratio to find time after drug administration. It is also our intention to extend the method to post-mortem blood samples and find out if a relationship exists between time of death and enantiomeric ratio.

Conclusions

The method developed is reliable for measurement of temazepam enantiomers in serum or blood. It can also be extended for oxazepam enantiomers. Low levels of each enantiomer can be detected with levels down to 10 ng/ml.

References

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[3] Cho AK, Segal DS (Eds.). Amphetamine and its analogs. Academic Press, 1994, p154.