AN INVESTIGATION OF THE PHARMACOLOGY OF SELECTED ANTI-MYCOBACTERIAL PHENAZINES

A dissertation submitted for the degree of
Doctor of Philosophy
by

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Under the supervision of Prof. Richard O'Kennedy

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Man with his burning soul
Has but an hour of breath
To build a ship of Truth
In which his soul may sail-
Sail on the sea of death
For death takes toll,
Of beauty, courage, youth,
Of all but Truth.

John Masefield

DECLARATION

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged with in the text of my work.

Signed: Robert O'Connor Date: 27/8/95

ACKNOWLEDGMENTS

At the outset, I would like to thank all those who instilled in me a thirst for knowledge which ultimately led to me undertaking this work. If I am anything now, it is because of their endeavours on my behalf. Of those no longer with me, I treasure the memories they gave.

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Finally, to Tracy, my best friend, you have kept me going through it all, and I dedicate this most of all to you.

ABSTRACT

The research presented in this thesis has centred on chemical and pharmacological investigations of the phenazine antibiotic clofazimine and certain substituted phenazine analogues.

A simple extraction system was developed with dichloromethane and sodium hydroxide which quantitatively extracted all of the agents tested from tissue, faecal and blood samples. The extracted drugs could then be quantified using a reversed phase HPLC method with a mobile phase of tetrahydrofuran/acetic acid/hexane sulphonic acid and U.V. detection at 285 nm. Purity and chemical structure of the agents studied was confirmed using NMR, TLC, PDA-HPLC, silica column chromatography and elemental analysis.

The tissue distribution of clofazimine (B663) and phenazines B749, B3954, B4090 and B4100 was investigated by oral gavaging these agents into mice for 3 weeks and measuring drug levels using the HPLC method described. B4100 and especially B4090 gave superior tissue levels to clofazimine in all tissues tested except fat. A simpler method of administering phenazines in food was developed for dosing rats. B663, B4090, B4100, B4103 and B4154 were incorporated into rat food which was given to rats in specially made metabolism cages which allowed the measurement of food and water intake and collection of uncontaminated faeces.

The absorption of these agents was estimated by giving groups of rats a single dose of drugged food containing the non-adsorbable dietary marker chromic oxide, with subsequent collection and analysis of faecal drug and chromium levels using HPLC and spectrophotometric assays respectively. Absorption levels were found to be different for all the agents B4090 giving the poorest absorption at 50.5% and B4103 giving the most absorption at 92%. The bio-distribution of these compounds was measured after 4 weeks of administration. Again B4090 gave the highest levels in all tissues excluding fat.

During this study potential toxicity of these agents was investigated using blood enzyme markers, blood cell counts, measurement of food and water intake, behavioural observation, urinary markers and *post mortem* tissue weights. A newly developed method of analysing animal urine by proton-NMR spectroscopy was also used to investigate toxicity. None of these tests provided evidence that any of the compounds tested were more toxic than clofazimine.

The absorption and distribution of complexes of clofazimine with β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (H-β-CD) were tested. The H-β-CD formulation gave a greater absorption of clofazimine and increased blood and tissue drug levels.

Phenazine conjugates to the proteins bovine serum albumin, thyroglobulin and keyhole limpet haemocyanin (KLH) were produced using phenazine derivatives with amino acid substituents. These conjugates were characterised using TLC, PDA-HPLC and spectrophotometric assays. The thyroglobulin and KLH conjugates were used to immunise rabbits to produce specific anti-phenazine antibodies which were purified and characterised.

ABBREVIATIONS

A.L.T.	alanine amino transferase
A.P.C.	antigen presenting cell
A.S.T.	aspartate amino transferase
Ab	antibody
Abs	absorbance
ATP	adenosine triphosphate
B.S.A.	bovine serum albumin
BCA	bicinchoninic acid
BS^3	*bis*(sulfosuccinimidyl) suberate
Clofazimine (p)	pharmaceutical grade clofazimine
Clofazimine (s)	synthesised clofazimine
C.V.	coefficient of variation
conc	concentration
DCM	dichloromethane
DDS	dapsone
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
EDC	N-ethyl-N'(dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
F(ab)	variable portion of IgG molecule
F(c)	constant portion of IgG molecule
GC	gas chromatography
H_2O_2	hydrogen peroxide
Hb	haemoglobin concentration
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HRP	Horseradish peroxidase
I.S.	internal standard
I.V.	*intra venous*
IgG	Immunoglobulin of the G class
IR	infrared
KBr	potassium bromide
KLH	Keyhole Limpet haemocyanin

LD_{50}	dose causing 50 % mortality
LDL	low-density lipoprotein
LED	light emitting diode
Log	logarithmic
m.w.	molecular weight
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MHC	major histocompatibility complex
NADH	reduced nicotinamide adenine dinucleotide
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
OPD	o-phenylenediamine
P.B.S.	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis
PCV	packed cell volume
PDA	photo-diode array
PEG	polyethylene glycol
PGE_2	prostaglandin E_2
PHR	peak height ratio
r	regression coefficient
RBC	red blood cell count
Rf	retention factor
RP	reverse-phase
rpm	revolutions per minute
RSD	relative standard deviation
Rt	retention time
RT	room temperature
RU	response units
SD	standard deviation
SDH	sorbitol dehydrogenase
SDS	sodium-dodecyl sulfate
SEC	size exclusion chromatography
SPR	surface plasmon resonance
THF	tetrahydrofuran
THYR	thyroglobulin
TLC	thin layer chromatography

TMAO	trimethylamine-N-oxide
TMP	tetramethyl piperidine
TRIS	tris(hydroxymethyl)methylamine
U	unit
U.P.	ultrapure
U.V.	ultraviolet
v/v	volume per unit volume
w/v	weight per unit volume
WBC	total white blood cell count

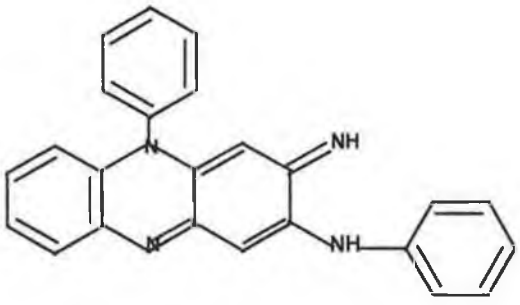
UNITS

(K)Da	(Kilo)Daltons
µg	microgram
µl	microlitre
µm	micrometre
^{0}C	degrees Celsius
cm	centimetre
cm^{-1}	wavenumber per centimetre
hrs.	hours
Kg	kilogram
L	litre
M	molar
mg	milligram
MHz	megahertz
mins.	minutes
ml	millilitre
mm	millimetre
mM	millimolar
mol	moles
ng	nanogram
nm	nanometre
ppm	parts per million

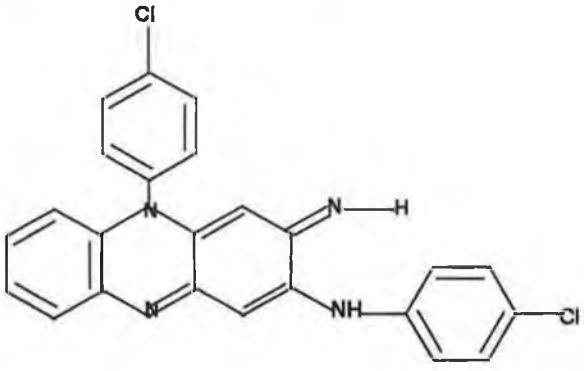
CHEMICAL STRUCTURES OF PHENAZINES USED

The chemical structures of rimino-phenazines B283, B628, B663 and B749

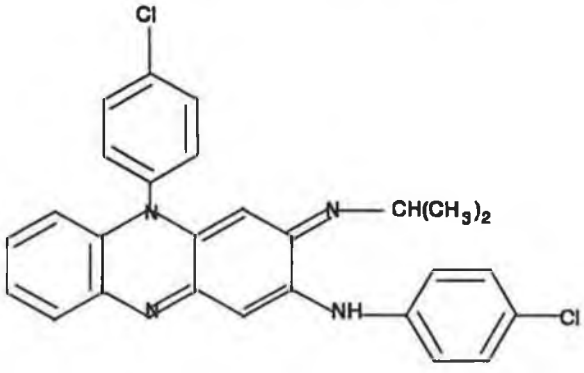
B283



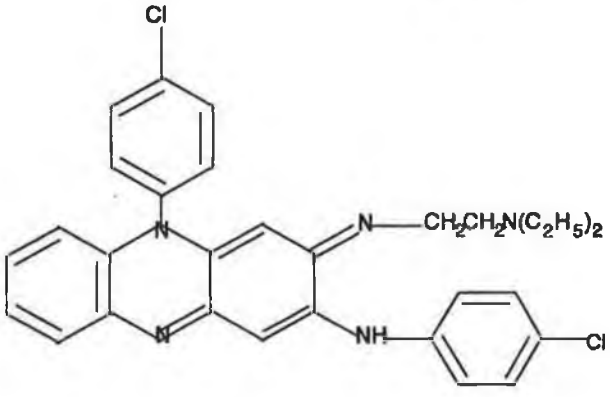
B628



B663
(clofazimine)
(Lamprene™)

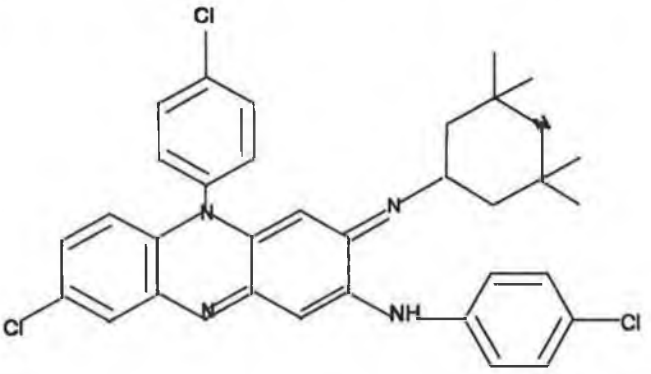


B749

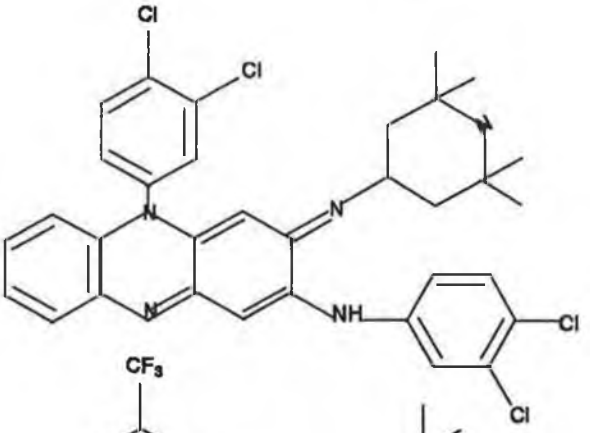


The chemical structures of the rimino-phenazines B4090, B4100, B4103 and B4154.

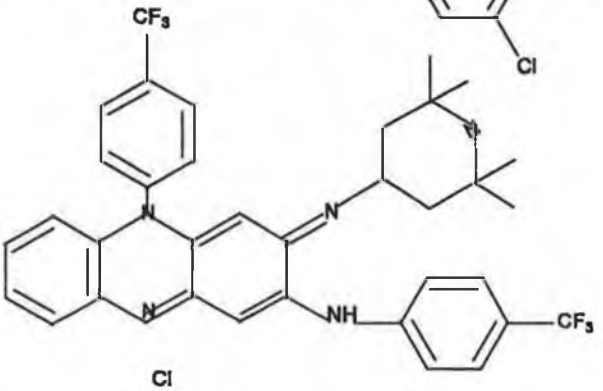
B4090



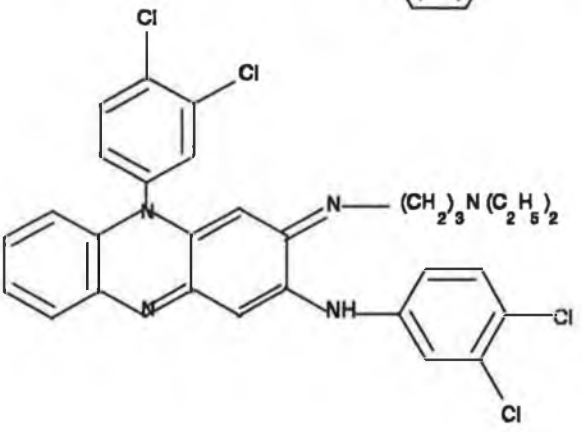
B4100



B4103

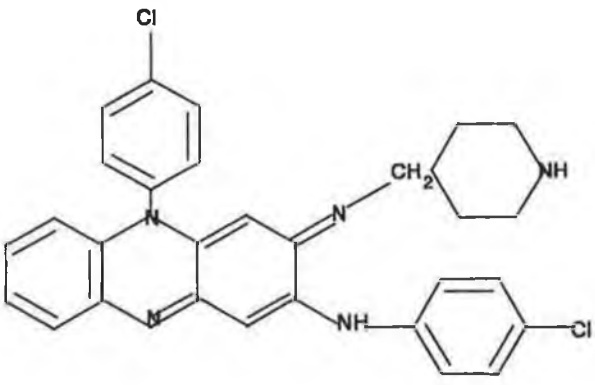


B4154

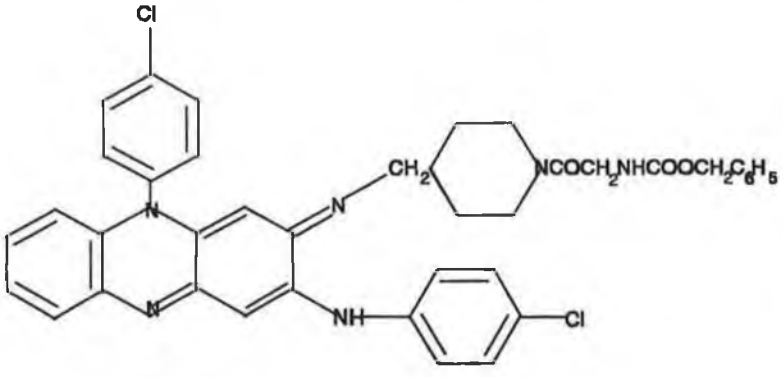


The chemical structures of the rimino-phenazines B3640, B3954, B3955 and B3976.

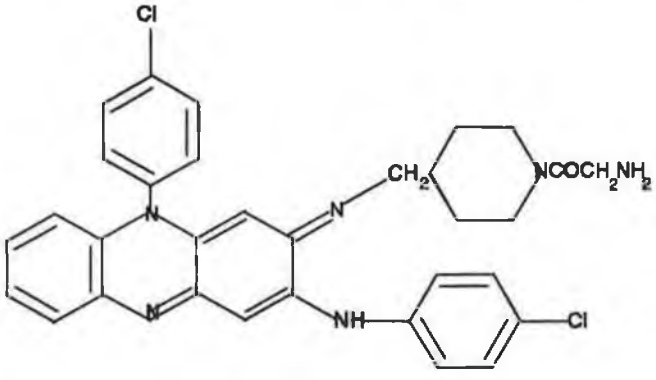
B3640



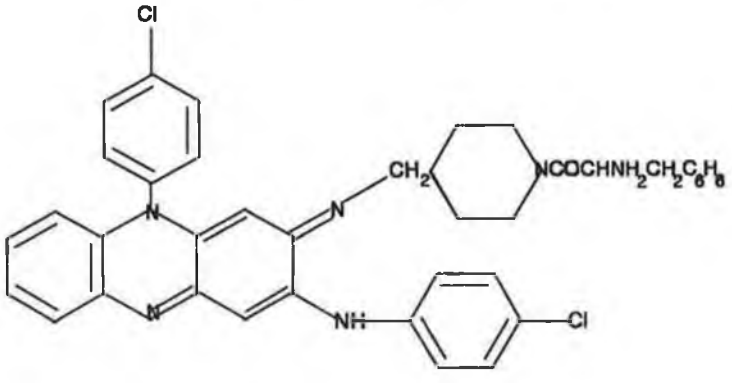
B3954



B3955



B3976



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CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

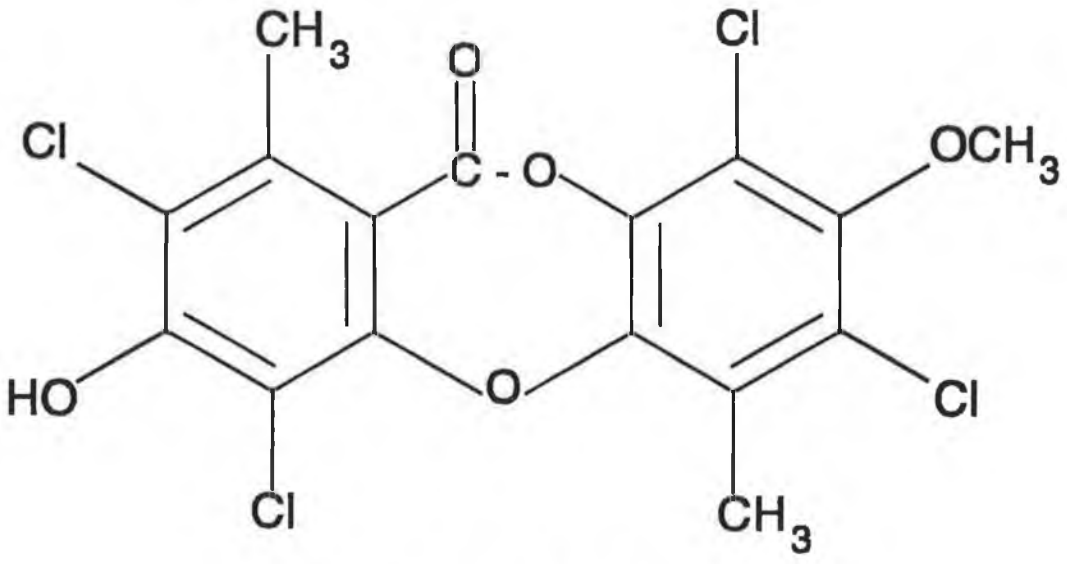
Clofazimine (3-(4-chloroanilino)-10-(4-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine) is an orange-red rimino-phenazine agent which has been used in the treatment of mycobacterial diseases including leprosy (Dollery, 1991; British Pharmacopoeia Commission, 1988; Barry *et al.*, 1957). Clinically it has been shown to be both safe and effective in the treatment of these diseases (Hastings *et al.*, 1976; Stenger *et al.*, 1970). This introduction describes the pharmacology, metabolism and chemistry of clofazimine, and recent advances in our knowledge of this drug, and thus, gives a clearer picture of our present understanding of its mode of action.

1.2. HISTORY

Clofazimine (B663 or Lamprene) emerged as the most active antimycobacterial agent of a class of compound, the riminophenazines, synthesised by the laboratories of the Medical Research Council of Ireland from 1944, as part of a project to find a treatment for tuberculosis.

This programme of compound development initially began with large scale screening of several hundred compounds which were available or produced in the chemistry department of University College Dublin. These substances included extracted constituents from lichens such as usnic acid, roccellic acid and diploicin. Diploicin (Figure 1.1) was the first organic chlorinated compound found to occur in nature, and on opening the lactone ring, the sodium salt of the resulting carboxylic acid was found to inhibit *Mycobacterium tuberculosis in vitro* at a 1/100,000 dilution (Barry, 1969). Diploicin proved to be inactive in animal models and its complete substitution prevented increasing activity with further alteration. Attempts to chemically imitate the opened diphenyl structure yielded an aminodiphenylamine compound which also had anti-bacterial properties. However, on standing, this compound oxidised to give a red crystalline precipitate which completely inhibited the growth of the H37Rv strain of *M. tuberculosis* at a dilution of 2×10^5. Following structural determination, this compound, which was termed B283, was shown to be the same as anilinoaposafranine which had been first synthesised in 1896 (Barry *et al.*, 1948; Barry *et al.*, 1957; Barry, 1969). *In vivo* activity against leprosy and urinary tuberculosis was demonstrated, but toxicity was also evident (Lane, 1951; Allday and Barnes, 1952). Chlorinated derivatives of B283 had far superior activities in rodent models of tuberculosis (T.B.), in particular one derivative, B663 or clofazimine (Barry and Conalty, 1958; Barry *et al.*, 1959; Barry *et al.*, 1960; Grumbach 1960; Steenken *et al.*, 1960; Noufflard and Berteaux, 1961; Barry and Conalty, 1965). Limited clinical trials of B663 in

Figure 1.1. The chemical structure of diploicin. This compound was extracted from a lichen and was one of the first compounds tested by Barry and colleagues which showed significant antitubercular activity.



Diploicin

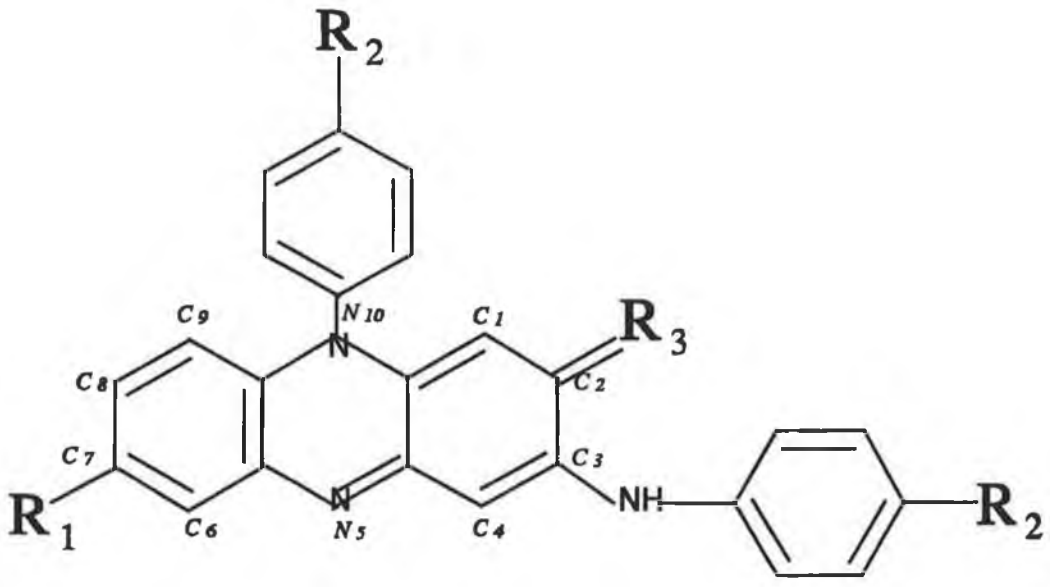
human tuberculosis produced poor results (Barry, 1969). When the formulation was changed from a coarse crystalline, to an ultrafine micronised preparation, models indicated excellent activity against leprosy, and clinical trials vindicated clofazimine's efficacy as an anti-leprosy agent (Browne and Hogerzeil, 1962; Chang, 1962; Lunn and Rees, 1964; Browne, 1965; Chang, 1966; Chang, 1967; Pettit *et al.*, 1967; Karat *et al.*, 1970). Currently, the major use of clofazimine is in the World Health Organisation Multiple Drug Therapy (MDT) for lepromatous leprosy (WHO, 1988).

1.3. CHEMISTRY

1.3.1. Properties

Clofazimine, $C_{27}H_{22}Cl_2N_4$, has a molecular weight of 473.14 and a melting point of 210-212^{0}C. It has a characteristic deep red to orange colour under normal condition due to its complex heterocyclic nature. Chemically it is a phenazine molecule and belongs to a group of phenazines with substituents on the N2, N3 and C7 (Figure 1.2) which were termed rimino-phenazines by Barry and co-workers who developed these compounds (Barry *et al.*, 1957; Barry, 1969). Clofazimine is a very hydrophobic molecule as indicated by its log P value (log of organic over aqueous partition) of approximately 7.48(octan-1-ol/water) and will not dissolve in non-acidic aqueous solutions (Morrison and Marley, 1976(a,b)). pKa values of 8.35 +/- 0.09 and 8.37 have been reported although the exact value is a matter of controversy since the compound's aqueous insolubility makes calculation difficult (Morrison and Marley, 1976(a); Canavan *et al.*, 1986). Clofazimine is, therefore, a basic drug and must exist in a charged form at physiological pH values. In alkaline environments and in organic solvents clofazimine is uncharged and has an intense orange-yellow colouration. However, as the pH drops the colour becomes more red and the aqueous solubility increases. In strongly acidic solutions this colour becomes violet. It is thought that these colour changes reflect the transition from an uncharged species (orange) in alkaline conditions, to a mono-protonated form (red) in dilute acid, to a di-protonated (violet) form in more concentrated acid to a triply-protonated molecule, which only partially exists, in concentrated acid and is colourless (Levy and Randall, 1970). While these transitions obviously reflect changes occurring over a very large pH range, clofazimine can also accept and donate protons as part of a redox system. The measured redox potential of B663 is -0.18v at pH 7 (E_{m7}) (Barry *et al.*, 1957; Barry *et al.*, 1960). Although the natural cellular environment cannot reach the pH needed to change the colour of clofazimine, it is seen as different colours *in vivo* and this obviously reflects the ability of the body to reduce this compound to various extents. The visual colouration represents an absorbance in the range 450-

Figure 1.2. The chemical structure of the basic phenazine nucleus, with the standard system for substituent numbering.



550 nm and is very dependent on the chemical environment of the molecule, whilst there is a stronger UV absorbance at 284-287 nm in all forms of clofazimine (Barry, 1969; Barry and Conalty, 1965; Levy and Randall, 1970; Banerjee *et al.*, 1974).

Clofazimine can also crystallise inside cells, although other rimino-analogues exist which have different substituents and do not crystallise *in vivo* (O'Sullivan *et al.*, 1992; Van Landingham *et al.*, 1993). X-ray studies by Rychlewska *et al.*, (1985) have shed some light on the structure of clofazimine. The phenazine plane is almost completely flat, with the N-10 chlorophenyl ring near perpendicular to this plane, and the phenyl ring on the anilino moiety inclined at 34° to the phenazine plane (Eggleston *et al.*, 1984; Humprey-Broom *et al.*, 1984; Rychlewska *et al.*, 1984; Rychlewska *et al.*, 1985).

1.3.2. Synthesis

Early methods of producing rimino-phenazines were quite difficult, involving the oxidation of derivatives of *o*-phenylenediamine with ferric chloride or *p*-benzoquinone with subsequent catalytic hydrogenation of the imidazophenazine produced (Barry *et al.*, 1958(a,b,c); Barry *et al.*, 1970). The same rimino-phenazines can now be prepared by the reduction of substituted anilinoaposafranines in the presence of a suitable ketone (O'Sullivan, 1984).

1.4. PHARMACOKINETICS

1.4.1. Absorption

The absorption of clofazimine is very variable, both between patients and with different pharmaceutical preparations (Barry, 1969; Vischer 1969; Banerjee *et al.*, 1974; Yawalker and Vischer, 1979). Initially clofazimine was produced as a crystalline preparation until studies indicated that only approximately 20% of an oral dose was absorbed. In a microcrystalline suspension of oil-wax, after a fat-rich meal, approximately 70% is absorbed (Yawalker and Vischer, 1979). Schaad-Lanyi *et al.*, (1987) have shown that the peak plasma concentration ($C_{max.}$) was 0.41 mg/l eight hours after a single 200 mg dose. Others have shown that, in patients on long duration therapy, the peak time ($t_{max.}$) lies between one and six hours (Banerjee *et al.*, 1974). It has also been shown that the absorption was increased by 30-60%, and the $t_{max.}$ decreased from twelve to eight hours, when clofazimine is administered after food. With a 50 mg daily dose, it was estimated that 70 days would be needed to reach a steady-state plasma

concentration, which correlates with the long duration needed for the clinical effects of the drug to be evident *in vivo* (Banerjee *et al.*, 1974; Venkatesan, 1989).

Absorption also varies between species, being good in mice, rats, and monkeys, poorer in rabbits and guinea pigs, and negligible in dogs. This may reflect some species specificity in the absorption mechanism of the drug (Barry and Conalty, 1958 and 1965; Barry *et al.*, 1960; Banerjee *et al.*, 1974).

Absorption after intra-muscular injection is very slow, probably due to the aqueous insolubility of the drug, and, hence, only the oral route has been used. The exact mechanism of absorption of clofazimine is uncertain (Barry *et al.*, 1948; Barry *et al.*, 1959; Vischer, 1969).

After an initial dose the drug can be seen to slowly build-up in the plasma and then starts to appear mainly in the cells of the reticulo-endothelial system and adipose tissue (Vischer , 1969; Conalty and Jackson, 1962; Conalty, 1966; Conalty *et al.*, 1971). Four explanations for the accumulation of the drug in these tissues were originally suggested by Barry *et al.*, (1959) 1) that B663 is present in the plasma attached to a carrier which transports the drug across the membrane of target cells. 2) B663 may be free in the plasma and accumulate via an active transport mechanism in the target cell. 3) B663 may be free in the plasma and diffuse passively into the target cell where it is bound and crystallises. 4) the drug may enter by phagocytosis or pinocytosis. The partition coefficient value of 7.48 mentioned in the previous section indicates that the molecule should be so lipophilic as to limit its capacity to traverse the cell membrane, since it would tend to stay in the lipophilic layer of the membrane (Morrison and Marley, 1976(a,b)). This observation precludes the idea of clofazimine being transported by any form of passive mechanism. Very little clofazimine seems to reach the excretory portions of the kidney since excretion in the urine is very slow, indicating that little or none of the plasma clofazimine is in free solution. A certain amount of B663 in the plasma is in solution in chylomicra which have reached the blood via the intestinal lymph (Barry, 1959). Conalty and Jina (1971) demonstrated that clofazimine is not taken up by macrophages in particulate form but rather enters in solution linked to some form of carrier (Conalty, 1966; Conalty *et al.*, 1971).

In an unpublished report, Barry and co-workers quoted results by Dr. L. H. Schmidt from monkeys treated with a twice daily dose of 100mg/kg for 7 days. Schmidt was able to measure serum levels of drug in the range of 2.3 -6.7 µg/ml but this level dropped to .07 µg/ml after the serum had been ultracentrifuged. Other results with human, mouse and guinea-pig serum showed that clofazimine bound appreciably to the α- and β- lipoproteins in serum,

particularly the β- lipoproteins, and that this binding was very firm but was saturated at approximately 10ug/ml. Binding to γ-globulin and albumin was negligible.

Unfortunately, no other work was performed to further identify the lipoprotein responsible for clofazimine transport in blood. It also appears likely that a specific carrier mechanism is responsible for the uptake of the clofazimine-lipoprotein complex which then yields free clofazimine within the cell by enzymatic cleavage of the complex within the lysosome. This intracellular clofazimine is often seen as crystal inclusions around osmiophilic rods (Conalty and Jackson, 1962; Conalty, 1966; Conalty and Jina, 1971; Conalty *et al.*, 1971; McDougall, 1974).

1.4.2. Distribution

The distribution of clofazimine throughout the body is slower than absorption and is very heterogenous (Vischer, 1969). Hence, the volume of distribution has never been calculated and would probably have no clinical relevance. Clofazimine has two main target areas consisting of certain cellular groups of the reticulo-endothelial system and fat cells in the adipose tissues, where it is avidly taken up (Conalty and Jackson, 1962; Vischer, 1969). This means that any intra-cellular effect of the drug cannot be correlated to plasma levels. Clofazimine has been detected (often as crystals), or measured, in other regions of the body including bone, muscle, skin, heart, eye, gallbladder and nervous tissue. The drug has also been found in urine, bile, sweat, milk, sebum, tears, and sputum. The highest levels of clofazimine are found in the spleen, liver, lung, adipose tissue, and mesentery (Vischer, 1969; Mansfield, 1974; Desikan *et al.*, 1975; Desikan and Balakrishnan, 1976; Kumar *et al.*, 1987). The lowest levels of drug are found in the brain. Mansfield, (1974) found levels below the limit of determination (<0.1mg/g) in the brains of humans at autopsy and coupled with the cerebral drug level measurements in pharmacology chapter (see section 5.8.1.) and that of Barry *et al.*, (1960), which showed measurable levels in the brains of rats and mice, indicates, that contrary to established belief, clofazimine can cross the blood-brain-barrier, although in very small amounts. In many tissues clofazimine is mainly found in crystalline form (up to 99%) and it is believed that in this way the drug can remain in the body for years after cessation of the dosage regime (Banerjee *et al.*, 1974).

1.4.3. Metabolism and Elimination.

A two compartment pharmacodynamic model has been suggested based on experimental models (Banerjee *et al.*, 1974; Levy, 1974; Hastings *et al.*, 1976). The first compartment is evident with short-term low dosages and has an elimination half-life of approximately 1 week, whereas with higher doses and/or longer duration therapy a second elimination half-life of 70 days or greater is seen. However, this slow elimination does not appear to be dependent on the presence of the drug in crystal form in the body (Banerjee *et al.*, 1974). Small amounts of clofazimine are eliminated in the sebum and sweat (Vischer, 1969). Urinary excretion of un-metabolised clofazimine has been shown to be in the range of 0.03 to 0.41% of a dose, daily (Levy, 1974). Three metabolites of clofazimine have been identified in patients urine (Feng *et al.*, 1981 and 1982). The proposed structures and routes of production of these compounds are shown in Figure 1.3. Metabolite I arises from hydrolytic dehalogenation, metabolite II by hydrolytic deamination followed by glucuronidation of the resultant hydroxyl group, and metabolite III by hydration at C4, followed by glucuronidation of the resultant hydroxyl group. Besides the actual structures of these compounds, very little else is known about them, i.e. there is precedent to assume that they are the product of hepatic metabolic pathways, but no hard evidence, and their therapeutic value, if any, is unknown. Like clofazimine, these compounds are found in very small concentrations in urine, representing 0.20%, 0.25% and 0.2% of a daily dose, respectively, for metabolites I, II and III assuming a 70% absorbance value of a 300 mg/day dose.

Studies to measure faecal or biliary metabolites have not been performed, and although faecal levels have been followed in volunteers for 3 days, these values only give an estimate of absorption rather than faecal elimination (Levy, 1974; Mathur *et al.*, 1985).

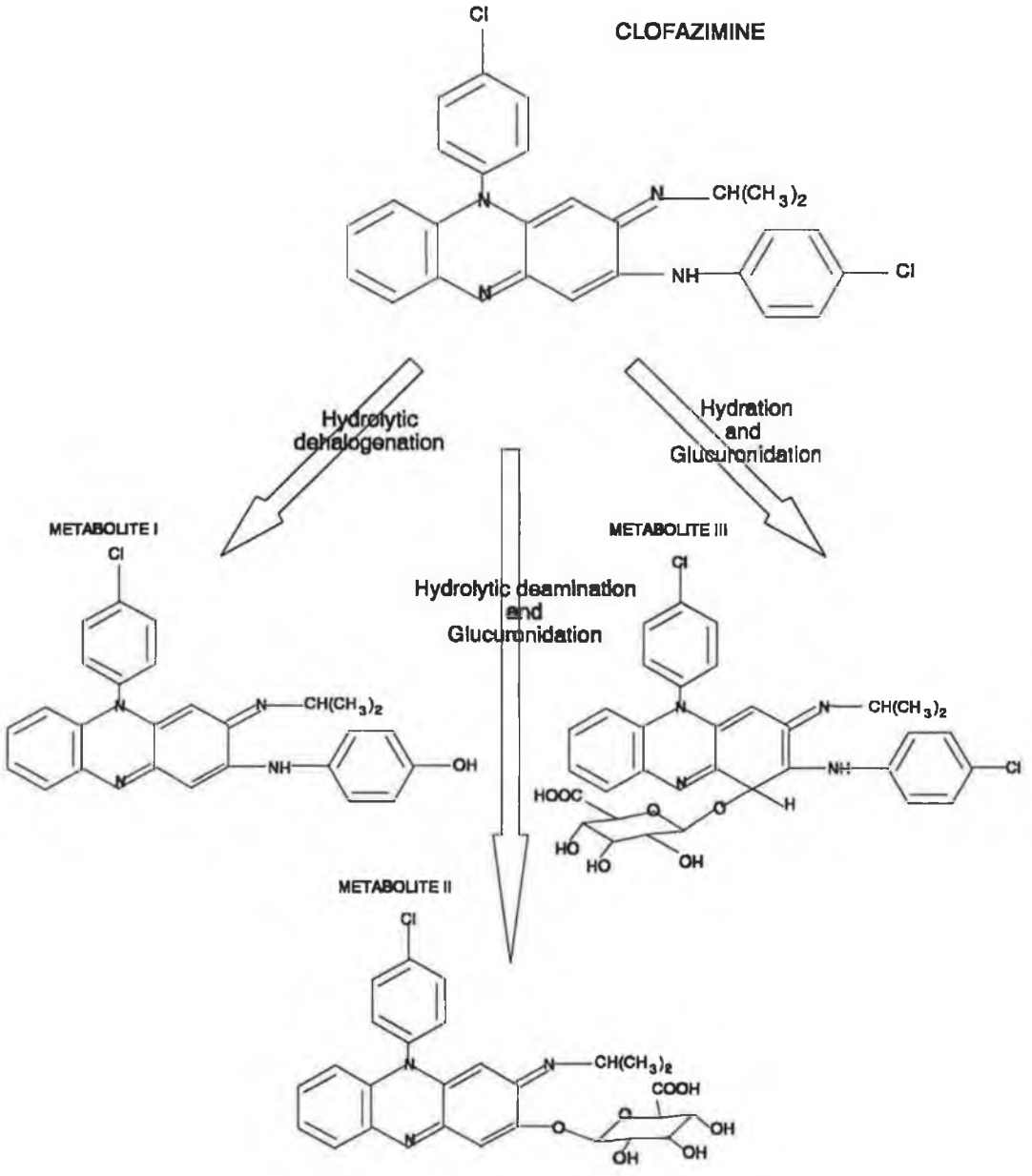
How clofazimine is able to remain largely un-metabolised is uncertain, but perhaps its necessity for a carrier to cross cellular membranes, as suggested earlier, infers that very little can enter the mitochondria of metabolically competent cells.

1.5. PHARMACOLOGY

1.5.1. Mechanism of action

Until recently there have been several different theories about how clofazimine works, with a limited amount of experimental observation to back up these findings. However, none of

Figure 1.3. The chemical structures and proposed metabolic routes of the three clofazimine metabolites which have been isolated and identified in human urine. (After Feng *et al.*, 1981 and 1982)



these ideas fully accounted for the broad range of effects seen with clofazimine *in vivo* and *in vitro*.

The first observation suggesting a mode of action was that clofazimine bound tightly to DNA (Morrison, 1972; Morrison and Marley, 1976(a,b) and 1977). At the high concentrations of drug used, Morrison *et al.*, showed that clofazimine selectively binds to runs of guanine and deoxyguanine, DNA with dG + dC bases and purified yeast tRNA. Mycobacteria have a high G + C content (67-69%), whereas human cells have a much lower G + C content. It was, therefore, suggested that clofazimine has a selective effect on mycobacteria due to differences in base pair content which allowed clofazimine to inhibit the template function of DNA in mycobacteria. With other analogues of clofazimine it was shown that DNA binding was increased with increasing chlorine substitution, which correlates with the increases in activity seen *in vitro* with these compounds. These experiments showed an interaction between clofazimine and isolated and synthetic strands of ribo- and deoxyribonucleic acids, where clofazimine had to be dissolved in 10% DMF or DMSO at concentrations in excess of its aqueous solubility. The implications of results so abstracted from the *in vivo* situation must therefore be very questionable, without further evidence, for example, the demonstration of *in vivo* binding of clofazimine to DNA.

It has also been suggested that clofazimine acts by inhibiting the respiratory chain of certain cells, since phenazine compounds are auto-oxidisable and it has been suggested they could act as artificial electron acceptors. Clofazimine is taken up (2 mg/g dry weight) and decolourised by living mycobacterial cells under anaerobic conditions but readily re-oxidised when re-exposed to air (Barry *et al.*, 1957; Rhodes and Wilkie, 1973; Delhanty *et al.*, 1974). Rhodes and Wilkie, (1973) reported that clofazimine was absorbing some of the terminal hydrogen transfer capability in yeast (*Saccharomyces cerevisiae*), fibroblast cells and rat liver mitochondria, i.e. the respiratory system was oxidising clofazimine instead of normal cellular substrates, such as NADH, causing a reduction in the amount of ATP available for all cellular processes. Interestingly, these authors were able to isolate mutant yeast strains resistant to the effects of clofazimine. The resistance appeared to be of two distinct types, the first involving a change in permeability to clofazimine since these organisms remained unstained by the drug. The second type had some form of intracellular change, probably an alteration in a mitochondrial element involved in drug reactivity, since the cells of yeast mutants in this group were stained by clofazimine. Clofazimine-resistant organisms were also cross-resistant to a number of anti-mitochondrial agents.

It has been shown in catalase-negative mycobacteria, that when the reduced form of clofazimine is re-oxidised, as with other redox compounds, hydrogen peroxide is produced (Barry *et al.*, 1957). This would be one explanation for the increased clofazimine sensitivity of isoniazid-resistant catalase-negative strains of *Mycobacterium tuberculosis*, and the observation that normal *M. tuberculosis*, which grows readily in an aerobic environment grows only anaerobically in the presence of inhibitory concentrations of clofazimine (Barry *et al.*, 1957).

While these authors have demonstrated that clofazimine can inhibit the growth of prokaryotic and eukaryotic cells by interfering with hydrogen transfer, it is not known how important this is clinically. If clofazimine interacts significantly with mitochondria *in vivo*, it should cause very serious side effects, especially in bone marrow cells where it can be found in high concentration (Desikan and Balakrishnan, 1976). However, these expected effects have never been seen, even at very high doses. Resistant prokaryotic and eukaryotic cells have been produced *in vitro* by continuous culture in a clofazimine-containing medium (Rhodes and Wilkie, 1973; O'Sullivan *et al.*, 1988). Although there are two questionably reliable reports of clofazimine resistance in patients (Warndorff-vanDiepen, 1982; Kar *et al.*, 1986), this has never emerged as a problem in clinical use.

1.5.2. Selective effects on the immune system

Clofazimine has been demonstrated to have several different effects on different aspects of the immune system. These include an increase in the number and size of lysosomes and phagolysosomes in isolated macrophages (Conalty *et al.*, 1971), an increase in the lysosomal level of cultured macrophages (Saracent and Finlay, 1982), an inhibition of complement-mediated solubilisation of immune complexes (Kashyap *et al.*, 1992), a dose-dependent inhibition of neutrophil motility and lymphocyte transformation (Gatner, *et al.*, 1982) and an enhancement of reactive oxidant production (Anderson *et al.*, 1986 and 1988a,b; Sahu *et al.*, 1991 and 1992). Based on a compilation of these observations, there is significant evidence to show that clofazimine has a potent effect on specific elements of the functioning of the immune system.

Several recent papers have suggested an explanation for the wide variety of effects caused by the interaction of clofazimine with the immune system and perhaps also its direct effects on bacteria (Anderson *et al.*, 1986 and 1988(a,b); Sahu *et al.*, 1991 and 1992; Van Rensburg *et al.*, 1992 and 1993). It has been shown by these authors that clofazimine, at concentrations within the therapeutic range (0.01-5 µg), stimulates reactive oxidant (specifically lysophospholipid) production in human polymorphonuclear leucocytes (PMNL) and gram-

positive bacteria. These substances are the products, or result from the products of, a selective stimulation of phospholipase A_2, since the activities of clofazimine could be blocked with the selective phospholipase A_2 inhibitors . One of the major targets of these products in lymphocytes is the enzyme Na^+, K^+-ATPase, whose inhibition causes a repression of lymphocyte proliferation (Anderson and Smit, 1993).

Combining this recent information, with established observations allows us to formulate a more comprehensive picture of the pharmacology of clofazimine. Early work has shown that clofazimine is transported by a lipoprotein carrier (β-lipoprotein now known as Low Density Lipoprotein LDL) through the body. LDLs are primarily concerned with the transport of cholesterol, and certain cells, particularly adipose and reticulo-endothelial cells, have specific receptors to transport LDL-cholesterol across the membrane with a subsequent lysosomal cleavage and recycling of the LDL to provide cholesterol in a suitable form within the cell (Goldstein and Brown, 1977; Goldstein *et al.*, 1979). This also allows for the selective entry of clofazimine into particular cells. In adipose cells, the level of clofazimine rises to macroscopic levels, where colour can be seen visually, but does not appear to have any other significant clinical effect; in reticulo-endothelial cells clofazimine also builds up, but by interacting with Phospholipase A_2, it causes several changes in the normal function of the immune system a whole. These stimulatory effects on Phospholipase A_2, coupled with a direct anti-bacterial action, and a selective concentration in one of the main targets of mycobacterial infection (phagocytic cells), explain how clofazimine produces its anti-mycobacterial effect.

1.5.3. Diseases where clofazimine has been used

As mentioned in the introduction, clofazimine is mainly used as part of the WHO recommended Multi-Drug Therapy (MDT) regime to treat lepromatous leprosy (WHO, 1982). Clofazimine is also used to control some of the acute reactionary phases which can occur with leprosy , especially erythema nodosum leprosum, and to reduce the dose of corticosteroids necessary to manage these episodes (Pettit, 1967; Morgan, 1970; Helmy *et al.*, 1972; Imkamp, 1981). Although largely inactive against tuberculosis, clofazimine is used to treat other rarer mycobacterial diseases, e.g. Beruli ulcer (Lunn and Rees, 1964; Oluwasani *et al.*, 1975). Clofazimine has been successfully used to control the mycobacteraemia common as an opportunist in Acquired Immune Deficiency Syndrome (AIDS), alone, and in combination with other agents, reversing the weight loss, night sweats and lethargy associated with these infections (Nunn and McAdam, 1988; Young, 1988; Polis and Masur, 1989; Garrelts, 1991; Goldschmidt and Dong, 1991). Burns and non-specific skin lesions (human and veterinary) have been

successfully treated by a topical cream formulation of clofazimine (Ellis, 1973; Knottenbelt *et al.*, 1989; Venkateswarlu *et al.*, 1992); in addition, as outlined in Table 1.1, clofazimine hasshown *in vitro* and/or *in vivo* activity in a number of other unrelated diseases and disease models. A patent has been registered for the use of a clofazimine derivative, B669, in the treatments for cancer and tissue rejection by the University of Pretoria, and the riminophenazines have also shown some potential in reversing multi-drug resistance in cancer cells (Anderson and Smit, 1993).

1.5.4. Structure-activity relationships

To date, several hundred rimino-phenazine agents have been synthesised, and certain relationships have been shown to exist between chemical substituents and biological and chemical properties, based on *in vitro* and some *in vivo* experiments with these compounds. These compounds can be grouped on the basis of substitutions of the phenazine ring, as shown in Figure 1.2.

1.5.4a. R_1 substitution

In general, substitution in this position with a chlorine, methoxy or ethoxy group, causes an increase in activated superoxide and arachidonate generation by neutrophils, gives increased anti-bacterial activity *in vitro*, and lipophilicity. Other substituents in this position have little or no effect (Barry, 1969; Barry *et al.*, 1970; Zeis and Anderson, 1986; Zeis *et al.*, 1987 and 1990; Savage *et al.*, 1989; Van Landingham *et al.*, 1993).

1.5.4b. R_2 substitution

Here, chlorination (to give dichloro-analogues) again increases anti-bacterial activity, although, the resultant elevation in lipophilicity is a major factor in fat retention as is well documented for clofazimine. The increases in activity associated with R_2 substitution appears to be due to the molecular size of the substituent, rather than its electronegativity. Bromine, ethoxy, and methoxy substitution all give increased activity, whereas hydrogen, or fluorine substituents produce compounds with reduced activity. Combined meta- or para- substitution with chlorine (tetrachlorinated compounds), have an even greater activity than their equivalent dichloro- compounds. Chlorinated compounds also have superior anti-tubercular activities. Unfortunately chlorination is linked to crystal formation in compounds not possessing a tertiary nitrogen in the R_3 position. It is this crystal formation which may be involved in some gastro-

Table 1.1: A list of various diseases, or disease models where clofazimine has been shown to possess some activity.

Disease where activity seen	**Reference**
Leishmaniasis	Evans *et al.*, (1989).
Malaria	Sheagren, (1968).
Scleroma	Shehata *et al.*, (1989).
Fistulous withers (horses)	Knottenbelt *et al.*, (1989).
Pyoderma gangrenosum	Michaelson *et al.*, (1976); Stone, (1990); Merret *et al.*, (1990); Kaplan *et al.*, (1992).
Annular elastolytic giant cell granuloma	Vehring *et al.*, (1991).
Discoid lupus erythematosus	Mackey, (1973 & 1976); Zeis *et al.*, (1989).
Pustular psoriasis	Chuaprapaisiep and Piamphongsant, (1978); Nair and Shereef, (1991).
Regressive ulcerative histiocytosis	Horiguchi *et al.*, (1989).
Vitiligo	Kumar *et al.*, (1987).
Nodulocystic acne	Mascaro *et al.*, (1991).
Oedematous complications of acne vulgaris	Helander and Aho, (1987).
Cutaneous malacoplakia	Herrero *et al.*, (1990).
Erythema dyschromicum perstans	Picquero-Martin, (1989).
Necrobiosis lipodica	Mensing, (1989).
Granulatomatous macrochelitis	Friedrich, (1989); Gall *et al.*, (1989); Cusano *et al.*, (1991).
Crohn's disease	Afdhal *et al.*, (1972); Pines *et al.*, (1993).

intestinal side-effects associated with clofazimine (Barry and Conalty, 1958; Barry, 1969; Barry *et al.*, 1970; Zeis *et al.*, 1987 and 1990; Franzblau and O'Sullivan, 1988; Franzblau *et al.*, 1989; Van Landingham *et al.*, 1993).

1.5.4c. R_3 substitution

This is the major substituent varied in studies to further develop this class of drugs. A substituted imino group is essential for activity . Imino alkyl substituents have various activities, but are generally quite active, as exemplified by clofazimine which has an isopropyl amine substituent in this position. Compounds with a basic nitrogen substitution in the group on the imino-nitrogen are active against organisms engineered to be resistant to clofazimine. Primary and secondary nitrogens in this class are poorly absorbed, with the exception of the tetramethylpiperidine group of substituents, whereas tertiary nitrogen-containing compounds are generally well absorbed. The compounds with nitrogen in the imino side chain also have reduced body fat solubility and do not crystallise in the cells of the body. Activity generally requires that the basic nitrogen be spaced at least three carbons from the imino- nitrogen, whether this basic nitrogen is primary, secondary or tertiary (Barry and Conalty, 1958; Barry, 1969; Barry *et al.*, 1970; Zeis *et al.*, 1987 and 1990; Franzblau and O'Sullivan, 1988; Franzblau *et al.*, 1989; O'Sullivan *et al.*, 1992; Van Landingham *et al.*, 1993).

1.5.5. Toxicity and side effects

1.5.5a. Common side effects

Clofazimine is generally a well tolerated and very safe drug. Nevertheless, like all drugs, there are some drawbacks and side effects associated with its use. The side effects seen are normally mild, dose related, and reversible (Yawalker and Vischer, 1979; Garrelts, 1991).

The most common side effect seen is a red-brown discolouration of the skin, which becomes visible 2-4 weeks after commencing treatment, and is evident in almost all patients on high doses (300 mg/day) (Browne *et al.*, 1981; Moore, 1983). This pigmentation is especially evident on the trunk and face, but other regions may be affected, particularly the conjunctiva, and discolouration has also been noted in sweat, hair, sputum, urine, faeces, tears, and the inner organs, with the exception of the central nervous system (Browne and Hogerzeil, 1962; Desikan and Balakrishnan, 1976; Desikan *et al.*, 1975; Kumar *et al.*, 1987; Kumar, 1991). Certain cultures, particularly some Asian races, find the associated colouration stigmatising and

unacceptable, and this is the major cause of non-compliance in treatment regimes (Pettit, *et al.*, 1967; Warren, 1968; Moore, 1983). Lepromatous lesions can tend to become hyperpigmented, becoming a dark brown-black colour (Browne and Hogerzeil, 1962). Whilst the initial pigmentation is obviously due to the strong colour of the drug itself deposited in the skin, the later appearance of a deep tan colour appears to be due to hypermelanosis associated with the chemical structure of clofazimine, similar to an effect seen with high/prolonged doses of chlorpromazine, an anti-psychotic agent with a similar heterocyclic nucleus (Satanove, 1965; Levy and Randall, 1970). Differences in the oxidation state of clofazimine in different areas of the skin may explain the tendency to colour uncovered regions of the body (Browne and Hogerzeil, 1962). The pigmentation may also be associated with a general dryness of the skin, xerosis, in approximately 30% of patients, which can progress to ichthyotic changes (scaly patches) (Moore, 1983; Kumar, 1991). The skin may also become pruritic (itchy) with a burning sensation which can extend to the eyes. There appears to be little correlation between these symptoms and dose. Other effects reported include phototoxicity, non-specific rashes, and, controversially, dimness of vision (Hastings and Trautman, 1968; Yawalker and Vischer, 1979; Moore, 1983; Kumar *et al.*, 1987).

Abdominal pain and transient digestive disturbances are also common side-effects, reported normally with high-dose therapies, but not usually severe (Yawalker and Vischer, 1979; Moore, 1983; Kumar *et al.*, 1987; Dollery, 1991; Garrelts, 1991). However, a more serious gastro-intestinal syndrome associated with very high-dose long-duration therapy, termed the Late Syndrome, has been reported. Symptoms of this syndrome include persistent diarrhoea, severe abdominal pain, nausea and weight loss (Jagadesan *et al.*, 1975; Mason *et al.*, 1977; Yawalker and Vischer, 1979; Venencie *et al.*, 1986; Hassan *et al.*, 1987). This syndrome appears to be more common in Indian patients, and, although occasionally fatal, can generally be reversed by removal of the drug, and may not recur with re-administration. It is currently thought that these symptoms reflect the deposition of large amounts of B663 in the cells of the intestinal mucosa. These crystals may cause irritation, leading to intestinal disturbances.

Various forms of clofazimine enteritis have been reported, and in some cases, although apparently initially triggered by clofazimine therapy, other causes e.g. gluten sensitivity appear to maintain the disease state (Jost *et al.*, 1986). Prostaglandins may play a role in these states, since clofazimine stimulates prostaglandin production, especially PGE2 synthesis (Zeis and Anderson, 1986), which, in the gut, increases permeability, smooth muscle contraction and can cause diarrhoea (Bennet *et al.*, 1968 and 1981; Hawkey and Rampton 1985). Supporting this

idea is the observation that anti-spasmolytics have been used with some success in treating uncomplicated B663-induced diarrhoea (Kumar *et al.*, 1987).

There has been no evidence to date to suggest that clofazimine is carcinogenic, teratogenic or mutagenic in humans (Peters *et al.*, 1983) despite limited evidence of clastogenicity in mice (Das and Roy, 1990; Dash *et al.*, 1990; Roy and Das, 1990).

1.5.5b. Biochemical and haematological side effects

A study by Hastings *et al.*, (1976) on patients receiving long-term B663 treatment reported no statistically significant changes in the following haematological parameters; SGOT (serum glutamine oxaloacetic transaminase), thymol turbidity, serum globulins, uric acid, alkaline phosphatase, white blood cell count, haematocrit, haemoglobin, BUN (blood urea nitrogen), serum creatinine, serum cholesterol, serum albumin, serum potassium and calcium and routine urinalysis. Significant, but clinically asymptomatic changes were seen in fasting blood sugar and total bilirubin. In addition, Bulak *et al.*, (1983), have shown that 12 months of clofazimine therapy caused a reversal of the liver dysfunction common in leprosy.

1.5.6. Drug Interactions

Mehta *et al.*, (1986) have shown an absence of any significant difference in various pharmacokinetic parameters of dapsone, when co-administered with clofazimine. With rifampicin, clofazimine delayed the time to reach its peak plasma concentration, and increased the half life ($t_{½}$) marginally. However, this had no effect on the area under curve (AUC) or peak plasma concentration (C_{max}) values. Hence, the relevance of these findings is unclear. One study has shown that isoniazid increased the serum concentration and urinary excretion of clofazimine, by as yet uncertain mechanisms (Venkatesan *et al.*, 1980).

In addition, there is evidence of clofazimine interacting with endogenous substances. Duncan *et al.*, (1983) reported a diminished oestrogen excretion in pregnant women receiving 300 mg clofazimine per week, with an uncertain clinical relevance. Bharadwaj *et al.*, (1982) have shown reduced skin and plasma levels of vitamin A in leprosy patients, and a more pronounced reduction of these levels in leprosy patients on clofazimine therapy.

1.6. SUMMARY

Although much still remains to be understood about the chemical and pharmacological properties of clofazimine and other phenazine agents, recent advances have provided a clearer picture of how and why these agents work. They also provide strong evidence that more useful compounds in this class can be produced, and that existing agents may be more broadly useful in a variety of disease states than was initially anticipated.

1.7. THESIS OUTLINE

The preceding sections have outlined much of the general information known about clofazimine and phenazine agents in general. The aim of this project was to provide further basic knowledge on selected properties of these agents. For clarity, the research undertaken has been divided into three chapters, with a separate chapter (chapter 2) for the materials and methods which were used.

Chapter 3 describes the investigations into some of the chemical properties of the rimino-phenazines which were selected for testing. The development and validation of a new method for quantification of phenazines in biological matrices is also described.

Chapter 4 reports the production and characterisation of phenazine-protein conjugates, their use in the production of polyclonal antisera and attempts to develop a quantitative assay system based on the antibody produced.

Chapter 5 describes the studies of some of the pharmacokinetic properties of the chosen agents which were undertaken using the methods discussed in chapter 3.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1. SOURCE OF PHENAZINE COMPOUNDS

All phenazine compounds used in this study, except the pharmaceutical grade clofazimine, were synthesised by Dr. Sean O'Sullivan in the Chemistry Department of University College Dublin, Dublin 4. Pharmaceutical grade clofazimine was supplied by Geigy pharmaceuticals, Horsham, West Sussex, England.

2.2. REAGENTS AND CHEMICALS

All reagents, unless specified otherwise were, purchased from the Sigma Chemical Co., Poole, Dorset, England. Chromium, cyclodextrins and deuterated solvents were bought from The Aldrich Chemical Co., The Old Brickyard, New Road Gillingham, Dorset, SP8 4JL, England. Silica and silica TLC plates and other listed reagents were supplied by Riedel-de Haën, Wunstorfer Straße 40, P.O. Box, D-3016 Seelze 1, Germany. Alumina was supplied by Macherey-Nagel GmbH & Co. KG, P.O. Box 101352, D-5160 Düren, Germany. Hexane sulfonic acid was purchased from Romil Chemicals Ltd., 63 Ashby road, Shepshed, Loughborough, Leicestershire, England. All organic solvents used were of HPLC grade and were supplied by LabScan,unit T26, Stillorgan Industrial Park, Co. Dublin. The water used in HPLC mobile phases was Ultrapure grade (10^{-18} Ω resistivity) produced by a still supplied by Millipore, 80 Ashby Rd., Bedford, MA 01730, USA. Sephadex and the BIAcore equipment and reagents were supplied by Pharmacia, Bjorkgaten 30, S-75182, Uppsala Sweden. KLH was purchased from Calbiochem, Behring Diagnostics, La Jolla, CA 92037, USA. All animal food was provided by W.M. Connolly and Sons Ltd., Redmills, Goresbridge, Co. Kilkenny.

2.3. CONSUMABLE LABWARE

BM-test-8 urine test strips were supplied by Boehringer Mannheim UK Ltd., Bell Lane, GB-Lewes, East Sussex, England. Maxisorb ELISA plates were supplied by NUNC, Postbox 280 - Kamstrup DK, Roskilde, Denmark. Amine Binding ELISA plates were purchased from COSTAR, 1 Alewife Ctr., Cambridge, MA 02140, USA. Emphase and BCA reagents were purchased from Pierce and Warringer (UK) Ltd., 44 Upper Northgate Street, Chester, Cheshire, England. Centricon SR3 and Centriplus centrifugal concentrators were purchased from Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire, England. Bondclone C_{18} and Biosep SEC-4000 HPLC columns were purchased from Phenomenex, Melville House, Queens Avenue, Hurdsfield

Ind. Est., Macclesfield, Cheshire SK10 2BN, England. 10 ml glass blood tubes were purchased from Medlabs Ltd., Unit 1C, Stillorgan Industrial Park, Stillorgan, Co. Dublin. 1.1 ml glass autosampler vials and 8 mm PTFE septae were supplied by Chromachol, P.O. Box. 293, Trumbull, CT 06611, U.S.A. 2500 um polyethylene mesh was produced by E.C. Stewart Filtration, 128 Botanic Avenue, Dublin 9, Ireland.

2.4. EQUIPMENT

All pH measurements were performed on a 3015 pH meter from Jenway Ltd., Gransmore Green, Felsted Dunmow, Essex CM6 3LB, England. I.R. analysis was performed on a Nicolet I.R. spectrometer supplied by The Nicolet Instrument Corp., 5225-1 Verona rd., Madison, WI 53711, USA. Electrophoresis was performed using gels supplied by the Atto Corp., 2 - 3 Hongo 7- chome, Bunkyo-Kui, Tokyo 113, Japan. NMR analyses were performed on a 400 MHz AC-400 NMR spectrometer from Brucker, Banner Lane, Coventry CV4 9GH, England. Samples were analysed in 5 mm glass NMR tubes supplied by the Wilmad Glass Co., Route 40, 1 Oak Road, Buena, N.J. 08310, U.S.A. Freeze drying was performed using Hetosicc, Hetofrig and Hetovac apparatus from Heto Lab Equipment A/S, Gydevang 17 - 19, DK 3450, Allerod, Denmark. The complete System Gold HPLC system, consisting of a 507 autosampler, 126 pump, 166 U.V. detector, 168 PDA detector and version 8 software were supplied by Beckman Instruments Inc., Bioindustrial Business Unit, Fullerton, CA 92634-3100, U.S.A. The photodiode HPLC system used for protein conjugate analysis was supplied by the Waters Corp., 34 Maple St., Milford, MA 01757, U.S.A. All spectrophotometric measurements were made on a U.V.-160A spectrophotometer from the Shimatzu Corporation, 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604, Japan. Centrifugation was performed on the specified Heraeus centrifuges supplied by Heraeus Instruments Inc., 111-A Corporate Blvd., South Plainfield, N.J. 07080, U.S.A. The absorbances in ELISA and BCA assays was measured using a Titertek Mulitscan plate reader produced by Flow Labs. Ltd., Woodcock Hill, Harefield Rd., Richmansworth, Hertfordshire WD3 1PQ, England. Faecal samples were oxidised in a high temperature oven supplied by Lenton Thermal Designs Ltd., Unit C2 Valley Way, Welland Industrial Estate, Market Harborough, Leicestershire LE1 7PS, England.

METHODS

2.5. LICENSING

All of the experiments involving animals were thoroughly vetted before initiation. Each investigation was performed with the appropriate licences and exemptions from the Department of Health. In every cases, all appropriate efforts were made to minimise distress and discomfort to the animals used.

2.6. DISTRIBUTION STUDIES IN MICE

Selected phenazine agents were administered to female Schofield mice housed in groups of six by oral gavage. Suspensions of each agent, at a concentration of 1.28 mg/ml, were made in 1 % (w/v) Carboxymethyl Cellulose 1 % (v/v) Tween 20 (Riedel-de Haën) and 0.4 ml administered using a modified spinal needle to each animal every day for 21 days. On day 22 the animals were sacrificed and organs and blood removed. The samples were stored at -18 °C and thawed just before analysis of drug levels measured by DCM extraction and HPLC as described in sections 2.9.1 - .4. and 2.10., respectively.

2.6. RAT EXPERIMENTS

2.6.1. Metabolism cage construction

For absorption, distribution and toxicity studies female Wistar rats were maintained in purpose built metabolism cages in groups of 3 animals. Each animal was identified by a series of rings or lines indelibly marked on its tail. Figure 2.1 (a) shows a side view of this cage. The cages consisted of standard polypropylene rat cages (41 x 24 x 13 x 20 cm) with "high type" stainless steel mesh lids and the bottom cut out. A stainless steel grid (39.5 x 23.5 cm) with a mesh of 4.75 x 0.8 cm provided the living surface at the bottom of the cage through which food, faeces, urine and water could freely fall. Faeces were directed by a 2.5 cm polyethylene mesh (E.C. Stewart) (see Figure 2.1 (b)), suspended at a 45^0 angle under the cage, to a detachable bag made from the same mesh. Urine was collected by a polythene plastic sheet (see Figure 2.2 (a)) suspended under the mesh at a similar angle. A 50 ml universal tube at the lowest angle of the bag was used to collect this urine.

Figure 2.1. A cut away, side view of the metabolism cage used for the absorption and distribution studies of phenazines in rats. The cage dimensions are outlined in the text. The dimensions of the mesh used for faecal collection are shown in part (b).

Figure 2.1 (a)

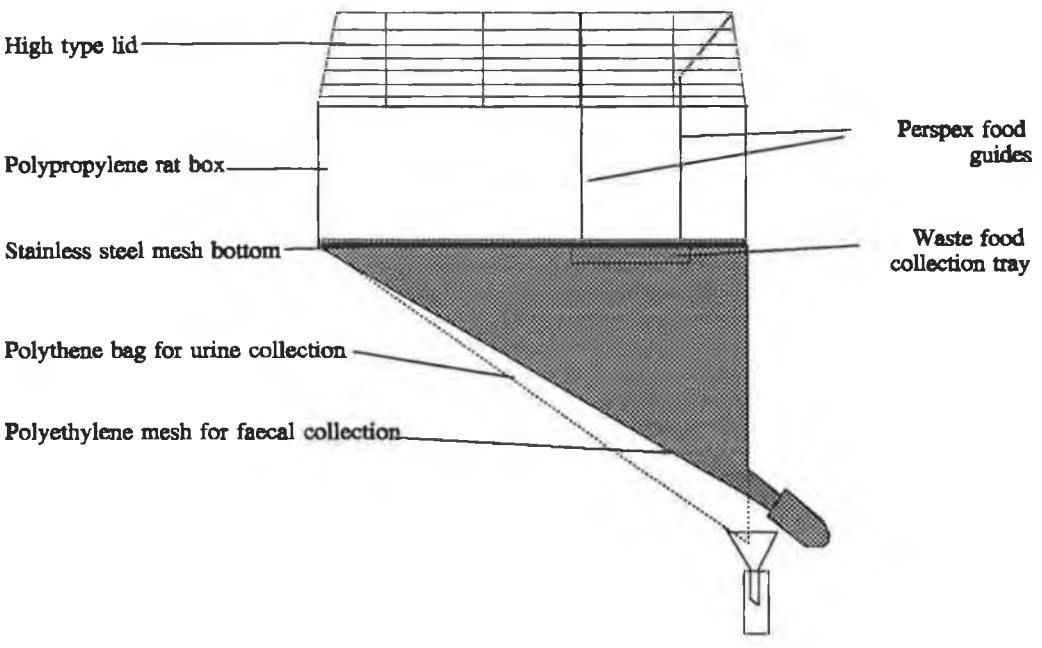
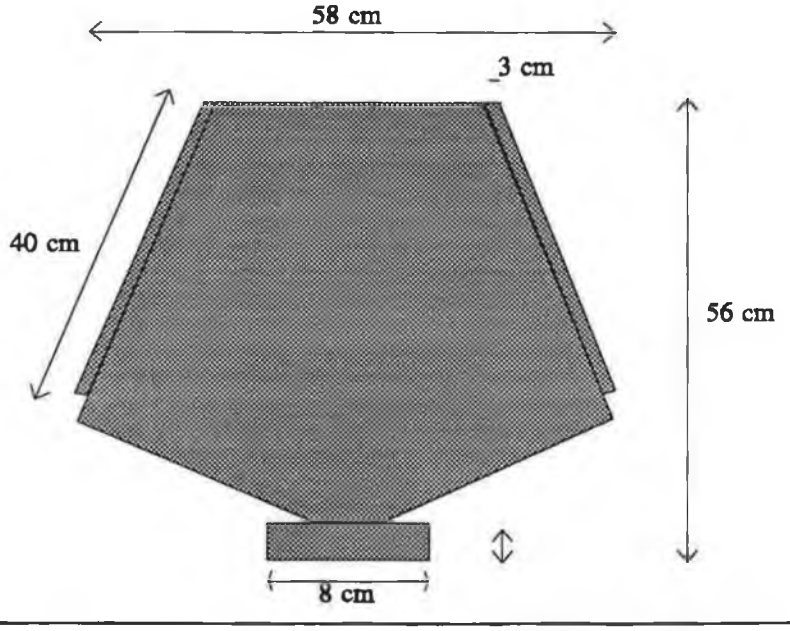


Figure 2.1 (b)



Weighed amounts of food were given into the feeding compartment of the high sided top which had a rectangle of perspex allowing the rats access to the food only from the side. Waste food was collected by a perspex box (24 x 8 x 2 cm) suspended underneath the food area using steel springs. Sheets of perspex either side of this food area bolted to the bottom mesh of the cage prevented food from falling anywhere but into this box (see Figure 2.2 (b)). Water was administered in a standard 250 ml water bottle in the water slot of the cage.

2.6.2. General method for absorption studies in the rat

Rats were maintained in the metabolism cages on normal rat food for one week before each study to allow the animals to acclimatise to their environment and allow baseline measurements of all parameters investigated. All food was removed from the cages 15 hrs. before the scheduled beginning of the study but animals still had free access to water. A known weight of drug-doped food was added to the cage to begin the experiment. All faeces were collected at this time to provide blank measurements. After 4 hrs. any remaining food and waste food were removed and weighed and the animals returned to their normal diet. Faecal samples were collected at intervals for 105 hrs.

2.6.3. Drug doping of food for absorption studies

Drug doped food was prepared by hydrating 20 g of rat pellets (W.M. Connolly and Sons Ltd.) in 40 ml of distilled water. 0.12 g of chromium (III) oxide powder (Aldrich), with a particle size less than 53 μm, and 7 mg of test phenazine dissolved in ethanol, or in the case of cyclodextrin complexes, 0.121 g of powdered complex, were thoroughly mixed into the food. The food was then fully dried overnight in an oven at 80 ^{0}C.

2.6.4. General method for distribution studies in rat

The animals were given a two week "washout" period after the absorption study, being maintained in the metabolism cages on normal rat pellets. Drugged food was prepared by hydrating 1.8 kg of rat pellets with 3.6 L of distilled water. 0.63 g of test phenazine dissolved in excess ethanol was thoroughly mixed in. As a control a batch of food was prepared in the same way except no phenazine was added. This food was given to one group to control for any anomalies caused by the hydration and heating processes. In the case of cyclodextrin complexes, 10.9 g of the powdered complex was mixed in. The food was dehydrated on oven trays in an oven at 80 ^{0}C for 24 hrs.

Figure 2.2. (a) The dimensions of the polythene sheet suspended beneath the metabolism cage to collect urine produced by the animal in the cage. (b) Shows a cut away, side view of the waste food collection apparatus in the metabolism cage. As shown in this diagram waste food can only fall into the collection box, allowing easy quantification.

Figure 2.2 (a)

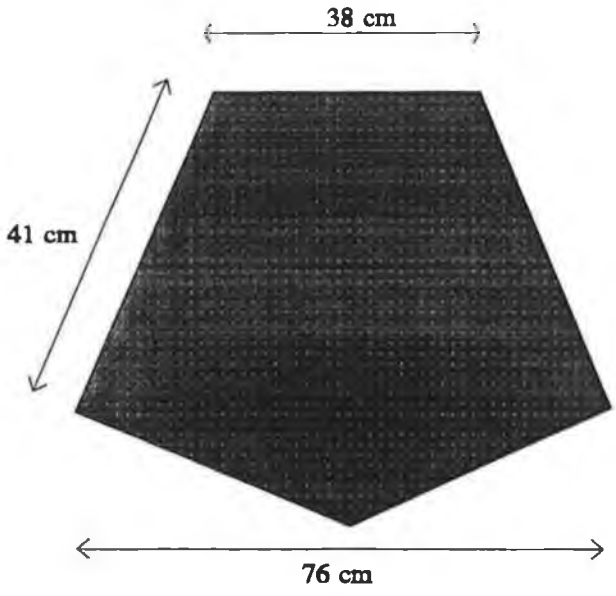
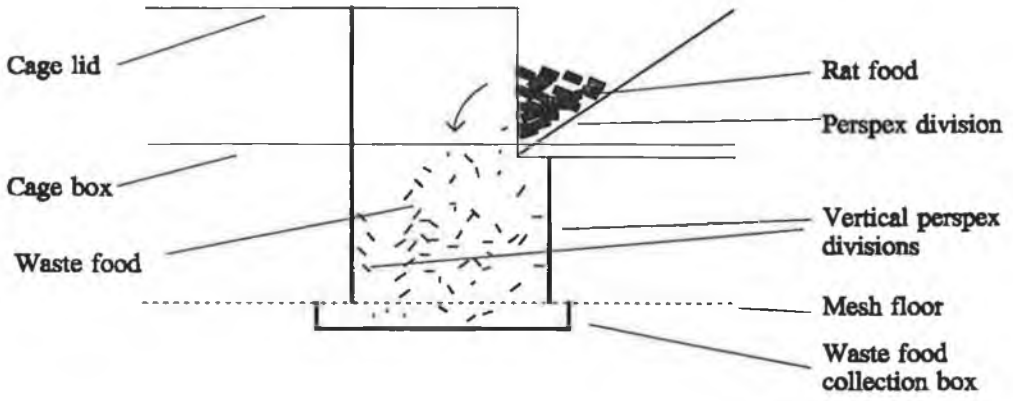


Figure 2.2 (b)



The treated food was given for 5 days of the week with normal untreated food being administered for the other two days of each week, to insure against possible nutritional deficiency due to the food treatment process, and allow measurement of normal feeding habits throughout the course of the experiment. This continued for 4 weeks with no period of normal food in the final week (ie 26 days total) as represented in the Figure 2.3.

The animals were killed by exsanguination on day 27 and drug levels were measured by the DCM extraction and HPLC methods as described in sections 2.9.1 - .4. and 2.10. respectively.

2.6.5. Toxicity assessment

During the absorption/distribution studies indicators of toxicity were measured for selected phenazines. After the initial settling in period, blood and clean urine samples were taken. The food and water consumption, behavioral characteristics, urinary parameters and individual animal weights were recorded regularly during the experiment. *Post mortem* tissue weights were also measured and compared between the groups.

2.6.5a. Haematological monitoring

1 ml blood samples were taken from the rats by tail bleeding, initially, and by cardiac puncture at termination, and clotting prevented by collection in potassium EDTA-coated tubes. Samples were immediately sent to Dr. Hugh Larkin in the Veterinary College of University College Dublin for routine analysis for the following parameters:- packed cell volume (PCV), haemoglobin concentration (HB), red blood cell count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), white blood cell count (WBC), protein content, neutrophil count, eosinophil count, lymphocyte count, monocyte count. Samples were also analysed for the enzymes Sorbitol Dehydrogenase (SDH), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) using clinical chemistry kits (Sigma Diagnostics).

2.6.5b. Urinary monitoring

Urinary samples were pooled and analysed with a combination test strip (Boehringer Mannheim) for the following indicators :- Nitrite, pH, protein, glucose, ketones, urobilinogen, bilirubin and blood (erythrocytes and haemoglobin).

Figure 2.3. A diagrammatic representation of the drugged food administration schedule for the rats on distribution studies.

2.7. PROTON-NMR ANALYSIS OF URINE

Urine samples taken before and after the drug distribution experiment, and from New Zealand rabbits being used to produce antibodies, were analysed for indicators of toxicity. This analysis was performed on a Brucker AC-400 Mhz NMR spectrometer with the help of Dr. Paraic James and Mr. Michael Burke in the dept. of Chemisty, D.C.U. Freshly collected urine samples were centrifuged at 13000 rpm on a Heraeus Biofuge 13 for 10 minutes and frozen, after which, 800µl aliquots were freeze dried in an eppendorf tube centrifuge connected to a freeze drier. These samples were reconstituted in 800µl of deuterated water (D_20) (Aldrich) and the pH adjusted to 7.0 with HCl/NaOH. The samples were analysed in the NMR machine using the presat water suppression programme to remove the strong ^1H_2O peak. As a positive toxic control urine was taken from a rat injected subcutaneously with 1 ml of carbon tetrachloride. Urinary results were compared with positive and negative controls to indicate areas of difference. Important peaks were identified by analysis of the purified compound in D_2O under the same conditions as the samples, and by comparison with published chemical shifts for urinary metabolites.

2.8. TREATMENT OF MURINE MALARIA WITH PHENAZINES

Selected phenazines were incorporated into mouse pellets at a concentration of 120 mg of drug per 1.2 kg (initial weight of food), with a food control prepared also, as described in section 2.6.4. for preparation of doped rat food. CD1 mice were housed in groups of six animals and given *ad libitum* access to drugged food and water for two weeks. A positive therapy group of mice given *ad libitum* access to the anti-malarial agent sulfasalazine, at a concentration of 30 mg/l in their drinking water, was concurrently dosed. On day 14 each animal was infected intraperitoneally with 250µl of mice blood with a 10 % *Plasmodium berghii*

parasitaemia (64 x 10^7 parasites/ml). Animals continued to receive their dosage regime until they became ill, when they were sacrificed, or, in the case of the sulfasalazine group, for a week longer, when the drug was removed, and the animals became susceptible to the infection.

2.9. DRUG EXTRACTION

2.9.1. Tissue samples

0.1 g of tissue was homogenised in a 2 ml mortar, with a motor-driven revolving teflon pestle (AGB), in 1 ml of ultrapure (U.P.) water (Millipore). The homogenate was poured into a 10 ml glass blood tube (AGB) previously coated with 2µg of an appropriate phenazine internal standard (I.S.). Remaining homogenate was washed from the homogeniser tube with 1 ml of 5 M sodium hydroxide (NaOH). 2 ml of dichloromethane (DCM) was added and each tube was mixed for 20 mins. on a blood tube mixer. Tubes were further mixed in a sonicating bath (Elma) for 5 mins. The tubes were centrifuged (Heraeus, Labofuge GL) at 4000 rpm for 15 mins. resulting in the bottom organic layer being covered by a solid disc of tissue debris in the aqueous layer. 1.1 ml of the organic layer was carefully removed to a glass autosampler vial (Chromachol) and allowed to evaporate off over 2 hrs. by heating at 40^{0}C on a heating block.

For spiked standards blank tissue was homogenised and the extraction tube was coated with I.S. and spiked amounts of drug.

2.9.2. Faecal samples

Faecal samples were collected from the metabolism cages in plastic scintillation tubes (Beckman) and dried overnight under vacuum. Each dried sample was powdered and mixed using a mortar and pestle. 0.1 g of powdered sample was then extracted as described above.

2.9.3. Fat tissue

0.1 g of sample was homogenised as per the tissue method (section 2.9.1.). After centrifugation, the aqueous layer was aspirated off and 1.5 ml of alcoholic sodium hydroxide (10 % (w/v) NaOH in ethanol) added to the DCM layer. The tubes were then heated in a heating block at 80 ^{0}C until bubbling stopped indicating complete evaporation of the DCM. 6 mls of cold U.P. water and 2 mls of DCM were then added. The tubes were mixed for 5 mins on the blood tube mixer and then centrifuged at 3,500 rpm for 10 mins. The aqueous soapy layer was

aspirated off and the DCM layer was washed twice more in the same manner. 1.1 mls of the DCM layer was removed and evaporated off in autosampler vials as described above.

2.9.4. Serum samples

1 ml of serum sample was added with 1 ml of NaOH and 2 ml of DCM to internal standard (I.S.)-coated blood tubes. Extraction was carried out as per the tissue extraction method (section 2.9.1.).

2.9.4a. Estimation of bound clofazimine fraction in serum.

1 ml of fresh serum sample was placed in centricon SR-3 centrifugal filters with a M.W. cut-off of 3,000 Da. The tubes were centrifuged in a Sorval SS-34 head at 7000 rpm for 2.5 hrs. The 100µl retained in the top (high m.w. fraction) and bottom 900µl (low m.w.) were placed in I.S.-coated blood tubes and the volumes brought up to 1 ml with U.P. water. Drug concentrations were measured using the extraction and HPLC method in section 2.9.4. and 2.10. respectively.

2.10. HPLC QUANTIFICATION OF PHENAZINES

After extraction, samples were reconstituted in 60µl of acidified tetrahydrofuran (THF) (60µl of acetic acid in 10 ml of THF). Fat samples were reconstituted in 120µl of acidic THF. Measurement was performed using a Beckman System Gold HPLC system comprising 507 autosampler, 126 pump, 166 U.V. detector, with data collected and analysed by computer using version 8 of the Gold software. The peak height ratio of analyte to I.S was used to quantify drug concentrations from spiked standards. The mobile phase consisted of 594 ml of U.P. water, 6 ml of acetic acid, 400 ml of HPLC grade THF and 0.471 g of hexane sulfonic acid (Romil chemicals). This was mixed and degassed in a sonicating bath for 5 mins. The system operated by pumping the mobile phase through the column at 1.5 ml per min. at ambient lab. temperature (18-21 ^{0}C) with the column eluent recycling into the mobile phase reservoir as part of a sealed recycling circuit of mobile phase flow. Separation occurred on a Phenomenex Bondclone C_{18} reversed phase column with dimensions of 300 x 3.9 mm and a particle diameter of 10 um with a C_{18} µBondapak cartridge precolumn. Absorbance of the column eluent was monitored at 285 nm. This wavelength was chosen because it was an average of the wavelengths at which all the agents gave their strongest absorbance.

To determine the intra-day precision and accuracy, blank tissue, fat and serum samples were spiked with B663 and I.S. (B4100) across the linear range. Five sets of samples spiked with identical concentrations of drug were prepared using a single group of standards and quantified using calibration standards. The inter-day precision and accuracy for these samples was determined by preparing and analysing spiked standards prepared on five different days. Inter- and intra-day variability was also measured in the same way for B4090 using B663 as the I.S.

2.10.1. PDA-HPLC analysis of phenazines

The same method of analysis was employed as described above, except a 168 System Gold PDA detector module was substituted for the U.V. detector.

2.11 CHROMIUM MEASUREMENT IN FAECES

2.00 g of the freeze-dried and powdered faecal samples from the absorption studies were completely oxidised at 600 ^{0}C in a high temperature oven overnight in ceramic crucibles. The ashed samples were then reweighed and transferred 10 ml blood tubes. The chromium content of the samples was then measured spectrophotometrically by a modification of the method of Czubayko *et al.*, (1977) 2 ml of conc. ortho-phosphoric acid and 3.5 ml of 4.5 % (w/v) aqueous solution of potassium bromate were added to each tube and standards of faecal ash and known masses of chromium (III) oxide powder. The tubes were heated to boiling in a heating mantle until no more bromine gas was being evolved (paper in vapour remained white) indicating complete oxidation of the sample to chromium (IV). The tubes were then centrifuged at 3,500 rpm for 10 mins to precipitate ash. Suitable dilutions of each supernatant were made in distilled water and the absorbance of all tubes was measured in quartz cuvettes at 346 nm in a spectrophotometer (Shimatzu).

2.12. CONJUGATE PRODUCTION

2.12.1 Glutaraldehyde conjugations

2.12.1a.One step conjugation

B3976 (phenylalanine derivative) (0.5 mg/ml) was mixed with solutions of BSA (20 mg/ml) or THYR (2.5 mg/ml) in various molar ratios in U.P. water at 4 ^{0}C. 0 - 1 ml of 1 % (v/v) aqueous glutaraldehyde was added to the mixtures and the solutions were reacted for 60 mins. Conjugates were dialysed overnight against U.P. water to remove unconjugated compound.

2.12.1b.Two step conjugation

10 ml of BSA solution (10 mg/ml) or THYR (2.5 mg/ml) were dialysed against 200 ml of 0.2 % (v/v) aqueous glutaraldehyde for 16 hrs at 4 ^{0}C. The activated carrier proteins were dialysed against U.P. water to remove free glutaraldehyde. The activated proteins were dialysed against 100 ml of 0.2 mg/ml aqueous B3976 for 16 hrs at 4 ^{0}C. 0.1 ml of 0.2 M lysine was added to each bag and incubated for 2 hrs. The bags were dialysed against U.P. water to remove free drug and lysine.

2.12.1c.Preservation and storage of glutaraldehyde conjugates

The dialysed samples were frozen to the side of a round bottomed flask using a freezer bath (Heto) and -80 ^{0}C freezer. Samples were then freeze dried on a Heto freeze drier overnight. Samples were kept frozen until use.

2.13. CARBODIIMIDE CONJUGATION

100 mg of Keyhole Limpet Haemocyanin (Calbiochem) solution (1.46 ml) was dialysed overnight against 50 mM phosphate buffer to remove the glycerol preservative. This solution was removed and made up to 4 ml with phosphate buffer. 9.77 mg of B3955 (glycine derivative), 3.33 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) and 6.67 mg of N-hydroxysuccinimide (NHS) were mixed together in 2 ml of dimethyl formamide (DMF) for a few seconds before the KLH solution was added. The reaction was mixed at 25 ^{0}C for 7 hrs followed by overnight dialysis against P.B.S., (0.15 M), pH 7.4, with 1 mg/ml lysine added. The

conjugate was concentrated by reverse dialysis against 6 KDa molecular weight polyethylene glycol (PEG). This conjugate was freeze dried and kept frozen until used as described in section 2.12.1c.

2.14. CHARACTERISATION OF GLUTARALDEHYDE CONJUGATES

2.14.1. SDS-PAGE analysis of conjugates

The freshly prepared samples were adjusted to a concentration of 1 mg/ml and diluted 1 : 1 with sample buffer (2 % (w/v) SDS; 0.08 M Tris/HCl, pH 6.8; 10 % (w/v) Coomassie Brilliant Blue). The samples were boiled for two minutes and 25µl aliquots applied to the wells of a 5 - 20 % polyacrylamide gel (Atto). The gels were run at a constant current of 20 mA per gel for approximately 2 hours. The electrode buffer for electrophoresis consisted of 0.025 M Tris, 0.192 M glycine and 0.1 % (w/v) SDS at a pH of 8.3. The gels were stained for 30 mins in 0.5 % (w/v) Coomassie Brilliant Blue in acetic acid : water :methanol (1:10:8, v/v/v) and destained overnight in the same solvent to visualise the protein bands.

2.14.2. TLC analysis of conjugates

Conjugate samples and controls were applied to silica TLC plates with a capillary tube and the spots developed with an 80 : 20 (v/v) methanol : water solvent. When the drug spot had migrated 2/3 of the plate length, the plates were removed and the protein present was stained in an iodine chamber.

2.14.3. HPLC analysis of conjugates

Protein conjugate samples were characterised using the Beckman system Gold HPLC system pump and detector as described in section 2.10. The proteins were separated using a Protein Pak (Millipore) SW 300 column with a particle diameter of 10 µm. The mobile phase was 0.1 M phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min with U.V. detection at 280 nm. 20 µl of sample was manually injected into the system.

2.14.4. PDA-HPLC analysis of conjugates

BSA, THYR and conjugates were analysed with the same column and mobile phase as described in the previous section. Peak characterisation was carried out using a Waters 990

pump, 990 PDA detector, 990 plotter and 990 30 chromatogram measure APC 3 computer.

2.14.5. Size exclusion chromatography of conjugates

Sephadex G-25 was swollen overnight in U.P. water. A 200µl sample of the conjugate was applied and washed through with water at a flow rate of 1 ml/min. A coloured fraction eluted in the void volume fraction and the amount of B3955 was estimated using a standard curve of dilutions of the drug in water. The concentration of protein present in this fraction was measured using the micro B.C.A. protein assay (section 2.17.3.) with standards of BSA. Calculation of the molar masses of protein and drug present gave an approximation of the conjugation ratio.

2.15. CHARACTERISATION OF EDC CONJUGATE

Sephadex G-100 (Pharmacia) powder was swollen overnight in P.B.S., (0.15 M), pH 7.4. The gel was poured into a 10 ml column and allowed to settle. A 200µl fraction of the P.B.S.-dialysed KLH conjugate was applied to the column and fractions collected with absorbance monitored at 488 nm. A coloured fraction eluted in the 10 - 12.5 ml fraction and the amount of B3955 was estimated using a standard curve of dilutions of the drug in P.B.S., (0.15 M), pH 7.4. The concentration of KLH present in this fraction was measured using the micro B.C.A. protein assay (section 2.17.3.) with standards of KLH. Calculation of the molar masses of protein and drug present gave an approximation of the conjugation ratio.

2.16. PRODUCTION OF ANTI-PHENAZINE ANTIBODY

2.16.1 Immunisation protocol

A 0.5 mg/ml solution of B3976-THYR conjugate was made by homogenising 1.5 mg of conjugate in 1.5 ml of P.B.S., (0.15 M), pH 7.4, and 1.5 ml of Freunds adjuvant in a mortar and pestle type homogeniser. 1 ml of this suspension was subcutaneously injected at several sites along the back of two female New Zealand rabbits. Control sera was taken before immunisation. The first immunisation was made with complete adjuvant and all subsequent immunisations were using incomplete adjuvant. Animals were boosted on day 28 and bled 11 days later from the marginal vein in the ear. This cycle was repeated until the antibody titre was sufficient.

2.16.2. Screening for antibody production

2.16.2a.Direct assay using one step glutaraldehyde immobilisation

Serum from immunised animals was screened for the presence of antibodies by enzyme-linked immunosorbent assay (ELISA). 25µl of 2 % (w/v) BSA in P.B.S. (0.15 M), pH 7.4, and 25µl of a 0.1 % (v/v) solution of gultaraldehyde in P.B.S. (0.15 M), pH 7.4, was added to each well of an Maxisorb ELISA plate (NUNC). The plate was incubated overnight at 37 ^{0}C. 50µl of 0.25 mg/ml B3955 was added and allowed to react for 2 hrs. at 37 ^{0}C. The plate was washed with U.P. water and blocked with 2 % (w/v) BSA for 1.5 hrs. After 4 washes with 0.15 M PBS, pH 7.4, containing 0.05 % (v/v) Tween-20, 50µl dilutions of test serum 2 % (w/v) BSA PBS were added for 1.5 hrs at 37 ^{0}C. After 3 washes with PBS-Tween and one PBS wash, 50µl of a 1 in 15,000 dilution of horseradish-peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin in blocking solution was added for 1 hr at 37 ^{0}C. The plate was washed 4 times in PBS-Tween. 50µl of peroxidase substrate (10 mg of *o*-phenylenediamine (OPD) dissolved in 25 ml of 0.15 M citrate buffer, pH 5.0, and 5µl of 30 % (v/v) H_2O_2) was added to each well and incubated at room temperature (R.T.) until colour developed. 50µl of 1 M H_2SO_4 was added to stop the reaction and the absorbance of each well was measured at 492 nm using a TiterTek Twinreader plus plate reader.

2.16.2b.Two step glutaraldehyde immobilisation

25µl of 2 % (w/v) BSA and 25µl of 0.2 % (v/v) glutaraldehyde was added to the ELISA wells and incubated overnight at 4 ^{0}C. The wells were washed and 50µl of phenazine added. The plate was incubated at 37 ^{0}C for 2 hrs. The plate was washed and antibody binding measured as described in the previous section.

2.16.2c.Competitive assay

The plate was coated with 250µl of 1 % (w/v) BSA for 5 hrs at 37 ^{0}C. Excess BSA was removed and aspirated out. 100µl of 0.2 % (v/v) glutaraldehyde was added and the plate incubated for 2 hrs at 37 ^{0}C. The plate was washed with water, 100µl of B3976 added and the plate was incubated at RT overnight. The plate was washed four times and 100µl of 1 % (w/v) lysine added for 1 hr. at 37 ^{0}C. The lysine was removed by aspiration and the plate blocked with 100µl of 1 % (w/v) BSA. The plate was washed four times and 50µl of a two times B3976 concentration added. 50µl of two times rabbit antibody concentration diluted in P.B.S. (0.15 M),

pH 7.4, was added and the plate incubated at 37 ^{0}C for 1 hr. The plate was washed and antibody binding measured as described in section 2.16.2a.

2.16.3. Measurement of amount of bovine serum albumin bound to ELISA plate

50µl, 25µl of 2 % (w/v) BSA in P.B.S. (0.15 M), pH 7.4, 25ul of 2 % (w/v) BSA and 0.2 % (v/v) of glutaraldehyde or 25µl of 0.25µg/ml glycine were added to the ELISA plate wells and the plate was incubated overnight at 4 ^{0}C. The plate was washed as described above and dried for 1 hr. at 60 ^{0}C. 10µl of water was added to each dry well and the amount of protein present determined using the micro BCA protein assay (section 2.17.3.).

2.16.4. Experiments using Amine binding plate

100µl aliquots of B3955 and B3976 in the range 0 - 1 mg/ml in water were added to wells of a Costar amine binding plate and left overnight at 4 ^{0}C. Concentrations of B3832 were made by diluting a stock concentration of 1 mg/ml B3832 in DMF with water and only concentrations of 0.2 mg/ml or less could be used due to the effect of DMF on the plastic. The wells were washed 4 times and blocked with 2 % (w/v) BSA for 1 hr. The blocking solution was washed out with four washes of P.B.S.-Tween and a 1 in 2000 dilution of rabbit serum added to the wells. After a one hour incubation the plate was washed and the antibody binding quantified (section 2.16.2a.).

2.16.5. EDC/NHS immobilisation of phenazine to ELISA plate

50µl of 2 % (w/v) BSA was added to the wells of a maxisorb ELISA plate and incubated overnight at 4 ^{0}C. The wells were washed and 10µl of 0.1 mg/ml of B3955 in U.P. water, 10µl of 0.5 mg/ml glycine, 35µl of 5 mg/ml NHS, 35µl of 5 mg/ml EDC and 10µl of 4.5µg/ml Na_2HPO_4 were added. The plate was incubated overnight at 4 ^{0}C and washed. 100µl of 0.5 mg/ml glycine was added to each well to cap any free reactive groups and the plate was incubated for an hour at 37 ^{0}C. The plate was then blocked with 200µl of 2 % (w/v) BSA for a further hour. The plate was washed and 100µl of the test anti-serum, diluted in 1 % (w/v) BSA in 0.02 % (v/v) P.B.S.-Tween added. For competitive assays 75µl of antibody dilution and 25µl of B3955 in P.B.S.-Tween was added. After an overnight incubation at 4 ^{0}C, the amount of antibody binding was assessed as per section 2.16.2a.

2.16.6. Experiments using Nunc covalink plate

50µl of a 1 mg/ml aqueous solution of bis(sulfosuccinimidyl) suberate (BS^3) was added to the wells of a Nunc Covalink ELISA plate. After incubation at R.T. for four hours, the plate was washed with water and 50µl of drug dilution was added. The plate was incubated at 4 ^{0}C overnight, washed and blocked with 2 % (w/v) BSA. Dilutions of antibody were added and binding assessed as described in section 2.16.2a.

2.16.7. Experiments using silica coated plate

30µl of 0.05 % (w/v) polyisobutylmethacrylate in cyclohexane was added to the wells of a maxisorb plate. Each well was filled with silica powder and the cyclohexane evaporated off at 56 ^{0}C. The excess silica was washed out with water and concentrations of rimino-phenazines from 0.002 to 1 mg/ml added in water or methanol as appropriate. Direct and competitive assays were performed in the conventional manner (sections 2.16.2a and 2.16.2c.).

2.17. ANTIBODY PURIFICATION

Frozen rabbit serum was thawed and filtered using 100 KDa molecular weight cut-off Centriplus centrifugal filtration tubes (Amicon) at 4000 r.p.m for 3 hrs. The retentate was washed twice by adding 5 ml of P.B.S. (0.15 M), pH 7.4, to it and centrifuging at the same speed for one hour.

2.17.1 Affinity chromatography of antibody

327 mg of B3832 was dissolved in 10 ml of DMF with 100 mg of biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Biotin-NHS) and 3 ml of 0.1 M borate buffer, pH 8.2, added. The reaction was mixed overnight at R.T. and characterised using T.L.C. and PDA-HPLC. Several solvent mixes were employed for separation of the resultant compounds including an 8 : 1 DCM/methanol mix and a mixture of butanol/acetic acid/water (93 : 5 : 5 (v/v/v)). However, even when coupled with a biotin visualisation agent, D.A.C.A, the results were not clear due to the similarity in colour between the phenazine and biotin-D.A.C.A product. Biotinilated compounds were visualised by the method of McCormick and Roth, (1980) using 0.2 % (w/v) *p*-dimethylaminocinnamaldehyde (D.A.C.A) in 2 % (v/v) sulfuric acid - ethanol sprayed onto the plates to produce a pink product indicating the presence of the biotin group. Samples were also analysed by PDA-HPLC using the method described for phenazine analysis

(section 2.10.1.).

35 mg of avidin was dissolved in 17.5 ml of a mix of 0.2 M carbonate buffer and 0.6 sodium citrate (1.68 g of sodium bicarbonate and 17.65 g of sodium citrate dissolved in 100 ml of water with pH adjusted to 9.0 with NaOH). This was added to 1.25 g of Emphase Biosupport Medium AB 1 (Pierce) with 7.5 ml of buffer used to wash out all the remaining protein. The protein and beads were mixed overnight. The B3832-Biotin-NHS reaction was then added to the swelled beads and allowed to mix for 2 hrs. The mixture was poured into a 10 ml column and unbound compounds removed by washing with water and DMF. Free reactive groups were quenched using a 1% (w/v) lysine solution. The column was stored in a 0.05 % (w/v) aqueous azide solution.

Before use the column was blocked with 2 % (w/v) BSA in P.B.S. (0.15 M), pH 7.4,. This was washed out with P.B.S. (0.15 M), pH 7.4, and the partly purified antibody solution was applied. The antibody solution was washed into the matrix with 4 ml of PBS and allowed to equilibrate and bind for 10 mins. Non-specific protein was eluted at 1 ml/min with PBS until the 280 nm absorbance of the eluent returned to baseline. 4 ml of 0.1 M glycine, pH 2.5, with 10 % (v/v) dioxane was added to the column for 15 mins. Eluted 2 ml fractions were then collected in tubes containing 110µl of 1 M 2-amino-2-(hydroxymethyl)1,3-propanediol (Tris), pH 10.9, to return the pH of the fractions to approximately neutral. Fractions from all stages of purification were collected and assayed for protein content using the BCA assay (section 2.17.3.). The samples were also analysed by HPLC using a Biosep SEC-4000 column and the same equipment and conditions described in section 2.14.3.

2.17.2. BIAcore™ analysis of antibody

Antibody samples were analysed using BIAcore technology. The machine and the immobilisation chemicals were supplied by Pharmacia. B3955 was immobilised to a sensor chip surface by the following procedure. 35µl of a 1 : 1 mix of 75 mg/ml EDC and 11.5 mg/ml NHS was applied to the chip surface at a flow rate of 5µl/min. 40µl of B3955 in 0.01 M sodium acetate, pH 4.5, was applied at 2µl/min and remaining reactive groups on the chip capped by 35µl of 1 M ethanolamine at 5µl/min. 20µl of antibody dilution at 0.2 mg/ml was added to the B3955 surface at 10µl/min.

2.17.3. Bicinchoninic acid (B.C.A.) protein assay

This assay is based on the reduction of Cu^{++} to Cu^{+} by proteins under alkaline

conditions, the Cu^{+} produced being quantitated by complexation with BCA which results in a coloured product. The BCA reagents (Pierce) were added together 1 part reagent B to 50 parts reagent A. 200µl of this solution was added to 10µl of sample or standard solutions in microtitre wells. The colour was developed at 37 ^{0}C for 30 mins. with absorbance being quantified at 562 nm using a plate reader . The protein concentration of samples was determined from standard curves plotted from the absorbance of concurrently run standards of the same protein.

2.18. CHEMICAL CHARACTERISATION OF PHENAZINES

2.18.1. Analysis by I.R. spectroscopy

Phenazine samples were mixed with desiccated potassium bromide (KBr) with a mortar and pestle. Part of this homogenous powder was transferred to a disc press. After 5 mins at 10 tons pressure, the resultant glass disc was carefully removed and placed in the slot of a Nicolet I.R. spectrometer which had been appropriately blanked. Spectral peaks were identified by comparison with reference spectra (Socrates, 1980; Pouchert, 1981; Kin-Vien, 1991).

2.18.2. Analysis by N.M.R. spectroscopy

N.M.R. analysis was performed on a Brucker 400 MHz N.M.R. spectrometer by Dr. Paraic James and Mr. Michael Burke in the Chemistry Dept. of D.C.U. Samples were dissolved deuterated chloroform or DMSO to saturation and transferred to glass N.M.R. tubes (Wilmad). After a sufficient number of scans the resulting spectra were recorded and analysed by comparison with reference spectra (Pouchert and Campbell, 1974).

2.18.3. Purification by Silica and alumina chromatography

Kieselgel S Silica (0.032-0.063 mm particle size) and neutral alumina (Brockmann Activity 1) columns were prepared by mixing the powders with the mobile phase used for the particular chromatographic separation. This slurry was transferred to a 20 cm glass column and allowed to drain until the liquid level was just above the gel level. Compounds to be separated were dissolved in the mobile phase and slowly applied to the top of the gel using a long glass pasteur pipette. Columns were eluted at atmospheric pressure and fractions were collected. Initially fractions were analysed by T.L.C. Fractions of interest were pooled and the solvent extracted on a rotary evaporator. Selected samples were further analysed by HPLC (section 2.10.) and N.M.R. (section 2.18.2.).

2.18.4. Analysis by thin layer chromatography (T.L.C.)

Samples to be tested were applied to silica coated aluminium plates in a line 10 mm from the end using capillary tubes. The plates were placed in mobile phase to a depth of 5 mm (below the sample line). The solvent was allowed to migrate to 4/5 of the length of the plate where they were removed and allowed to dry. Phenazines were visualised by eye and the retention factor (R_f) was measured.

2.19. PRODUCTION OF CYCLODEXTRIN-CLOFAZIMINE COMPLEXES

Clofazimine-cyclodextrin complexes were prepared by Ms. Bernie Brady in the Chemistry Dept. of University College Dublin. 5.5 g of clofazimine was dissolved in sufficient acetone to allow mixing with an aqueous solution of cyclodextrin, 89.67 g of β-cyclodextrin or 90.00 g of hydroxypropyl-β-cyclodextrin (Aldrich). The mixtures were stirred for 48 hrs. with acetone being removed by vacuum. The mixture was then freeze-dried and powdered.

2.19.1. Intravenous (I.V.) administration of clofazimine-cyclodextrin complex

The central ear artery of a New Zealand White rabbit was cannulated and a 3 ml control blood sample taken. The I.V. dose was prepared by adding 1 g of hydroxypropyl-β-cyclodextrin complex to 10 ml of 0.9 % (w/v) saline and mixing. The animal was given a bolus I.V. dose into the ear vein of 5 ml of the solution, which was filtered with a 0.22 μm filter fitted between the syringe and the infusion needle. A 1 ml sample of the filtered solution was also removed for measurement of the clofazimine content by HPLC. 3 ml blood samples were taken immediately and after time periods of 5, 15 and 30 mins, and 1, 2, 4, and 8 hours. Two further samples were taken at 24 and 48 hrs after the dose. These samples were then analysed for the presence of clofazimine using the extraction and HPLC method (section 2.9.4. and 2.10.).

A 15 ml bolus dose of saturated clofazimine-hydroxypropyl-β-cyclodextrin was administered I.V. to the same rabbit to test if the complex was being sequestered within tissues in the body. The animal was sacrificed and tissue samples were taken for analysis by the extraction and HPLC method (section 2.9.1. and 2.10.).

CHAPTER 3

CHEMICAL STUDIES ON RIMINO-PHENAZINES

3.1. INTRODUCTION

This chapter is concerned with investigations undertaken to study the purity, some of the chemical properties and the development of an accurate and precise method for the quantification of phenazine agents used in this project. A new liquid extraction method and an improved HPLC measurement procedure were developed and validated for these compounds. The HPLC method, coupled with TLC, IR and NMR spectroscopy, was also used to investigate the purity of selected phenazines.

3.2. NEED FOR SUBSTITUTED PHENAZINES AND METHODS FOR THEIR ANALYSIS

The anti-leprosy agent, clofazimine, was produced as a result of a directed structure-activity investigation to produce a more active anti-mycobacterial agent from a non-substituted phenazine (B283). B283 was the first phenazine to show signs of anti-mycobacterial activity, as outlined in section 1.2. of the introduction. Initially only a limited number of substituted phenazines were tested as part of the development programme. Clofazimine (B663) was the most effective anti-tubercular (*in vitro*) and anti-leprosy (*in vitro* and *in vivo)* agent of this chemical series (Barry, 1957) and became an established drug for the treatment of leprosy. Although efficacious and generally clinically safe (Vischer, 1969; Stenger, 1970; Hastings, 1976; Bulakh, 1983; Peters, 1983), use of clofazimine has a number of shortcomings (Hastings, 1976; Moore, 1983) (see section 1.5.5. in introduction), which can be generalised as failings in activity and pharmacokinetics. To try and overcome these problems, many phenazines with various different substituents have been synthesised by Dr. Sean O'Sullivan in the Chemistry Dept. of University College Dublin (O'Sullivan *et al.*, 1988, 1990 and 1992). Examination of the properties of the various substituted derivatives synthesised has produced the outline for an approximate structure activity relationship. The activity of these agents *in vitro* has been established elsewhere (Franzblau and O'Sullivan, 1988; O'Sullivan *et al.*, 1988 and 1992; Byrne *et al.*, 1989; Franzblau, 1989; Savage and O'Sullivan, 1989; Zeis *et al.*, 1990), but measuring these agents in biological samples for pharmacokinetic evaluation has been problematic due to endogenous interfering compounds and the physico-chemical properties of phenazines.

3.3. EXISTING PHENAZINE EXTRACTION METHODS

3.3.1. Extraction difficulties

To allow accurate quantification by methods such HPLC or spectroscopy, one must isolate phenazines from the interfering substances present in the body. Extraction of phenazines poses particular difficulties due to their inherent physicochemical characteristics.

3.3.2. Properties relevant to extraction

The phenazine ring is inherently hydrophobic due to its aromatic carbon backbone. The various substituent groups present also contribute to the strong organic solubility of all these agents. These include groups such as the phenyl, anilino, or chlorphenyl and chloroanilino groups, present on all the agents produced in this programme, and also many of the various carbon chain rimino- substituents. For example, clofazimine, is a very hydrophobic molecule with a calculated log P value of approximately 7.48(octan-1-ol/water) (Morrison and Marley, 1976). As a result, rimino-phenazines without any hydrophilic substituents have extremely low aqueous solubilites. The presence of ionisable atoms especially nitrogen in all of the rimino-phenazines introduces the added complication of the net charge on the phenazine molecule being dependent on the pH of the environment. These nitrogen atoms are generally alkaline. A pKa value of 8.35 +/- 0.09 has been reported for clofazimine (Morrison and Marley, 1976), although the exact value is a matter of controversy since the compound's aqueous insolubility makes calculation difficult. Hence, in neutral pH environments, rimino-phenazines are both strongly hydrophobic and polarised. As the pH decreases the nitrogens in the phenazine heterocycle can become ionised increasing the aqueous solubility until, in strong acid solutions, all phenazines become fully soluble. In compounds such as B3955, B3954 and B3976 the aqueous insolubility at neutral pH is partly overcome by rimino- substitution with water soluble amino acids which greatly increase net water solubility of the whole molecule.

As a result of the polarised hydrophobic properties of rimino-phenazines at physiological pH, these agents are found tightly bound to lipids and proteins or in many cases, crystallised within cells. Therefore, any extraction system must be able to remove rimino-phenazines from these pockets into free solution. Treatment with strong acid will solubilise all phenazines, but is not applicable to the whole range of compounds due to the acid lability of certain derivatives in light (Barry *et al.*, 1960). Consequently, acid solubilisation is not suitable as a general method for extracting phenazines.

3.3.3. Existing liquid extraction methods

Most of the phenazines developed initially were not significantly acid labile and hence acidic and organic solvents could be used to extract these agents. Barry *et al.*, (1960) extracted rimino-phenazines from biological matrices using benzene. Although tissue samples were homogenised to allow complete equilibration with benzene, 25 % acetic acid was also needed to quantitatively extract these agents from proteins present. The benzene layer was removed and phenazines re-extracted, using 10 M hydrochloric acid, for spectrophotometric quantification. This method was modified by others (Byrne *et al.*, 1989; O'Sullivan *et al.*,1990) who used chloroform (as the organic solvent) and 50 % phosphoric acid. Extraction via this method suffers from a number of drawbacks including :- the need for large volumes of a toxic chemical, benzene or chloroform, in the extraction, co-extraction of interfering contaminants, suspected to be porphyrins, variability in the final drug estimates, time consumption, due to the need for several transfers of extraction liquor, and variability due to the light-sensitivity of certain of the tested phenazines in acidic solution (Barry, 1960). The neutralisation of acetic acid in the mother homogenate liquor with 40 % (w/v) sodium hydroxide was also necessary for the more basic phenazines due their insolubility in benzene - acetic acid mixes. In addition, this method is also only suitable for later quantification by spectroscopy, since the drug is dissolved in strong acid, and hence restricts the limit of quantification.

Other liquid-based extraction methods have been developed for extraction of clofazimine from plasma. Gidoh and Tsutumi, (1981) redissolved clofazimine from evaporates of serum using ammonium sulfate and a mix of chloroform/DMF. Peters *et al.*, (1982) extracted clofazimine from plasma by mixing it with phosphate-citrate buffer, pH 6.0, and chloroform - methanol (4:1, v/v). The clofazimine present was further extracted by removing the organic layer, evaporating it under nitrogen, and extracting the resulting solution by mixing in 0.0425 M phosphoric acid in 81 % (v/v) methanol and hexane. Hence the clofazimine extracted into the methanol - phosphoric acid. Dill *et al.*, (1970) developed a method which could extract clofazimine from plasma and tissue homogenate. The sample was mixed with a degradative enzyme, "maxatase" (Pfizer) in 2 % (w/v) borax and incubated for 30 mins. This was extracted with n-heptane followed by extraction of the heptane with 1 M citric acid for quantification by further treatment to produce a fluorescent derivative.

3.3.4. Solid phase extraction

Liquid extraction of hydrophobic compounds involves using large volumes of organic solvents. However, new extraction systems have been developed which have a solid organic phase, which reduces this problem. A method of extracting clofazimine from plasma samples into such a solid phase was developed by Krishnan and Abraham, (1992). For the first time this allowed the inclusion of an internal standard (I.S.) for more accurate and precise analysis. The cyano solid phase extraction (SPE) columns used were conditioned by treating sequentially with methanol, water, and 0.1 M phosphate buffer, pH 6.0. Plasma samples, diluted in 0.1 M phosphate buffer, were applied and allowed to percolate through the columns. After drying and addition of I.S., both compounds were eluted with a solution of THF, acetonitrile and methanol in the ratios 2:2:1 (v/v/v) containing 2.5 mM hexane sulfonic acid. The eluent was evaporated to dryness under a stream of nitrogen gas and reconstituted for HPLC analysis.

3.3.5. Shortcomings of existing methods

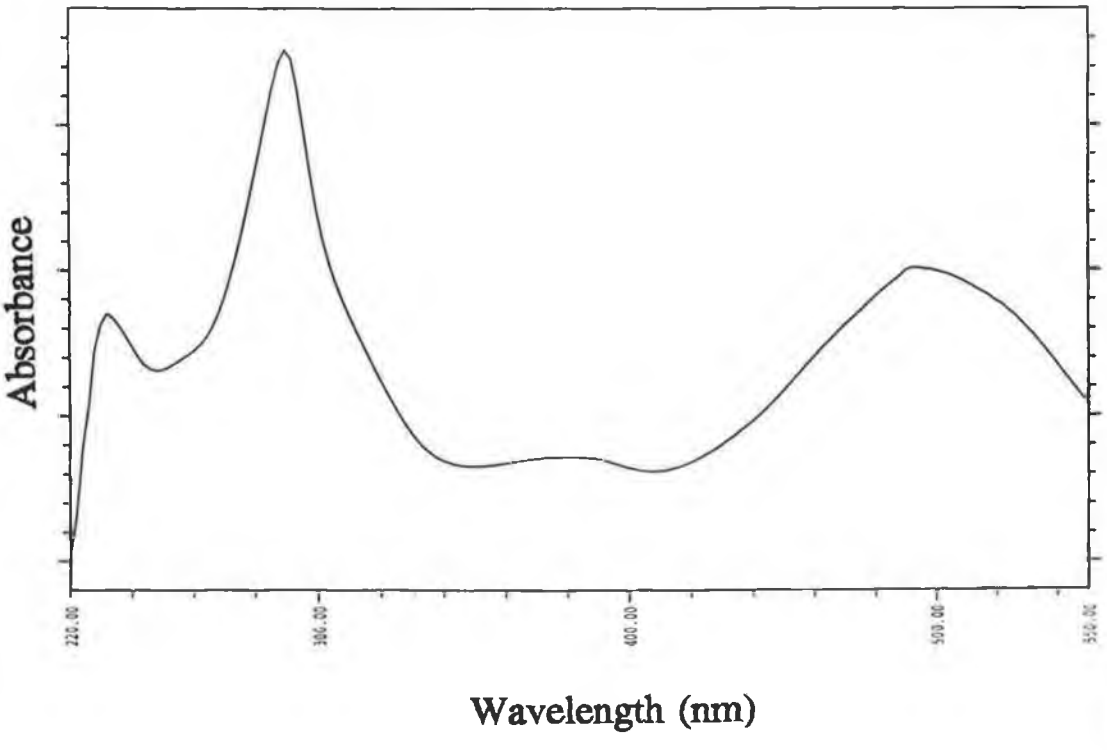
All existing extraction methods have a number of drawbacks which limit their use in the analysis of phenazines. In short, many methods use large volumes of toxic solvents, are limited in the range of rimino-phenazine they can extract, are only suitable for small numbers of samples and are not readily automated for routine analysis. The use of large volumes of organic solvents also tends to magnify errors in compound measurement.

3.4. METHODS FOR THE QUANTIFICATION OF PHENAZINES

3.4.1. Spectrophotometric quantification

Once extracted, the strong U.V. and visible absorbances of phenazines can be readily used to quantify these compounds spectrophotometrically (see Figure 3.1. for a typical spectrum). The spectrum of rimino-phenazines is dependent both on the chemical environment (Levy and Randall, (1970) especially pH, and the exact type and position of chemical substituents, but, in general there is a strong extinction coefficient at about 285 nm with a lesser absorbance around 490 nm (very variable) giving rise to the visual colouration of these agents. This visible absorbance was used by Barry *et al.*, (1960) to quantify phenazines from 5 to 0.2 µg/ml. This method was also used by Mansfield *et al.*, (1974) and in practice gave a maximum limit of detection of 100 µg/g of tissue.

Figure 3.1. The spectrum of clofazimine (p) in the HPLC mobile phase. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min. The spectrum was taken using a PDA detector. Absorbance maxima are seen at 290 and 492 nm with an absorbance ratio of 1.97 between the UV and visible peak.



3.4.2. Fluorescent quantification

Dill *et al.*, (1970) used a fluorometric method to quantify clofazimine with a similar limit of detection. This method converted clofazimine to a fluorescent product by adding 3 % (w/v) titanous chloride and 6 N sulfuric acid to a citric acid extract of the drug with heating to 100 ^{0}C for 10 mins. The resulting product was extracted with 2-ethyl hexanol and the fluorescent emission measured at 366 nm.

3.5. HPLC AND TLC METHODS OF QUANTIFICATION

The extracted solvent generally contains interfering impurities from the biological sample. Therefore, measurement systems need to separate these substances from rimino-phenazines to give more accurate and sensitive results. To date, this has been accomplished using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

3.5.1. Principle of reversed-phase HPLC

Normal-phase HPLC consists of a high-pressure pump capable of accurately pumping volumes of organic solvent at high pressure. The mobile phase is pumped through an injection system which introduces the sample to be analysed into the flow stream without interrupting the flow. The sample is then separated over a polar column generally consisting of silica or alumina with some form of detector system to quantify the sample components as they elute from the column. The mobile phase typically consists of a non-polar organic solvent with polarity and solubility modifiers flowing over a stationary phase of a polar matrix, usually silica. The separation of the sample components is a result of the different polarities of each component in the mixture. However, in reverse phase (RP) HPLC the situation is reversed (hence the name) and the stationary phase is hydrophobic, due to chemical derivatisation of the silica typically with an 18 carbon hydrophobic chain. The mobile phase is polar and generally water based with an organic modifier to fully solvate the stationary phase. Compounds added to the column elute on the basis of their net hydrophobicity. Poorly lipophilic, polar compounds elute first due to their small affinity for the stationary phase, whereas more hydrophobic agents take longer to elute.

3.5.2. Principle of TLC

Basic TLC methodology involves depositing a small spot of the sample of interest onto a plate of glass or aluminium coated with an adsorbent, usually consisting of silica or alumina. The spot is placed at a point so that when the plate is placed into a tank of a solvent it is just above the solvent level. As the solvent travels up the adsorbent it separates out the various components present in the sample which can be visualised, usually visually or under U.V. light, as discrete spots. This methodology is quick, simple and cheap as a guide to purity or the progress of a reaction, and can give useful information for column chromatography or HPLC.

Compounds separate as they travel with the solvent front on the basis of their polarity and their relative affinity for the solvent or adsorbent. Adsorbents are generally polar (although non-polar adsorbents exist) and silica is most commonly used. If a compound is not particularly polar it will be moved up the silica by a relatively non-polar solvent such as hexane due to a low affinity for the adsorbent. More polar substances will tend to remain at the point of application and will need a more polar solvent such as methanol to overcome the charge attraction and move up the plate with the solvent front. A mixture of solvents is normally employed which optimally separate the spots of interest from each other.

3.5.3.HPLC and TLC based methods for quantitation of clofazimine

Lanyi and Dubois, (1982) developed a sensitive TLC method with quantitation of serum clofazimine by densitometry. Serum clofazimine was extracted into toluene and applied to HPTLC silica gel 60 plates predeveloped in 1:1 chloroform - methanol. The plates were developed with a 50:50:4 toluene - acetic acid - water mix. Densitometric scanning with absorption at 545 nm allowed quantitation down to a limit of 5 ng/ml.

Three other methods based on HPLC have also been reported for the analysis of serum or plasma levels of clofazimine, all based on reversed-phase C_{18} columns. Peters *et al.*, (1982) used an ultrasphere-octyl column at 40 ^{0}C with a mobile phase of 0.0425 M phosphoric acid in 81 % methanol, pH 2.4, flowing at 1.5 ml/min, to analyse plasma clofazimine extracted into the same solvent mix as the mobile phase. Clofazimine was quantified by integration of the peak area produced by monitoring the column eluent at 285 nm. Gidoh and Tsutsumi, (1981) developed another HPLC method for quantitation of serum clofazimine levels as part of an investigation to measure serum levels of all anti-leprosy agents and their metabolites. They used two different methods with a µBondapak C_{18} column at ambient temperature and monitoring at

287 nm to measure clofazimine levels. The first method used a 40:60 mix of THF - 0.5 % (v/v) acetic acid flowing at 1.5 ml/min. The authors also modified this mobile phase to a mix of 50:50 THF - water containing PIC B-5 (pentane sulfonic acid in acetic acid), the exact levels of reagents being a commercial secret. Using the second system they reported a practical minimum measurable quantity of clofazimine of approximately 5 ng/ml. The final and most recently reported HPLC method was developed by Krishnan and Abraham, (1992). These workers measured plasma clofazimine levels extracted using SPE columns into a solvent mix of 2:2:1 THF - acetonitrile -methanol containing, 0.7 mM hexane sulfonic acid. The samples were analysed on a μBondapak C_{18} column at room temperature monitoring at 280 nm, with a mobile phase 8:11:1 THF - 0.5 % (v/v) acetic acid - methanol containing 2.5 mM hexane sulfonic acid. This method had a reported limit of detection of 3 ng/ml and, as mentioned in section 3.3.4., for the first time included an internal standard for more accurate and precise measurement.

3.6. LIMITATIONS OF ESTABLISHED QUANTITATION METHODS

With the exception of the method employed by Barry *et al.*, (1960), all of the methods mentioned in the preceding sections have been developed for the analysis of clofazimine, and their use in biological measurements of other phenazines, was never investigated. Sensitivity has been increased by 10 to 20 fold compared to the original quantitation methods, allowing the measurement of typical blood levels of clofazimine, but the extraction and measurement methods are laborious and relatively time consuming, with limiting sample volumes and throughput. All of the methods outlined involve the use of volatile organic solvents, and with the exception of the method of Krishnan and Abraham, (1992) do not employ an internal standard. Therefore, they are prone to the inaccuracies inherent in the use of such solvents. In short, investigation of biological levels of several phenazines necessitated the development of a new measurement system.

3.7. INTRODUCTION TO CHEMICAL STUDIES ON RIMINO-PHENAZINES.

All the rimino-phenazines investigated were synthesised by Dr. Sean O'Sullivan in the Chemistry Dept. of U.C.D. When synthesised these agents were purified by recrystallisation and analysed by elemental analysis to confirm the correct ratio of atoms, indicating a largely pure substance. When many of these agents were analysed by TLC, at the outset of this project, a major spot was noted in all cases, but minor spots with different retention factor (r_f) values were also evident. To quantify and further investigate the purity of these compounds samples were

analysed by HPLC. Column chromatography of B4100 was used to investigate if further purification was possible by this method. TLC, HPLC and proton NMR spectroscopy were used to investigate this process. To further confirm the basic chemical structure and since many of these agents were new, IR scans were also taken.

3.9. HPLC AND TLC ANALYSIS OF PURITY

The principles of these two methods have been described in sections 3.5.1 and 3.5.2. TLC gives a lot of qualitative information on the purity of test compounds. Impurity, and conversely, purity, is indicated by the number of spots and their relative intensity. This gives a good visual estimate of purity but quantification is difficult due to variation in the absorption spectrum between the different components present. The variety of spot sizes present on a plate also prevents accurate quantification, even when scanned with a densitometer, since this scans at one wavelength along a narrow lane. HPLC can also be used to investigate the purity of a compound. The number of peaks present in a chromatogram give a good indication of purity. Using a photo-diode detector allows the complete spectrum of the impurities present to be investigated. If these impurities have similar absorption spectra, then the purity can be measured by integration of the chromatographic peaks at the maximum absorption wavelength. Careful investigation of the spectra can also provide information as to the likely structure of any contaminant present.

If these methods provide evidence of the presence of impurities, these will normally have to be removed, since the pharmacological assessment of a drug will be hampered if other agents are present. Many different methods are available for compound purification but one of the more common is column chromatography.

3.10. PRINCIPLE OF COLUMN CHROMATOGRAPHY

Column chromatography involves the separation of compounds as they are carried through a glass column filled with a solvated polar adsorbent (generally silica or alumina) by an organic solvent mix, and is the forerunner of HPLC. The compounds emerge with the solvent and are collected as separate fractions. Generally these fractions are initially checked for purity by TLC and compounds of interest are separated from the solvent on a rotary evaporator. The principle of compound separation is the same as that for TLC separation. Since there is a much larger volume of silica to travel through and equilibration between solvated compound and adsorbent is complete, a more polar solvent mix is necessary to develop the

plates in a reasonable time.

3.11. METHODS OF VERIFYING DRUG PURITY

The purity information from HPLC and TLC is all based on the UV-visible absorbance of compounds and is, therefore, limited. To completely verify the purity and chemical structure of a drug necessitates the use of NMR and IR spectroscopy. These methods provide analysis on the basis of the atoms present in a molecule and thus describe the chemical structure present as well as indicating if any contaminating organic molecules are present.

3.11.1. Principle of proton NMR analysis

Nuclear magnetic resonance (NMR) is a sensitive technique for the measurement and identification of atoms with a magnetic moment, particularly hydrogen (H^1) and carbon (C^{13}). In chemical analysis proton NMR is routinely used for the identification of chemical compounds and can also indicate the presence of impurities. Samples are generally dissolved in a deuterated organic solvent, i.e. one in which all the hydrogen atoms present have been exchanged for non-magnetic deuterium (H^2), since the presence of H^1 in the solvent generally swamps the compound absorbances. The samples are placed in a narrow glass tube and spun in a very strong varying magnetic field which causes alignment of the magnetic atomic nuclei present (protons in hydrogen since neutrons are absent from the hydrogen nucleus). As the field varies the molecular motions of the nuclei can resonate and at specific frequencies they absorb energy in the radio frequency band due to movement from one magnetic alignment to another. This absorption is detected by a sensitive radio frequency detector. The energy absorbed is proportional to the mass of protons present and its exact frequency is dependent on the local electromagnetic environment of the constituent protons, thereby giving information both on the relative concentrations of protons and the nature of atoms in their immediate vicinity.

3.11.2. Principle of IR spectroscopic analysis

The constituent atoms in a molecule are joined together by bonds which generally allow the atoms to move relative to each other as opposed to the common perception of atomic bonds as fixed and inflexible. In this way these bonds can be visualised as having properties similar to a spring allowing movements like stretching, bending and twisting. Each type of movement, known as a vibrational mode, has its own frequency of oscillation and distinct frequencies of electromagnetic energy in the infra red (IR) region of the spectrum can interact and cause these

modes to resonate if the vibration produces an oscillating dipole moment that can interact with the electric field of the radiation. This interaction with the vibrational modes causes compounds to absorb IR radiation at characteristic frequencies dependent on the available vibrational modes of constituent bonds and the electric field associated with other elements of the whole molecule. The IR absorbance spectrum, therefore, gives information on the presence of particular chemical groups in a compound and can be used as a quick and simple method to confirm chemical structure.

In practice, powdered samples such as drugs, are analysed by IR spectroscopy generally in the form of mulls (pastes), or, more readily, as glass discs formed by compressing a ground mixture of the compound and KBr powder in a press at several tons of pressure. The disc becomes transparent under this pressure and when placed in the light path of the spectrometer only absorbances due to the compound present are evident. The frequency of absorbance is generally expressed in terms of wavenumbers (cm^{-1}) which are the reciprocal of the wavelength ($1/\lambda$) and therefore proportional to the frequency.

3.12. CHEMICAL ANALYSES OF PHENAZINES

The methods outlined in the previous sections were all combined in this project to give a broad picture of the purity and chemical properties of selected phenazines. The methods outlined are those which are used in the initial chemical investigations of a new pharmaceutical agent. When compared to the mass of information needed for the acceptance of a drug developed in the present age, very little background information is available on clofazimine and, especially, the other phenazines. While the extent of their application, as described in the results and discussion sections, was limited, this work provides the outline for the further studies which would be necessary for the pharmaceutical and pharmacological investigation of new phenazine agents.

RESULTS

3.13. HPLC ANALYSIS OF PHENAZINES IN BIOLOGICAL SAMPLES

3.13.1. Percentage recovery and reproducibility of extraction

Comparison of the chromatographic peak height ratios of extracted standards with those of paired unextracted standards in Tables 3.1.-3.3.shows that the recovery of clofazimine was 100 % across the linear quantification range 50 - 0.01 µg/ml (µg/g) for serum and tissue samples and 100 - 0.02 µg/g for fat samples. 100 % recovery was seen with B4090 in the linear range 50 -0.02 µg/ml (µg/g). Complete recovery for the other phenazines tested was also observed. However, this was not investigated with a large scale recovery and reproducibility study on every agent.

3.13.2. Difficulties with phenazine extraction

Extraction of phenazines from fat and faecal samples proved particularly difficult. For fat extractions this was largely overcome by heating the extract in alcoholic sodium hydroxide. This reduced the interference from co-extracting lipids. With faecal extractions a mixture of contaminants were found, which could not be selectively removed. The spectrum of this extract (Figure 3.2.(i)) indicated that an extracted mixture probably consisted of faecal porphyrins and bile salts (Fasman, 1976). As shown in the chromatogram in Figure 3.2.(ii), these compounds interfered with the analysis of low faecal phenazine levels by eluting around the same time as several of the phenazines. Photo-diode array (PDA) analysis of these peaks also indicated that a heterogenous mixture of substances was present (Figure 3.2.(ii).(b)).

3.13.3. Effect of component variations of the mobile phase

Several mobile phase variables were modified to try to optimise the chromatography and Figure 3.3. and Table 3.4. show the effect of these alterations on peak parameters. As shown, a 40 % (v/v) THF content in the mobile phase gave a compromised optimum between r_t and peak width, and the acid content was also critical. Increasing the strength of the acetic acid from 0.5 to 1 % (v/v) also increased the speed of elution without causing damage to the stationary support. Increasing the acetic acid content might further reduce the retention time. However, the associated drop in pH might also be expected to hydrolyse the silanol-C_{18} bond.

Table 3.1.

The percentage recoveries of B663 and B4090 from serum determined across the linear range (n=5). Recovery was determined by dividing the peak height ratio of the extracted spiked serum samples by the ratio obtained by injecting equivalent amounts of unextracted standards. The ratio was determined five times on a single day from samples prepared from the same standards.

DRUG	Conc. added (µg/ml)	% Recovery (± SD)	RSD (%)
B663	0.01	102.8 ± 24.7	24.0
	0.02	95.2 ± 6.9	7.2
	0.05	87.1 ± 9.4	10.6
	0.2	94.4 ± 5.8	6.1
	0.5	80.2 ± 8.2	10.3
	2	96.3 ± 9.6	9.9
	5	101.7 ± 2.8	2.8
	20	104.2 ± 4.3	4.1
	50	99.1 ± 1.4	1.4
B4090	0.02	95.4 ± 5.9	6.2
	0.05	99.1 ± 6.1	6.2
	0.2	103.2 ± 7.6	7.4
	0.5	100.1 ± 3.2	3.2
	2	96.9 ± 6.8	7.0
	5	101.7 ± 3.9	3.8
	20	102.5 ± 4.4	4.3
	50	99. ± 1.3	1.4

Table 3.2.

The percentage recoveries of B663 and B4090 from liver determined across the linear range (n=5). Recovery was determined by dividing the peak height ratio of the extracted spiked liver samples by the ratio obtained by injecting equivalent amounts of unextracted standards. The ratio was determined five times on a single day from samples prepared from the same standards.

DRUG	Conc. added (µg/ml)	% Recovery (± SD)	RSD (%)
B663	0.01	97.6 ± 9.2	9.4
	0.02	137.6 ± 28.7	20.8
	0.05	103.6 ± 14.0	13.6
	0.2	100.8 ± 2.2	2.2
	0.5	89.4 ± 12.4	13.9
	2	98.4 ± 1.3	1.3
	5	99.2 ± 5.4	5.4
	20	100.1 ± 3.7	3.7
	50	102.2 ± 5.0	4.9
B4090	0.02	95.2 ± 10.3	10.8
	0.05	118.6 ± 12.4	10.5
	0.2	106.2 ± 7.9	7.4
	0.5	98.4 ± 1.3	1.3
	2	97.9 ± 2.7	2.7
	5	100.7 ± 1.7	1.6
	20	99.6 ± 4.1	4.1
	50	99.2 ± 3.2	3.3

Table 3.3.

The percentage recoveries of B663 and B4090 from fat determined across the linear range (n=5). Recovery was determined by dividing the peak height ratio of the extracted spiked fat samples by the ratio obtained by injecting equivalent amounts of unextracted standards. The ratio was determined five times on a single day from samples prepared from the same standards.

DRUG	Conc. added (µg/ml)	% Recovery (± SD)	RSD (%)
B663	0.02	100.3 ± 5.5	5.5
	0.05	101.8 ± 10.2	10.1
	0.2	102.0 ± 5.3	5.2
	0.5	99.5 ± 9.0	9.0
	2	96.8 ± 5.6	5.8
	5	99.9 ± 4.0	4.0
	20	97.2 ± 6.9	7.1
	50	95.3 ± 5.17	5.4
	100	100.0 ± 1.0	1.0
B4090	0.02	96.2 ± 10.8	11.2
	0.05	90.0 ± 3.2	3.6
	0.2	93.7 ± 4.9	5.2
	0.5	98.5 ± 10.5	10.7
	2	99.3 ± 1.5	1.5
	5	101.0 ± 9.5	9.4
	20	98.1 ± 2.9	3.0
	50	115.0 ± 15.0	13.0

Figure 3.2.(i) A spectrum of a DCM extract from blank faecal material. The visible and UV absorption indicate that this spectrum is most likely the result of extraction of a mixture of porphorins and bile salts. The chromatogram of this extract is shown in Figure 3.2.(ii).

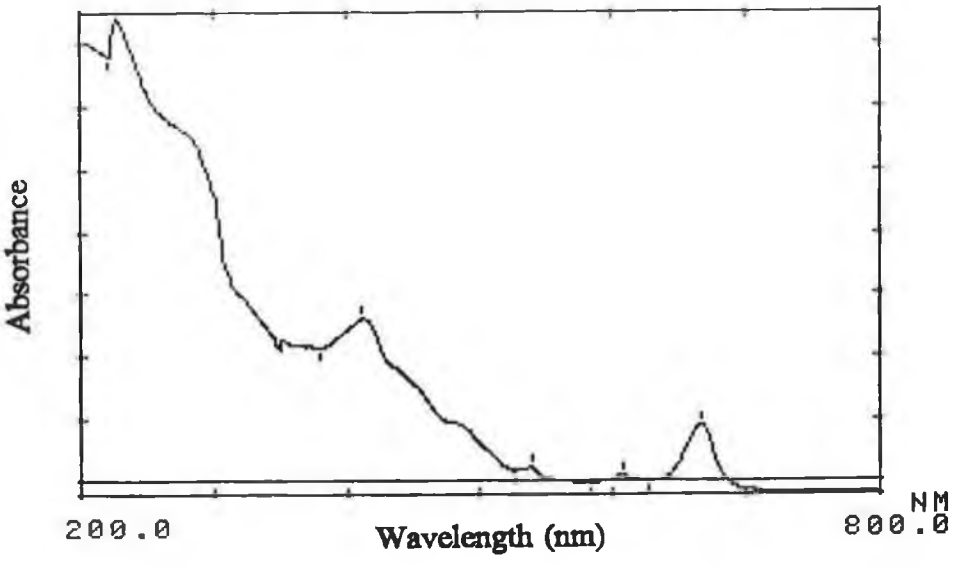


Figure 3.2.(ii) (a) The HPLC chromatogram of an extract from blank faeces. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min. The presence of a number of compounds is indicated by the peaks shown. These peaks interfer with phenazine peaks resulting in a reduced limit of quantification. The spectra of four of these peaks is shown in part (b). These spectra were taken using a PDA detector in the HPLC system.

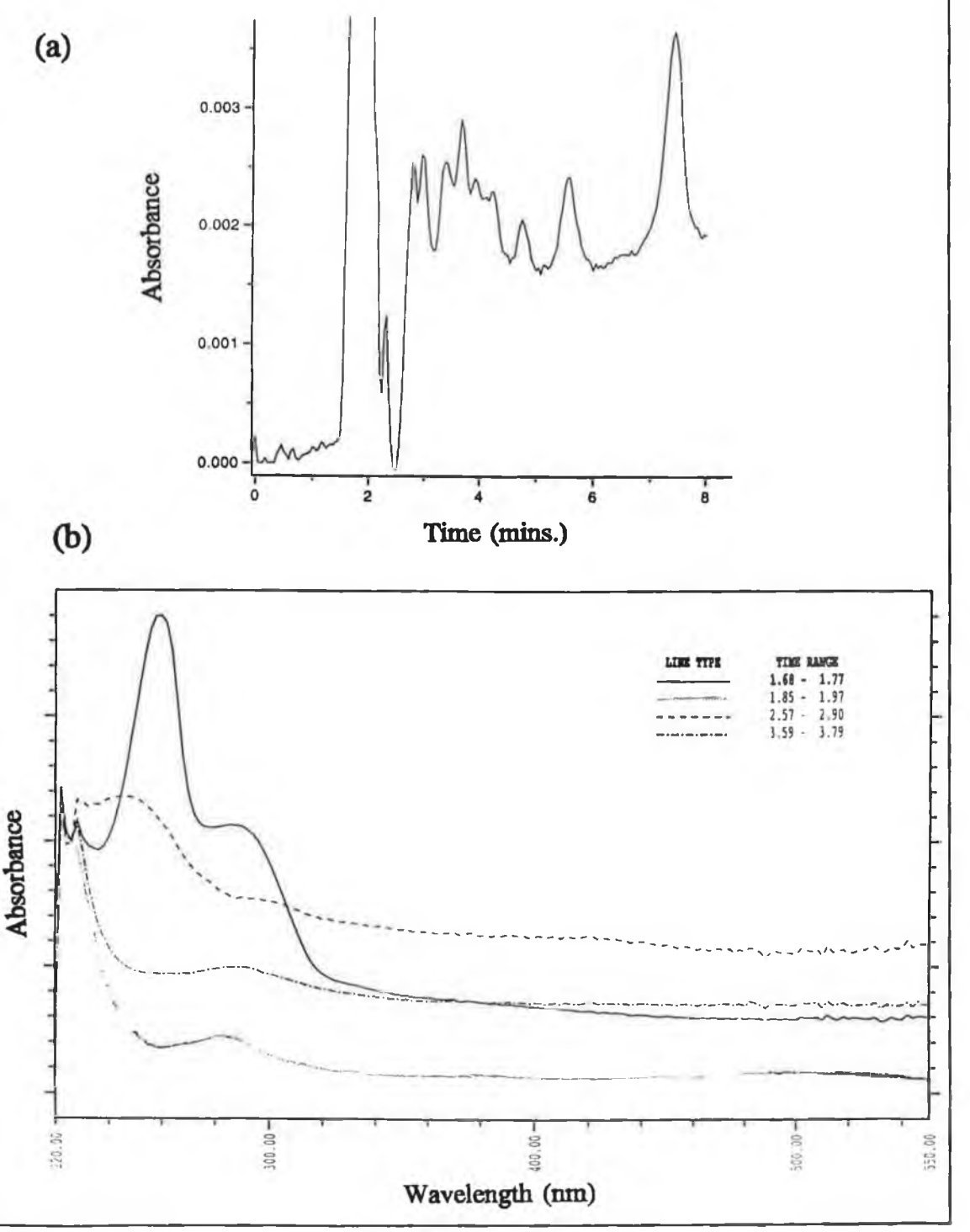


Figure 3.3. The effect of varying the water content of the HPLC mobile phase on peak width, retention time and peak height. The HPLC conditions were a Bondclone C_{18} column, with the amount of concentrated acetic acid at 6 ml/L and the hexane sulfonic acid concentration at 2.5 mM, kept constant in the mobile phase. The flow rate was 1.5 ml/min. The effect of the variation of the mobile phase on peak width is shown in (a), retention time, in (b), and, peak height, in (c). A 60 % (v/v) water content (40 % (v/v) THF) can be seen to be optimal for all three parameters.

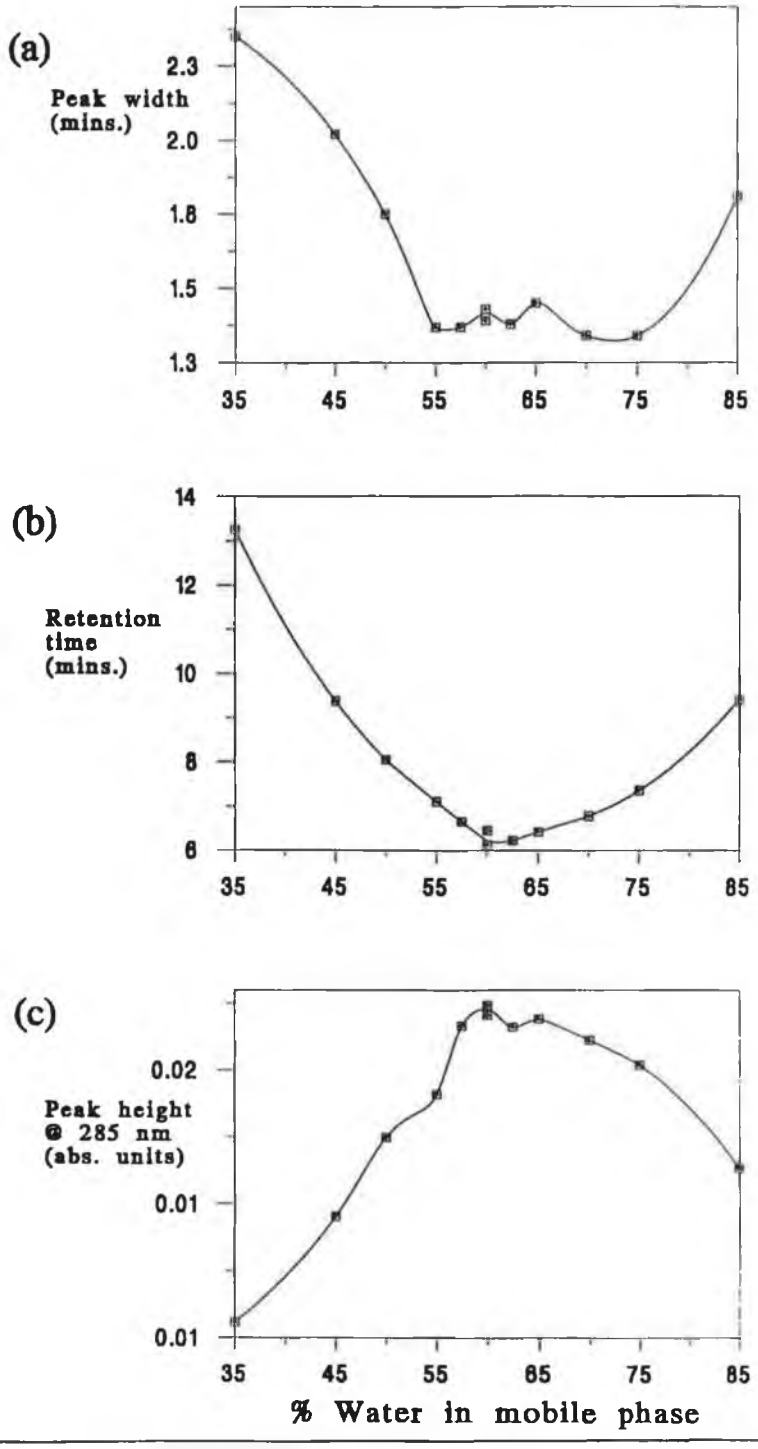


Table 3.4.

The effect of mobile phase pH on retention time, peak width and peak height of a standard clofazimine peak. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) water and 2.5 mM hexane sulfonic acid, at a flow rate of 1.5 ml/min, with detection at 285 nm. The pH represents the pH of the aqueous solution, adjusted with different concentrations of acetic acid, before addition of THF. Peaks with a retention time greater than 30 mins. could not be quantified. Decreasing the pH leads to improved chromatographic parameters.

Aqueous pH	Retention time (mins.)	Peak width (mins.)	Peak height (abs. units)
2.8	6.14	2	0.124
3.4	18.52	5	0.045
3.9	> 30	-	-
5.1	> 30	-	-

Although retention times became more reliable, drifting was still noted in the retention time of phenazines over several hours and was variable between batches of mobile phase. Ultimately this problem was traced to the evaporation of THF from the mobile phase. Figure 3.4. demonstrates this evaporation by showing the reduction in mass of 1 g of mobile phase in an open container over time. By sealing the eluent tubing into the mobile phase reservoir, a closed liquid flow circuit was produced where the free reservoir head space was rapidly saturated with THF and no further evaporation could take place. A 1.5 ml/min mobile phase flow rate was chosen as this gave a fast resolution of all phenazines whilst maintaining a back pressure (around 2,800 P.S.I.) within tolerances for maximum column lifetime.

The µBondapak C_{18} column was also changed for a Bondclone C_{18} produced by Phenomenex which gave almost identical chromatography.

This system proved to be successful because all the synthetic phenazines dissolved in high concentration in the mobile phase, each phenazine gave a well resolved peak clear from contaminant peaks and eluted rapidly from the column, giving a rapid analysis. Figures 3.5.(i) - (iii) shows typical chromatograms for the phenazines analysed. The retention times of these compounds are shown in Table 3.5. The individuality of retention times also meant that mixtures of phenazines could be chromatographed. This allowed phenazines to be used as internal standards for one another, increasing the accuracy and precision of the analysis. Figure 3.6. shows chromatograms of 5 µg of clofazimine with I.S., extracted from serum, liver and fat. These chromatograms show that the I.S. produces a separate and distinct peak for all samples.

3.13.4. Limits of detection and quantification

Samples containing 10 ng/ml produced peaks readily resolved from the background noise of the U.V. detector giving reproducible results for quantification and this value was taken as the limit of quantification for the analyses. The peaks produced by concentrations down to 5 ng/ml were also resolved from baseline noise. However, the underlying noise produced a peak height variability which made accurate quantification difficult. Injection of samples from standards containing 70 µg/ml or more in 60 µl produced peaks which were poorly resolved due to broader peak width and peak tailing. This form of peak abberation is consistent with column saturation. The peak broadening and tailing causes the analyte peak to no longer be proportional to its concentration. As a result, standards of 50 µg/ml were taken as the maximum limit of quantification, implying column saturation above approximately 9 µg of phenazine on the

Figure 3.4. The mass of a 1 ml sample of HPLC mobile phase in an open vessel, at room temperature, over 30 mins. This sample consists of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid. The mass steadily decreases over time due to evaporation of the THF component.

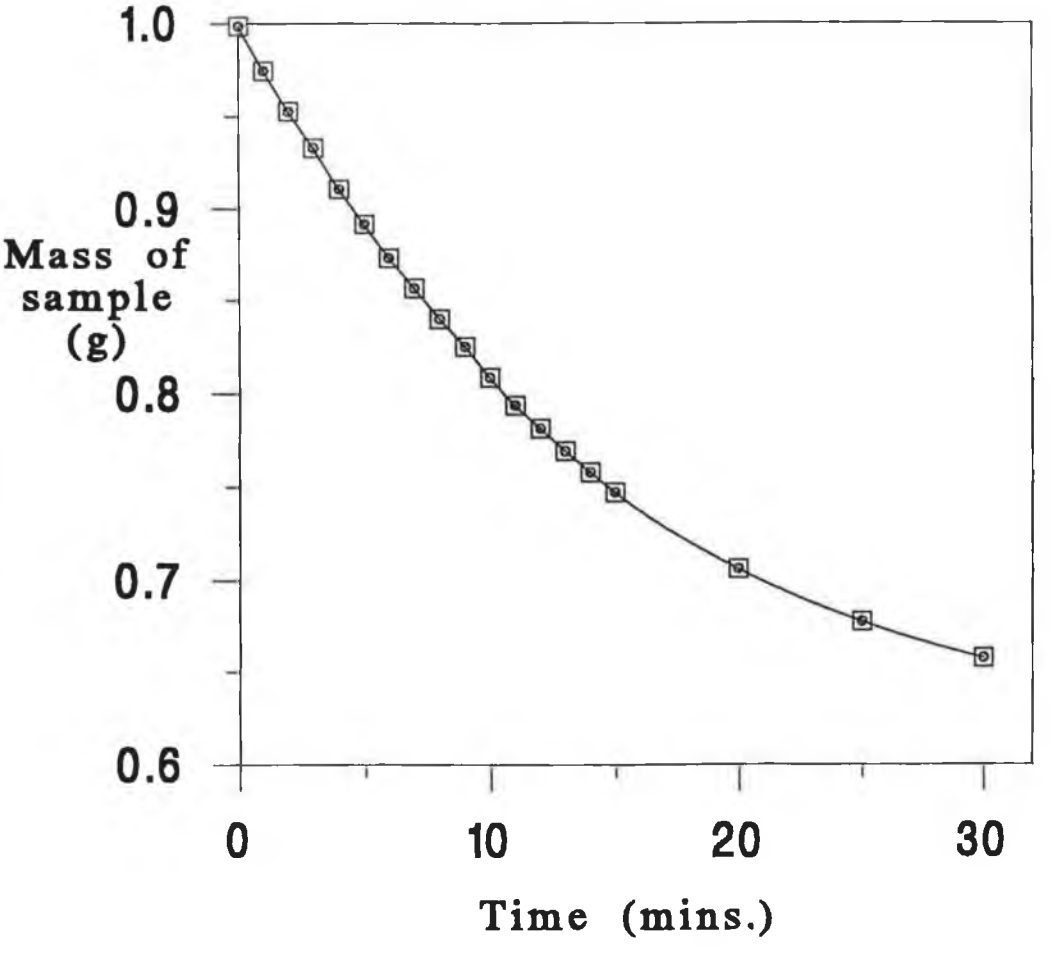


Figure 3.5.(i). HPLC chromatograms of the rimino-phenazines investigated in this project. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. The spectra are of: (a) clofazimine (p), (b) clofazimine (s), (c) B749 and (d) B4154. The retention time of each compound is shown in table 3.5.

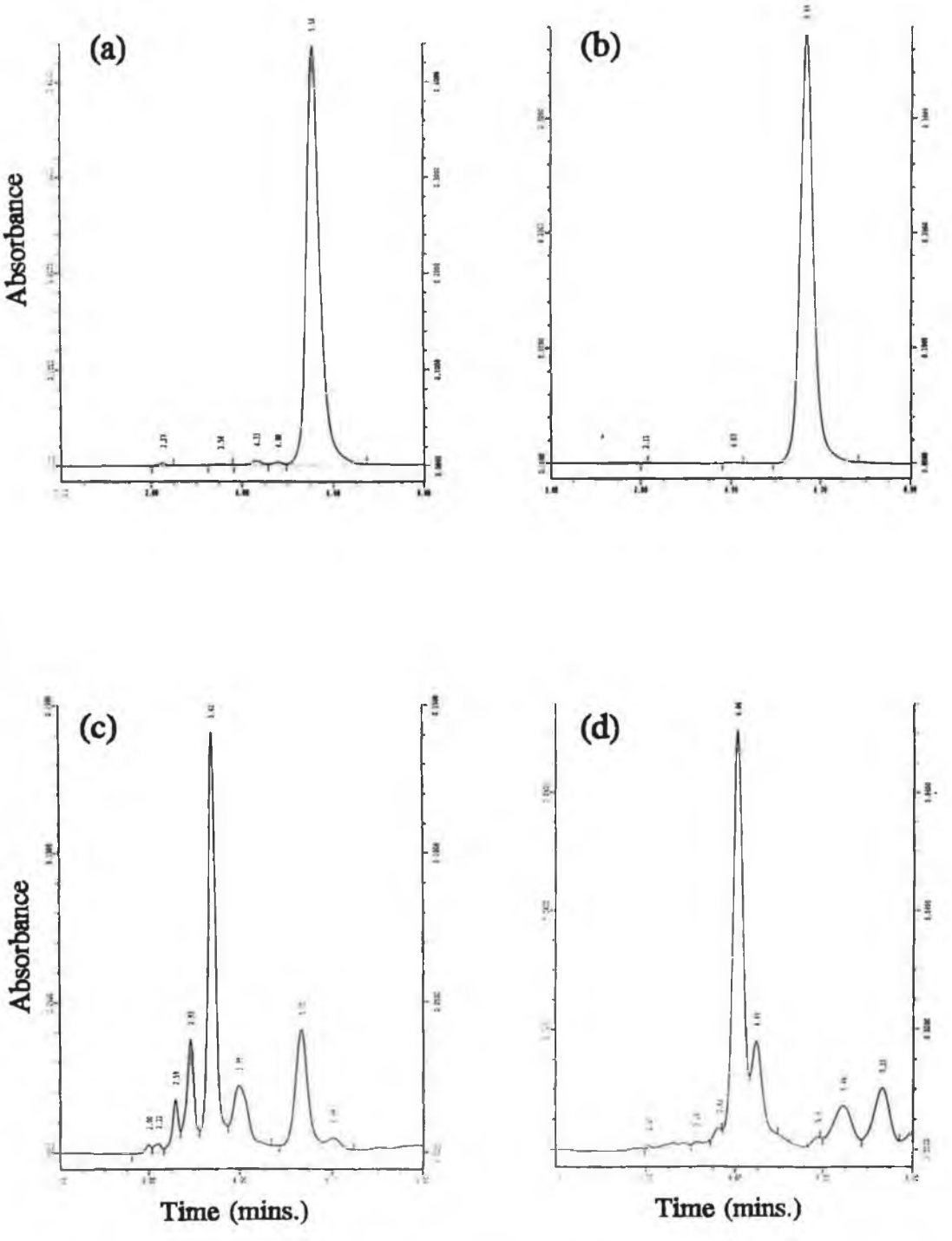


Figure 3.5.(ii). HPLC chromatograms of the rimino-phenazines investigated in this project. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. The spectra are of: (a) B3640, (b) B3954, (c) B3955 and (d) B3976. The retention time of each compound is shown in table 3.5.

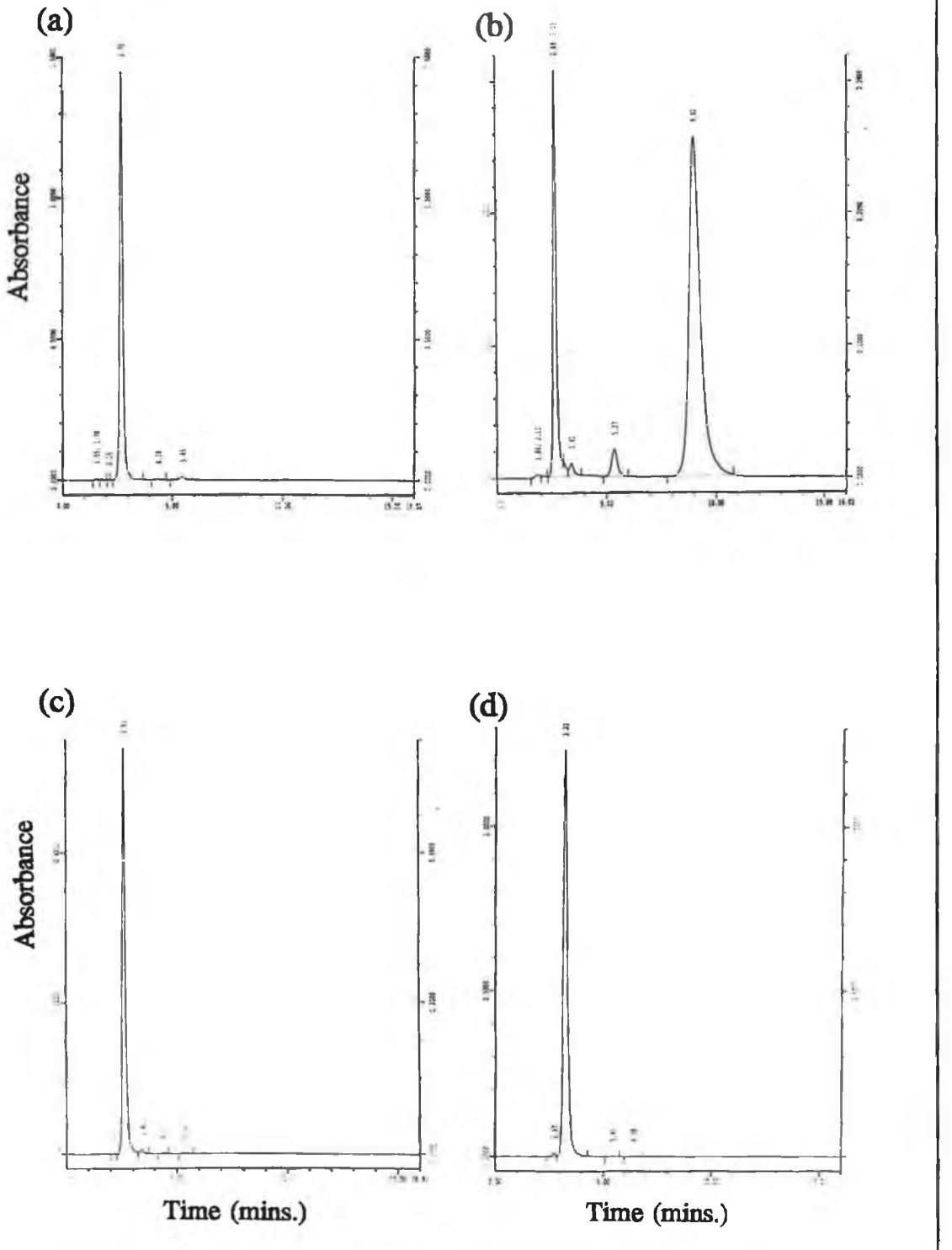


Figure 3.5.(iii). HPLC chromatograms of the rimino-phenazines investigated in this project. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. The spectra are of: (a) B 4090, (b) B4100 and (c) B4103. The retention time of each compound is shown in table 3.5.

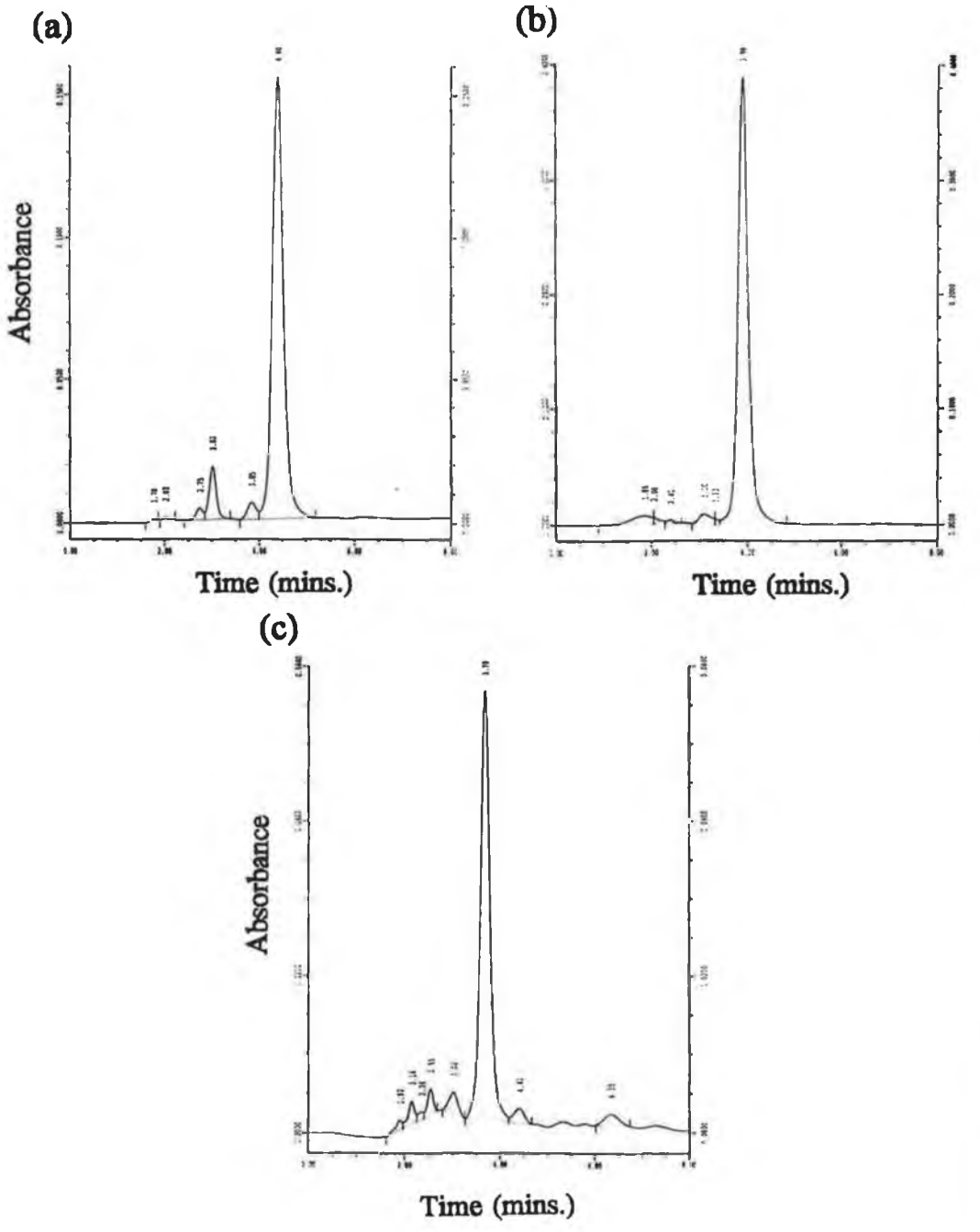
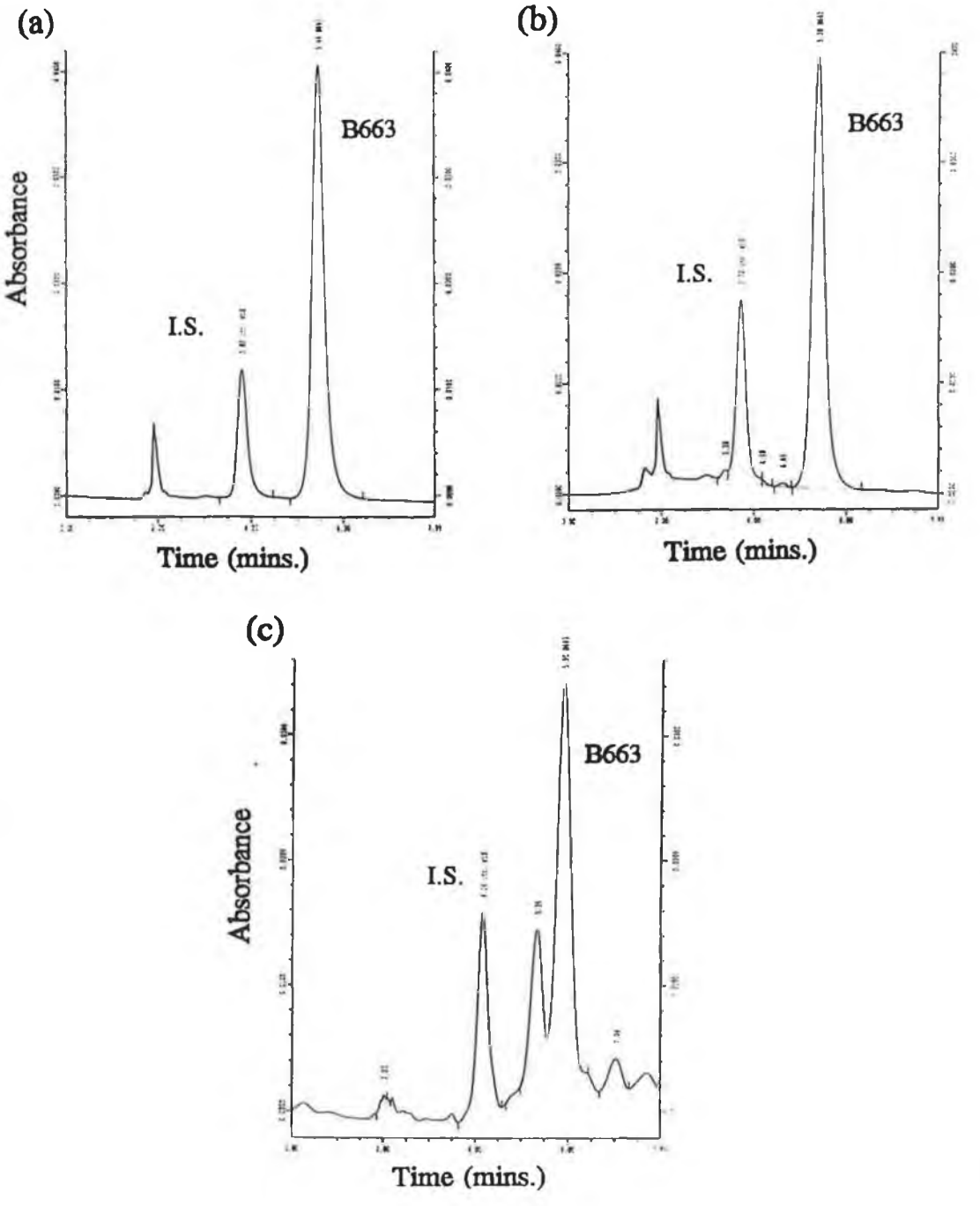


Table 3.5.

The chromatographic retention time of the rimino-phenazine compounds analysed. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. The chromatograms of each compound are shown in Figure 3.5.(i). - (iii).

Compound	Retention time (mins.)	Compound	Retention time (mins.)
clofazimine	5.6	B3955	2.6
B749	3.4	B3976	3.2
B4154	4.1	B4090	4.4
B3640	2.7	B4100	3.9
B3954	9.0	B4103	3.7

Figure 3.6. Representative HPLC chromatograms of clofazimine and internal standard (I.S.) extracted from; (a) serum, (b) liver and (c) fat samples. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. In each case the I.S. gives a sharp peak well resolved from the analyte peak.



column. The high levels of clofazimine found in fat were often in excess of this concentration. This problem was overcome by reconstituting samples and standards in 120 µl of THF - acetic acid, thereby diluting the amount loaded on to the column. This dilution increases the maximum measurable concentration but doubles the limit of quantification to 20 ng/ml.

The compound B4090 had a higher limit of quantification of 20 ng/ml due to a reduced extinction coefficient. As Figure 3.7. shows the spectrum of this agent is very similar in shape to that of other phenazines, however comparison with the spectrum of an equal concentration of B4100 which is the most similar rimino-phenazine shows the reduced absorption across the whole spectrum. Besides additional chlorination on the phenyl and anilino rings, the only difference between these compounds is that B4090 is chlorinated in the 7-position. It would, therefore, seem likely that this chlorine atom significantly affects the electron flow in the phenazine backbone responsible for its U.V.-visible absorption spectrum. Comparison of the spectra and spectral derivatives of B3954, B3955 and B3976 with that of the parent B3640 showed that although the retention times of these agents were different, the spectra were exactly identical. This indicates that substitutions distant from the phenazine ring had no effect on its spectrum (see Figure 3.8.).

3.13.5. Accuracy and precision of the HPLC method

To determine the intra-day accuracy and precision of the combined extraction and HPLC quantification method, blank liver, serum and fat samples were spiked with clofazimine standards using an I.S. of 2 µg of B4100, or B4090 standards using an I.S. of 2 µg of B663 (clofazimine). Five sets of standards were prepared using the same stock concentrations. These standards were analysed with a set of calibration standards overnight in a single chromatographic run. The concentrations of the standards were determined from the log plot of PHR to concentration of the calibration set. Tables 3.6. - 3.8. show the mean, standard deviation and % relative standard deviation (RSD) of these spiked standards for both compounds in the serum, liver and fat during the intra-day analysis.

The inter-day accuracy and precision of this method were also determined for B4090 and B663 in the same samples. A set of spiked standards were prepared daily on 5 different days from freshly prepared stock concentrations and the drug concentrations determined from the log plot of PHR vs. concentration of a calibration set. Tables 3.9. - 3.11. shows the inter-day mean, standard deviation and % RSD of the spiked standard values for these analyses.

Figure 3.7. An overlaid comparison of the spectra of 20 ug/ml B4090 and B4100 under HPLC conditions. These conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with PDA detection. The spectrum of B4090 is enlarged on the absorbance axis since it has a lower extinction coefficient than B4100. The comparison shows that the spectrum of B4090 is slightly different from that of B4100. This is due to chlorination of the phenazine ring in B4090.

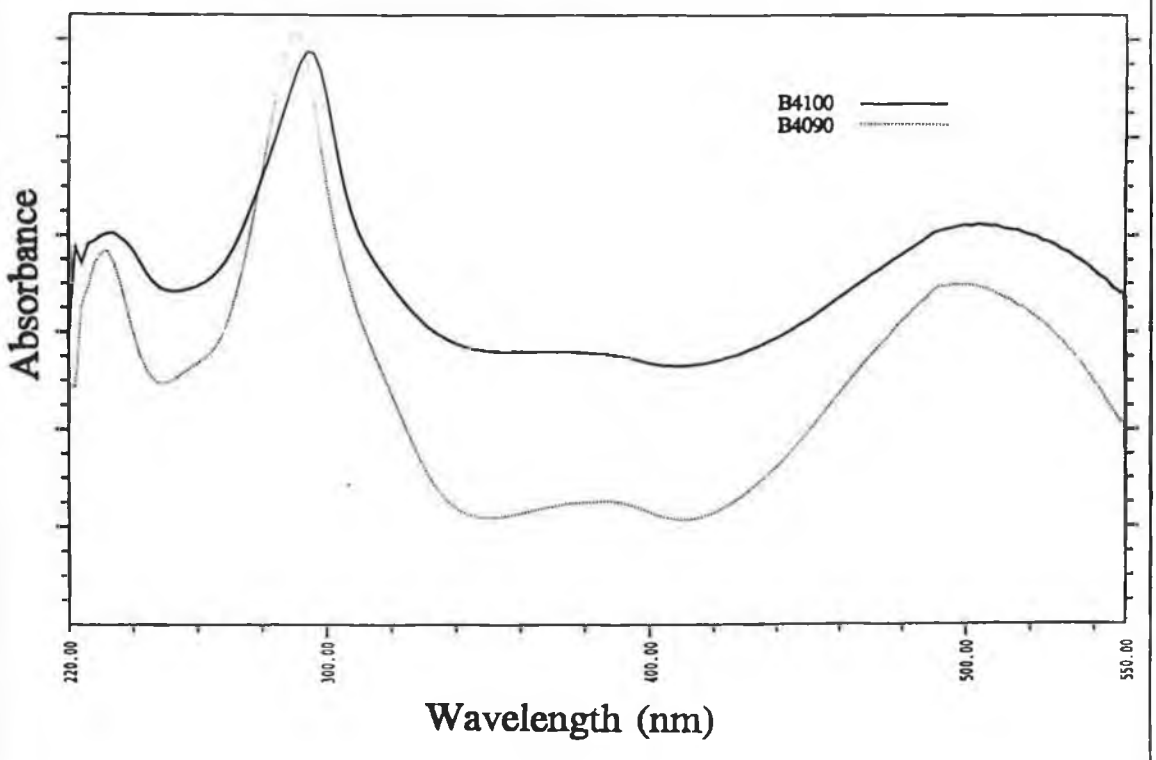


Figure 3.8. An overlaid comparison of the spectra of 20 ug/ml B3654, B3955, B3976 with the parent compound B3640 under HPLC conditions. These conditions were a Bondclone C_{18} column, with a mobile phase of 40 % v/v THF, 60 % v/v of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with PDA detection. Although the compounds have individual chromatographic retention times, the spectra are identical with the same nodal points when derivatised and a correlation coefficient of 0.998. This indicates that substituents distant from the phenazine ring have no effect on the phenazine spectrum.

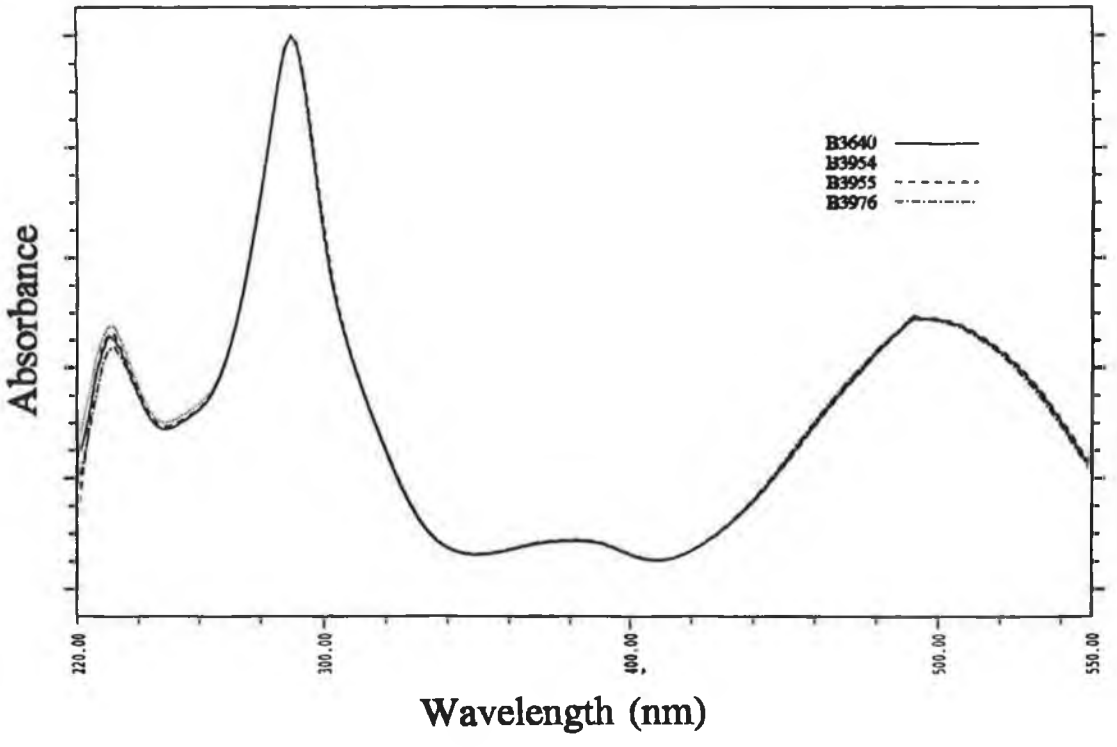


Table 3.6.

Intra-day precision and accuracy for spiked serum samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking blank serum samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on a single day from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.01	0.013 ± 0.003	27.6
	0.02	0.021 ± 0.001	8.0
	0.05	0.051 ± 0.005	11.3
	0.2	0.21 ± 0.01	6.2
	0.5	0.58 ± 0.06	10.3
	2	1.9 ± 0.1	5.7
	5	4.9 ± 0.1	3.1
	20	22.0 ± 0.9	4.2
	50	49.1 ± 0.7	1.4
B4090	0.02	0.019 ± 0.003	19.2
	0.05	0.051 ± 0.004	9.4
	0.2	0.20 ± 0.01	7.7
	0.5	0.51 ± 0.01	2.3
	2	2.0 ± 0.1	7.0
	5	5.0 ± 0.1	3.6
	20	20.0 ± 1.0	5.4
	50	50.1 ± 0.3	0.7

Table 3.7.

Intra-day precision and accuracy for spiked liver samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking blank liver samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on a single day from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.01	0.009 ± 0.003	34.6
	0.02	0.027 ± 0.005	21.2
	0.05	0.041 ± 0.012	31.2
	0.2	0.20 ± 0.01	3.7
	0.5	0.47 ± 0.06	13.5
	2	1.9 ± 0.1	1.9
	5	4.9 ± 0.3	6.8
	20	23.0 ± 1.4	6.1
	50	48.2 ± 6.1	12.8
B4090	0.02	0.019 ± 0.003	16.0
	0.05	0.052 ± 0.005	10.3
	0.2	0.20 ± 0.01	6.5
	0.5	0.49 ± 0.02	4.2
	2	1.9 ± 0.1	2.2
	5	4.9 ± 0.1	1.5
	20	19.8 ± 0.7	3.5
	50	49.4 ± 1.4	2.8

Table 3.8.

Intra-day precision and accuracy for spiked fat samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking fat samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on a single day from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.02	0.017 ± 0.002	13.2
	0.05	0.053 ± 0.007	14.7
	0.2	0.19 ± 0.01	5.8
	0.5	0.46 ± 0.04	10.6
	2	1.9 ± 0.1	6.6
	5	4.9 ± 0.2	4.1
	20	20.0 ± 1.4	7.1
	50	50.2 ± 2.8	5.5
	100	99.3 ± 1.0	1.0
B4090	0.02	0.020 ± 0.001	9.5
	0.05	0.051 ± 0.003	6.5
	0.2	0.21 ± 0.01	2.8
	0.5	0.50 ± 0.01	2.0
	2	1.9 ± 0.1	4.1
	5	5.0 ± 0.1	2.5
	20	21.0 ± 0.7	3.5
	50	50.3 ± 1.1	2.2

Table 3.9.

Inter-day precision and accuracy for spiked serum samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking blank serum samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on five different days from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.01	0.0097 ± 0.000	6.3
	0.02	0.019 ± 0.006	35.1
	0.05	0.054 ± 0.009	17.1
	0.2	0.20 ± 0.01	7.7
	0.5	0.52 ± 0.01	2.6
	2	1.9 ± 0.1	8.4
	5	4.7 ± 0.1	3.5
	20	20.6 ± 1.1	5.4
	50	49.7 ± 0.6	1.3
B4090	0.02	0.021 ± 0.002	11.6
	0.05	0.046 ± 0.009	19.7
	0.2	0.20 ± 0.01	3.2
	0.5	0.49 ± 0.02	5.7
	2	2.0 ± 0.1	6.0
	5	4.9 ± 0.1	1.5
	20	23.0 ± 1.6	7.3
	50	48.6 ± 3.4	7.1

Table 3.10.

Inter-day precision and accuracy for spiked liver samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking liver serum samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on five different days from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.01	0.013 ± 0.002	19.4
	0.02	0.019 ± 0.003	15.6
	0.05	0.051 ± 0.014	27.4
	0.2	0.19 ± 0.01	5.3
	0.5	0.49 ± 0.01	2.8
	2	2.0 ± 0.1	4.9
	5	5.2 ± 0.1	2.7
	20	21.5 ± 0.6	2.8
	50	50.3 ± 0.6	1.3
B4090	0.02	0.018 ± 0.004	22.0
	0.05	0.049 ± 0.001	2.7
	0.2	0.2 ± 0.01	3.4
	0.5	0.49 ± 0.01	3.8
	2	2.0 ± 0.1	2.8
	5	4.9 ± 0.1	1.7
	20	19.9 ± 0.7	3.7
	50	49.2 ± 1.4	2.9

Table 3.11.

Inter-day precision and accuracy for spiked fat samples extracted across the linear range (n=5). Precision and accuracy were determined by spiking fat samples with B663 and 2µg of B4100 as IS, or B4090 with 2µg of B663 as IS as appropriate. The concentration was determined five times on five different days from samples prepared from the same standards and quantified by log vs. log plots of peak height ratio (PHR) vs. concentration prepared from a calibration set.

DRUG	Conc. added (µg/ml)	Mean conc. measured (µg/ml ± SD)	RSD of mean (%)
B663	0.02	0.019 ± 0.001	5.8
	0.05	0.053 ± 0.004	9.1
	0.2	0.20 ± 0.02	10.1
	0.5	0.48 ± 0.02	6.0
	2	1.9 ± 0.1	9.1
	5	4.9 ± 0.3	7.8
	20	21.1 ± 3.3	15.9
	50	52.5 ± 4.4	8.5
	100	98.4 ± 6.1	6.2
B4090	0.02	0.020 ± 0.001	5.0
	0.05	0.051 ± 0.005	9.9
	0.2	0.20 ± 0.01	6.0
	0.5	0.50 ± 0.01	2.0
	2	1.9 ± 0.1	5.2
	5	5.0 ± 0.1	2.0
	20	20.8 ± 0.9	4.6
	50	52.5 ± 4.1	7.8

Although a graph of the PHR vs. concentration gave apparently straight lines with very good linear regression ($r \geq 0.995$ for all agents) use of the resultant equation with the ratios from lowest concentrations produced apparently anomalous results. This was due to the inherent bias of linear regression for larger values (i.e. the points with higher concentration-PHR values have a greater effect on the linear regression than lower value points). The quantification range was very broad (5000 fold difference between smallest and largest concentrations) so results were quantified using a plot of the log of PHR to the log of the concentration. By this method all points are given approximately equal bias in the linear regression allowing the use of a single equation to quantify phenazines across the whole range. Figure 3.9.(i) illustrates the errors involved with a conventional plot and Figure 3.9.(ii), how this is overcome using logarithmic plots.

3.13.6. Linearity of quantification for all rimino-phenazines

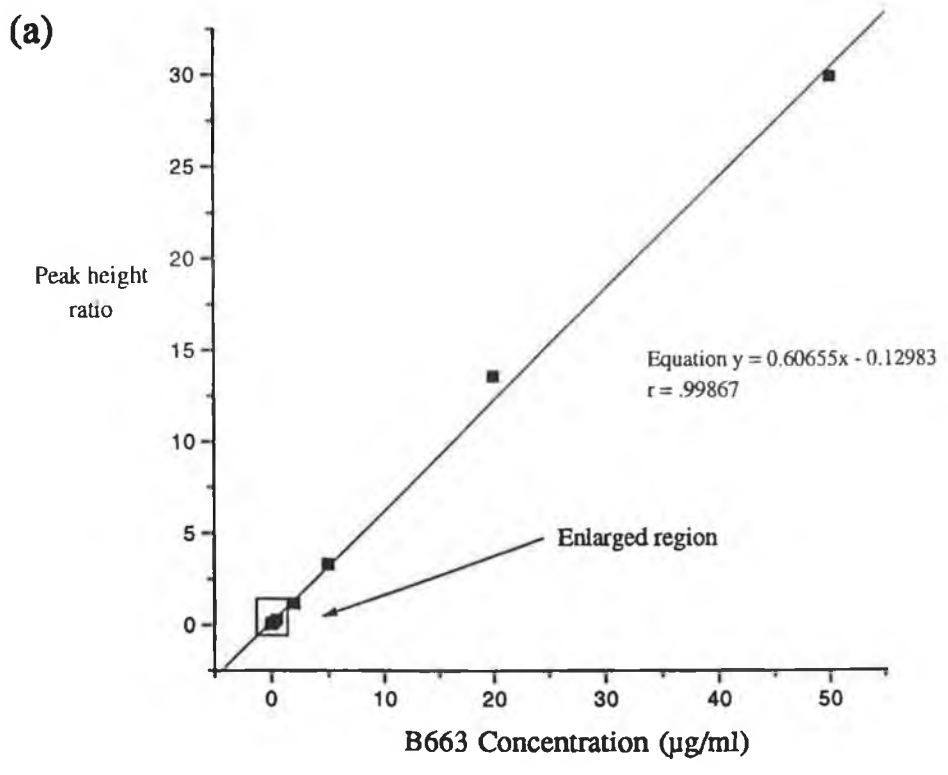
The DCM extraction and HPLC quantification method was also used for the analysis of all the other rimino-phenazine compounds tested in animals. Although a large scale investigation of inter- and intra-day variability was not undertaken for these compounds, standard log plots were prepared for all these agents as shown in Figures 3.10.(i). and 3.10.(ii). In all cases the resultant lines had regression coefficients of 0.997 or greater.

3.13.7. Compatibility with other anti-leprosy drugs

To investigate if other anti-leprosy drugs would interfere with this HPLC analysis system, dapsone (DDS) and rifampicin, two of the most used anti-leprosy agents, were extracted in a tube with clofazimine. All three compounds produced peaks in a PDA chromatogram.

Figure 3.11. shows the chromatogram of an extracted mixture of DDS, rifampicin and clofazimine with spectra of each peak included. By comparing the spectra produced with reference absorbances (Budavari, 1989; British Pharmacopoeia, 1980) and with analysed standard controls, each peak was identified. The first peak at 2.9 mins. is due to dapsone as indicated by strong absorbances at 262 and 300 nm. The small peak at 3.43 mins. appears to be a derivative of rifampicin due to its very similar spectrum and presence in the chromatogram of rifampicin on its own. Rifampicin appears at 4.03 mins. and its presence is confirmed by its spectrum and since the extinction ratio at 334 to 475 nm, 1.75, is the same as the quoted value (Budavari, 1989). Clofazimine appears at 5.49 mins. As shown in this figure, there is no interference between the peaks of rifampicin and clofazimine. However, since rifampicin

Figure 3.9.(i) (a) A plot of the peak height ratio versus drug concentration for clofazimine. Although all points appear to be very close to the linear regression line, enlargment of the region around the points of lowest value (b), shows that the regression line is very distant from these points. (See figure 3.9.(ii) for the log plot of this data.)



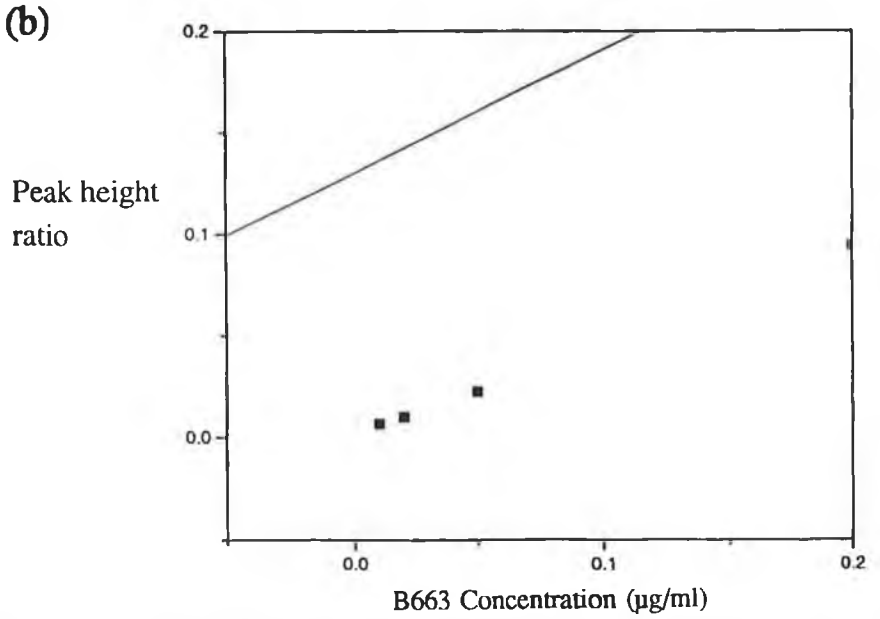


Figure 3.9.(ii) A log plot of the peak height ratio versus concentration data for clofazimine used in figure 3.9.(i). Since the log value is used, the resulting linear regression is not biased towards the larger values as was the case in figure 3.9.(i)(b). Therefore this regression line can be used across the full concentration range.

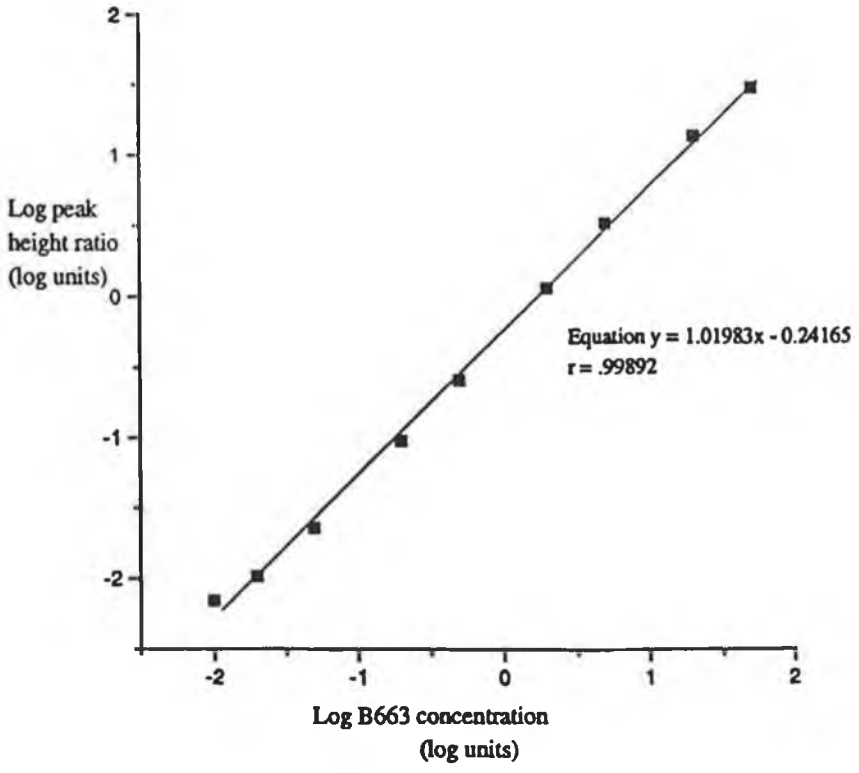


Figure 3.10.(i) Log plots of PHR vs. drug concentration for; (a) clofazimine, (b) B749, (c) B3954 and (d) B4090. The linearity across the whole range is indicated in each case by the proximity of each regression factor (r) to a value of 1.

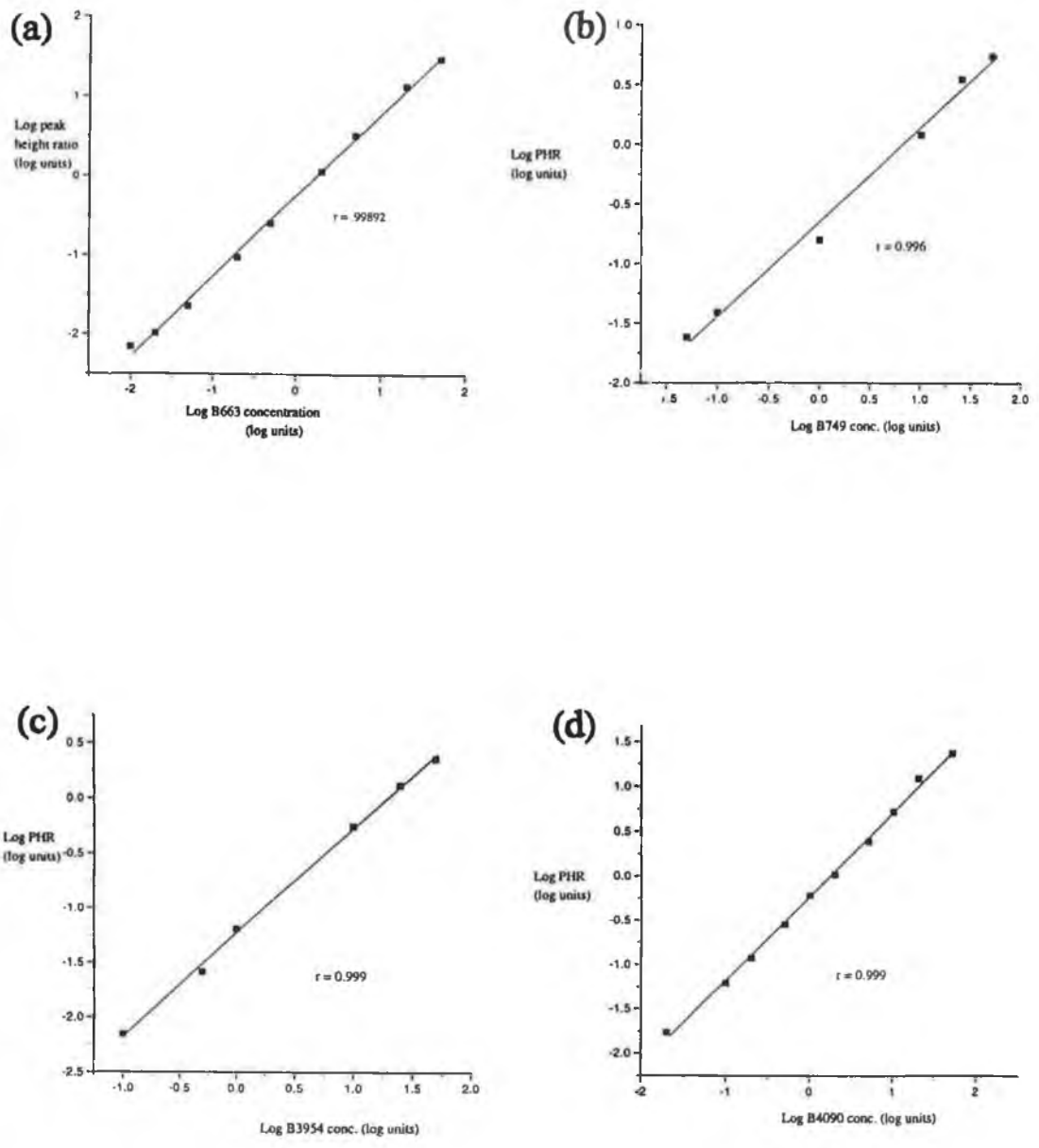


Figure 3.10.(ii) Log plots of PHR vs. drug concentration for; (a) B4100, (b) B4103 and (c) B4154. The linearity across the whole range is indicated in each case by the proximity of each regression factor (r) to a value of 1.

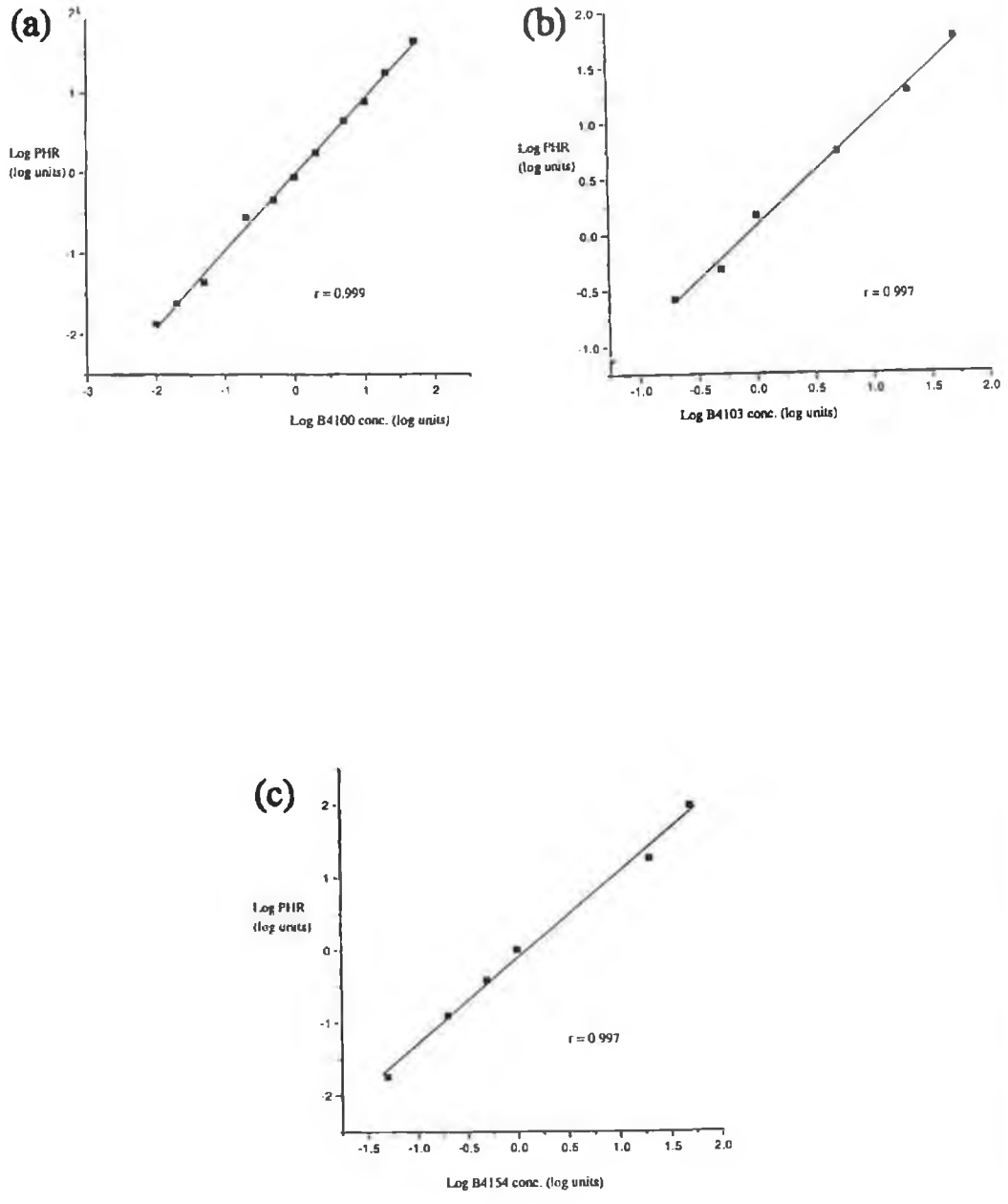
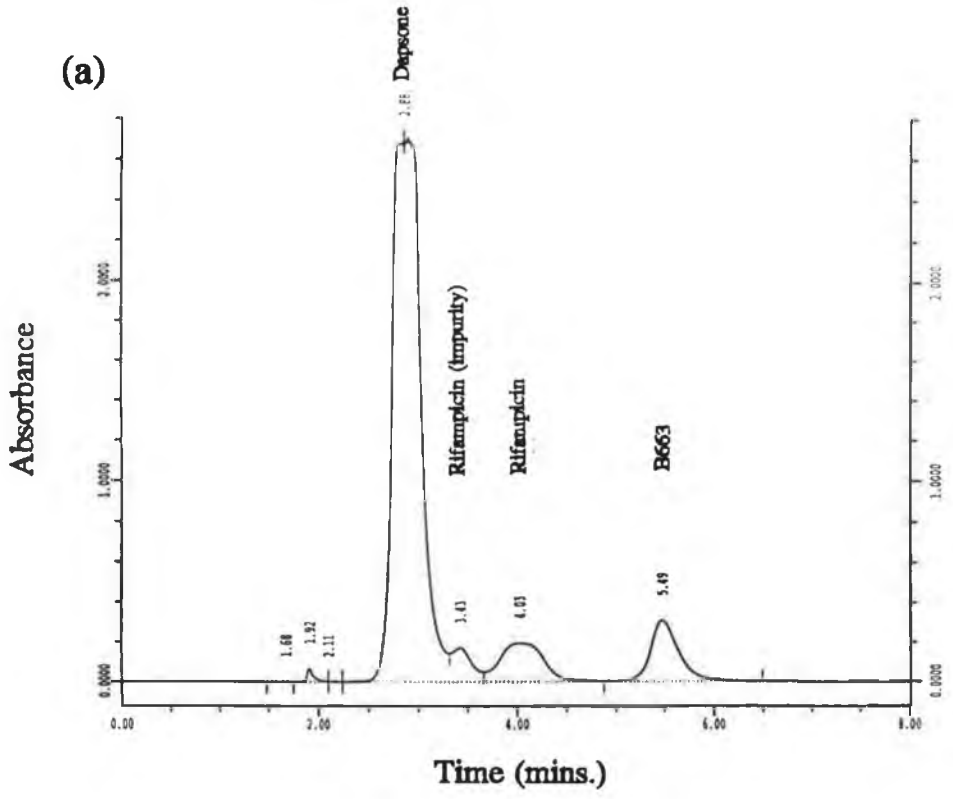


Figure 3.11. (a) The HPLC chromatogram of an extracted mixture of dapsone, rifampicin and clofazimine. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min, with PDA detection. The peaks and their retention times are shown on the chromatogram. The spectra of the 4 major peaks are also included (b).



appears as a broad peak at 4.03 mins. and has a spectrum quite similar to phenazines (see Figure 3.12.) under the chromatographic conditions, the IS used in this analysis would be unsuitable.

3.14. RESULTS FROM CHEMICAL ANALYSES OF PHENAZINES

3.14.1. TLC analysis of rimino-phenazines

Initially, compounds were spotted onto silica plates and various mixtures of non-polar and polar organic solvents were employed for development. A mix of DCM with methanol (7:1 v/v) was found to be optimal and Figure 3.13. shows some typical results seen with phenazines on silica plates. The r_f values for these compounds are shown in Tables 3.12(i) - (iii). The silica plate used are coated with a fluorescent dye to aid visualisation. However, no clear additional spots were visible under a short- or long-wave U.V. lamp.

Pharmaceutical grade clofazimine (clofazimine (p)), supplied by Ciba Geigy, and purified B4100 gave single spots on these plates but all other rimino-phenazines tested gave a major spot with several minor spots, indicating the presence of unknown impurities in these compounds. Certain compounds can be unstable on silica potentially producing additional spots so samples were also spotted onto alumina plates. Alumina is less polar than silica and so a mix of DCM - methanol 32:2 (v/v) was found to give good resolution for separation. Figure 3.14. shows typical TLC results with alumina plates, with the r_f values for the compounds analysed shown in Table 3.13. In general similar patterns of resolved spots were seen with silica and alumina plates giving no evidence for silica instability of these compounds. Additional alumina spots seen with some compounds may reflect differences in the amount of compound spotted onto the plates.

3.14.2. Determination of purity by HPLC

TLC gives a lot of qualitative purity information, but these results are generally difficult to quantify. The HPLC method developed was employed to give additional quantitative information on purity. Analysis of the rimino-phenazines with the PDA detector showed that impurities had similar spectra to that of typical phenazines, indicating that these were likely other forms of phenazine derivatives produced as side reactions in the original synthesis (see Figure 3.15.). Since all the compounds had a maximal absorbance around 285 nm, the relative percentage of each present could be quantified by integrating the absorption area of each peak at 285 nm. Figure 3.16. shows a chromatogram of B4100 with the main peak off scale to show

Figure 3.12. A comparison of the spectra of rifampicin and clofazimine under HPLC conditions. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min. The comparison shows how both compounds have absorbances in similar regions of the UV and visible spectrum.

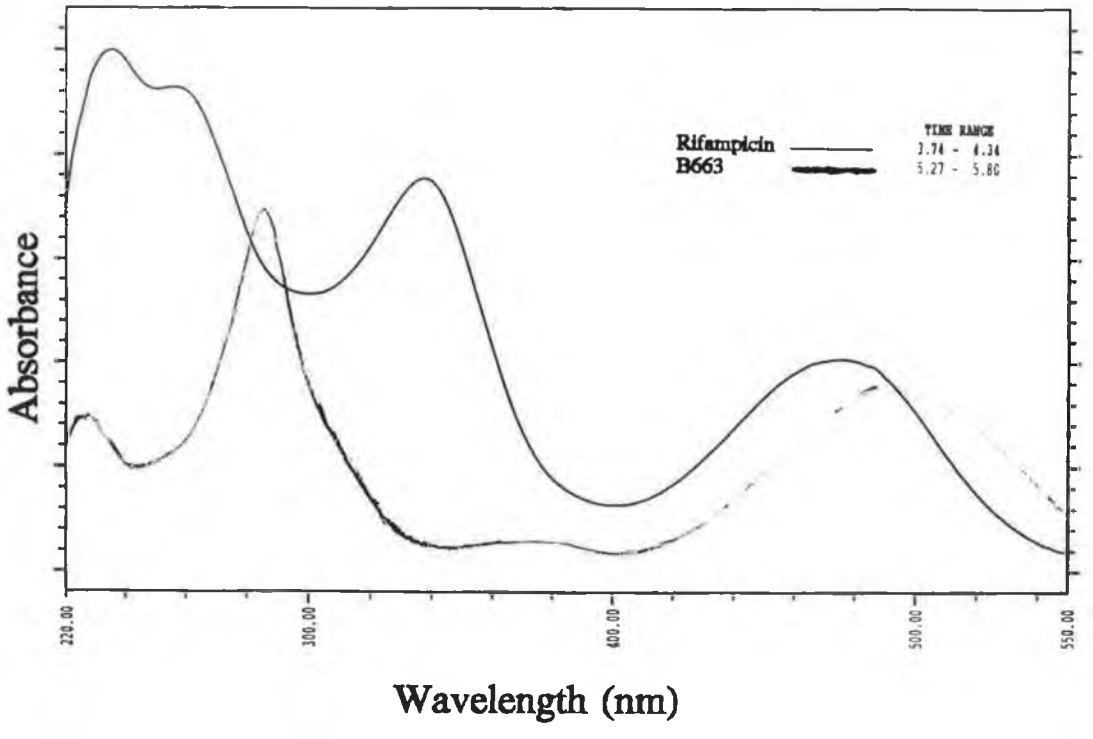


Figure 3.13. A picture of silica TLC plates used to indicate the presence or absence of impurities in the rimino-phenazine compounds. Compounds were spotted onto the baseline of a silica TLC plate and developed with a mixture of 12 % (v/v) methanol and 88 % (v/v) DCM. The outlined areas indicate the presence of spots visible when the plate is wet with solvent.

From left to right each plate shows the following compounds separated.

Plate (i) shows B749, B3832, clofazimine (s), clofazimine (p), and B628.

Plate (ii) shows B4090, B4103, B4154 and B4100.

Plate (iii) shows B3955, B3976, B3954, and B3640.

Plate (iv) shows multiple spots of purified B4100, impure B4100, purified, impure and purified B4100 with a solvent of 90 % DCM to better resolve each spot.

Plate (v) shows B3955, B3976, B3954, and B3640 with a solvent of twice the original methanol concentration to migrate the baseline spots further.

The r_f values of each spot are reported in table 3.12 (i) - (iii).

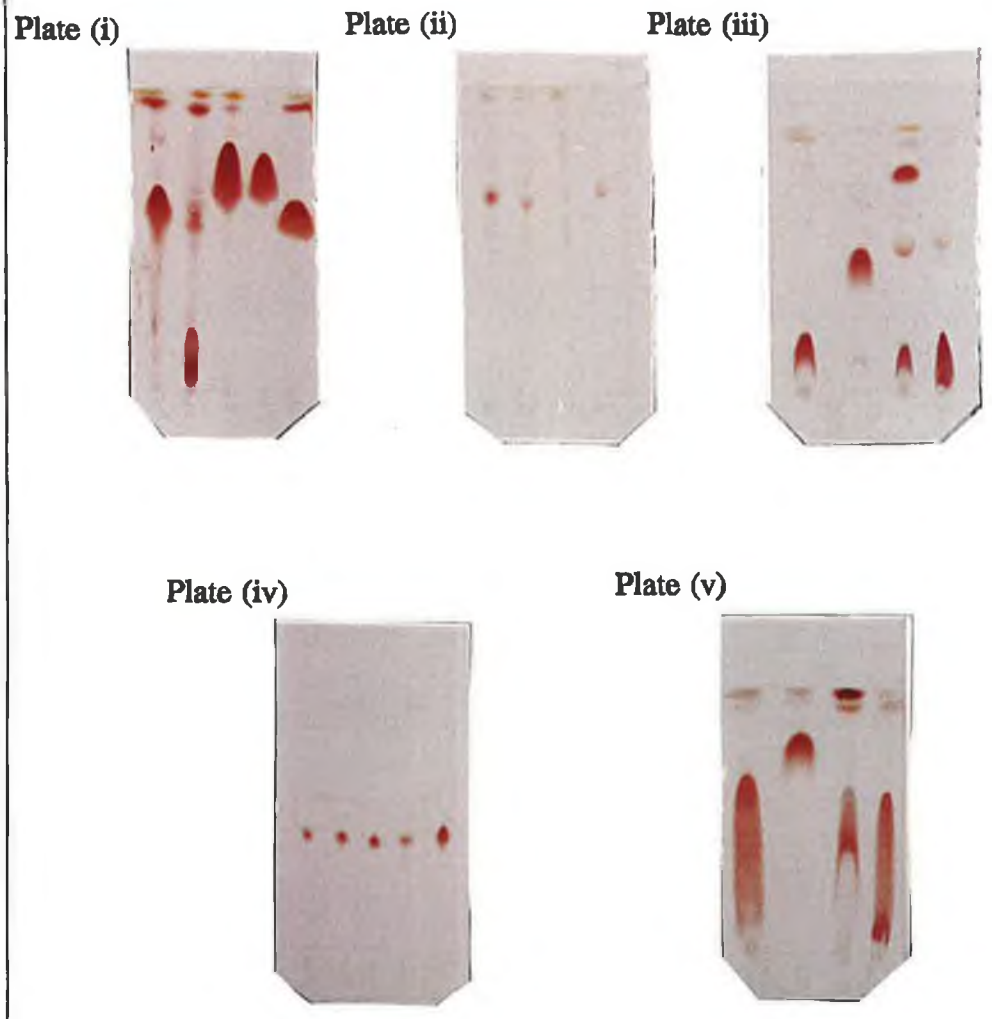


Table 3.12.(i).

The r_f values of spots resolved under silica TLC. Samples were spotted onto the baseline of a silica TLC plate and developed with a solvent mix of 12 % (v/v) methanol and 88 % (v/v) DCM. Purity is indicated by the presence of a single spot. The actual TLC plates are shown in Figure 3.13 (plates i-iii).

Compound	Main spot	Other spots
B749	0.60	0.23, 0.81, 0.93, 0.95.
B3832	0.80	0.16, 0.54, 0.59, 0.64, 0.90, 0.95.
clofazimine (s)	0.70	0.91, 0.95.
clofazimine (p)	0.70	(only single spot)
B628	0.58	0.91, 0.94.
B4090	0.60	0.55, 0.71, 0.89, 0.93.
B4103	0.57	0.48, 0.64, 0.69, 0.92.
B4154	0.92	0.49, 0.60, 0.77.
B4100	0.61	0.70, 0.94.
B3955	0.23	0.00, 0.64, 0.84, 0.94, 0.97.
B3976	0.51	0.13, 0.70, 0.97.
B3954	0.81	0.00, 0.16, 0.52, 0.91, 0.97.
B3640	0.16	0.55, 0.93, 0.97.

Table 3.12.(ii).

The r_f values of spots resolved under silica TLC. Samples were spotted onto the baseline of a silica TLC plate and developed with a solvent mix of 10 % (v/v) methanol and 90 % (v/v) DCM. This mixture was different from that described in Table 3.12.(i). to improve spot resolution. Purity is indicated by the presence of a single spot. The actual TLC plates are shown in Figure 3.13 (plate (iv)).

Compound	Main spot	Other spots
B4100	0.43	0.51, 0.94.
Purified B4100	0.43	(only single spot)

Table 3.12.(iii).

The r_f values of spots resolved under silica TLC. Samples were spotted onto the baseline of a silica TLC plate and developed with a solvent mix of 24 % (v/v) methanol and 76 % (v/v) DCM. This mixture was different to that described in Table 3.12.(i). to improve spot resolution. Purity is indicated by the presence of a single spot. The actual TLC plates are shown in Figure 3.13 (plate (v)).

Compound	Main spot	Other spots
B3955	0.62	0.66, 0.92, 0.96, 0.97.
B3976	0.79	0.93, 0.97.
B3954	0.58	0.92, 0.96.
B3640	0.58	0.92, 0.96.

Figure 3.14. A picture of alumina TLC plates used to indicate the presence or absence of impurities in the rimino-phenazine compounds. Compounds were spotted onto the baseline of an alumina TLC plate and developed with a mixture of 6 % (v/v) methanol and 94 % (v/v) DCM. The outlined areas indicate the presence of spots visible when the plate is wet with solvent.

From left to right each plate shows the following compounds separated.

Plate (i) shows B749, B3832, clofazimine (s), clofazimine (p), and B628.

Plate (ii) shows B3955, B3976, B3954, and B3640.

Plate (iii) shows B4154, B4103, B4100, and B4090.

Plate (iv) shows multiple spots of purified B4100, impure B4100, purified and impure B4100.

Plate (v) shows multiple spots of pure, impure, pure and impure clofazimine.

The r_f values of each spot are reported in table 3.13.

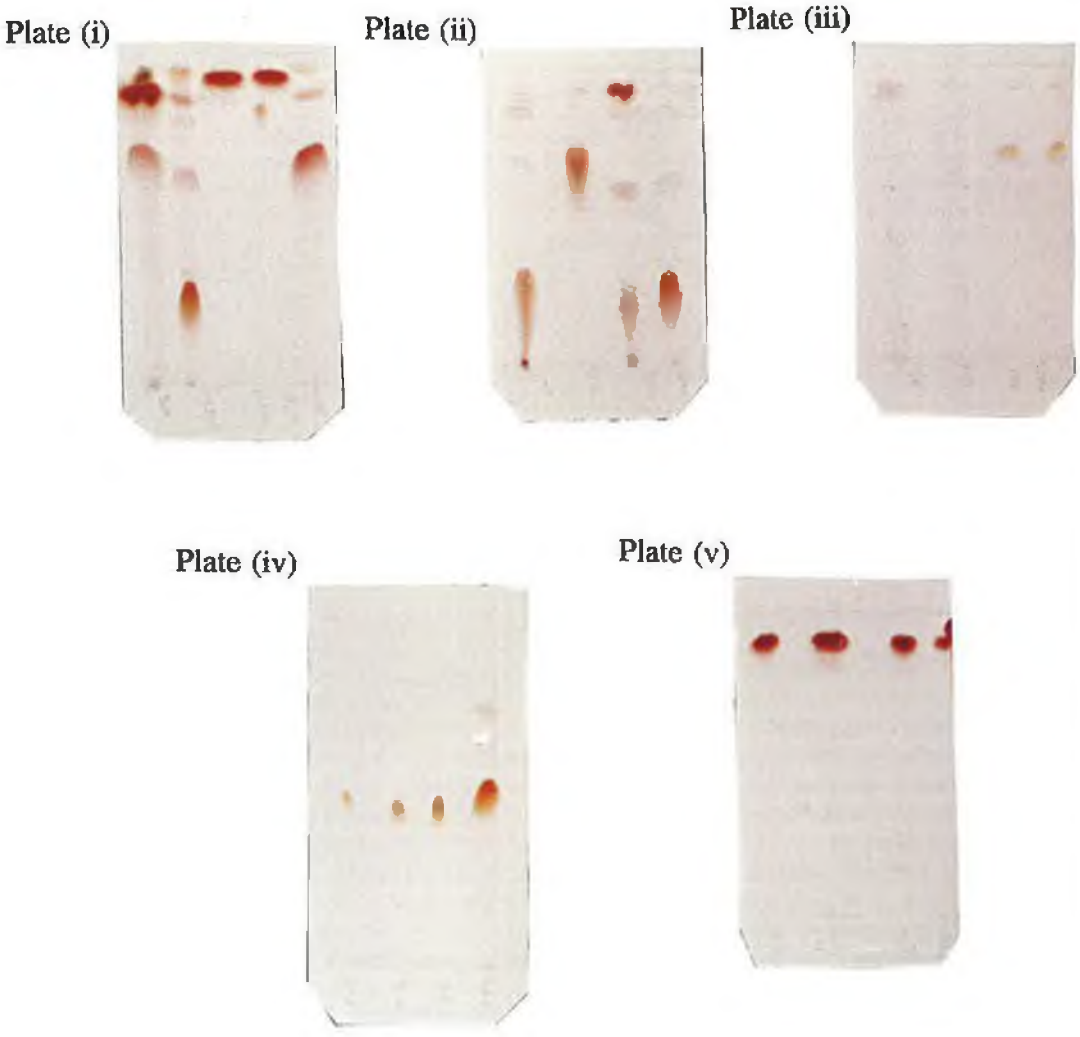


Table 3.13.

The r_f values of spots resolved under alumina TLC. Samples were spotted onto the baseline of an alumina TLC plate and developed with a solvent mix of 6 % (v/v) methanol and 94 % (v/v) DCM. Purity is indicated by the presence of a single spot. The actual TLC plates are shown in Figure 3.14 (plates (i) - (v)).

Compound	Main spot	Other spots
B749	0.87	0.63, 0.69, 0.92.
B3832	0.28	10.19, 0.63, 0.79, 0.86, 0.91, 0.94.
clofazimine (s)	0.92	0.85, 0.95.
clofazimine (p)	0.92	(only single spot)
B628	0.69	0.87, 0.94, 0.96.
B3955	0.28	0.00, 0.69, 0.87, 0.91.
B3976	0.71	0.93.
B3954	0.93	0.00, 0.21, 0.26, 0.60, 0.85.
B3640	0.28	0.62, 0.85, 0.94.
B4154	0.91	0.10, 0.41, 0.56.
B4103	0.62	0.22, 0.93.
B4100	0.69	0.93.
B4090	0.71	0.93.
B4100	0.56	0.23, 0.80, 0.89.
Purified B4100	0.56	(only single spot)
clofazimine (p)	0.92	(only single spot)
clofazimine (s)	0.92	0.82.

Figure 3.15. Spectra of the impurity peaks in a B4100 chromatogram. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min, with PDA detection. This figure illustrates that the impurities have a typical phenazine absorption spectrum. (See figure 3.16 for the chromatogram of these compounds and B4100).

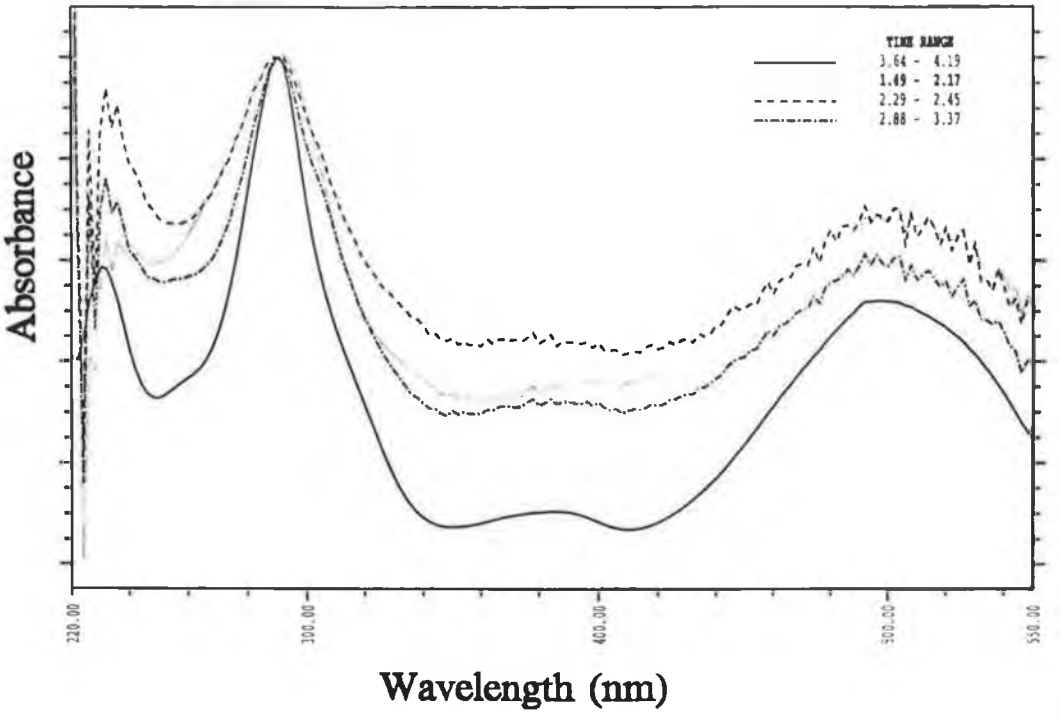
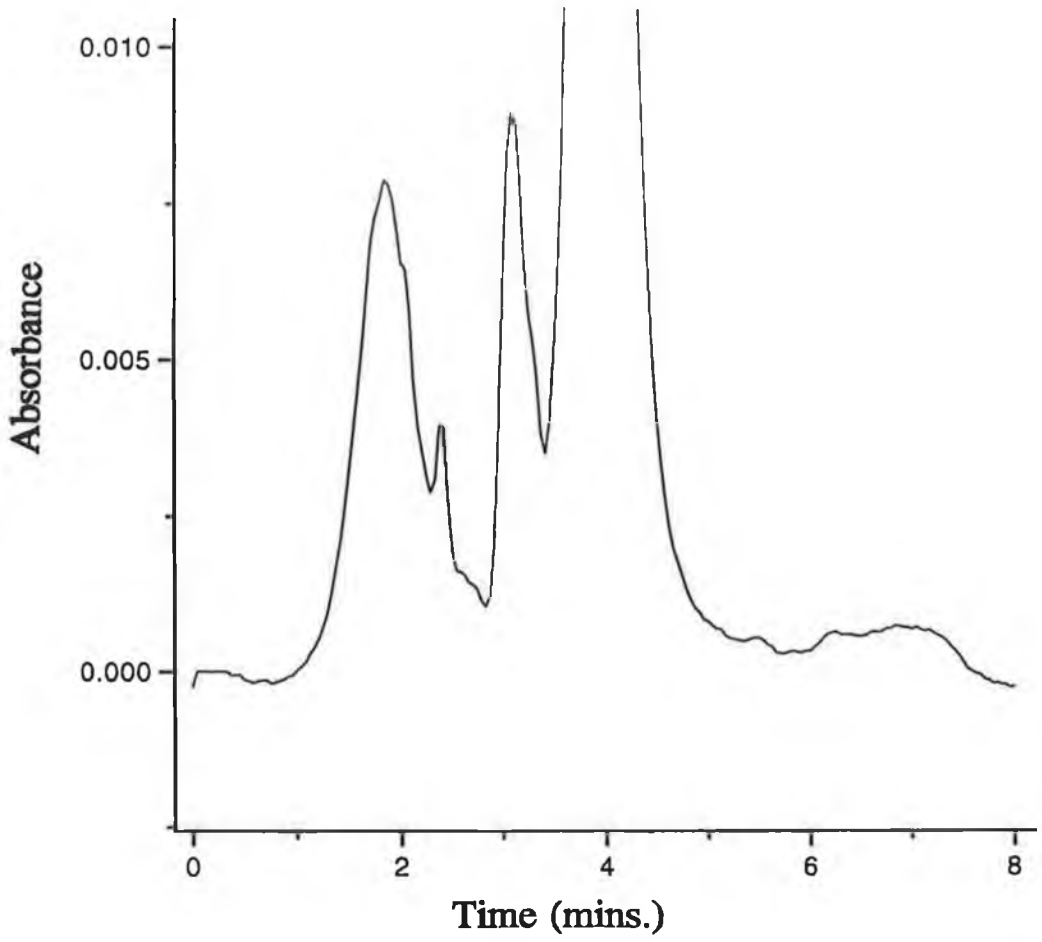


Figure 3.16. The HPLC chromatogram of B4100 with an expanded view of the baseline absorbance showing impurity peaks. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min, with absorbance monitored at 285 nm. (See figure 3.15 for the spectra of each peak.)



the impure peaks. Table 3.14. shows the purity determinations of these compounds derived by monitoring absorption at 285 nm. With the exceptions of B4154 (64 % pure) and B749 (46 % pure), which are particularly impure, all the compounds used in animal studies were relatively pure (greater than 80 %). Comparison of the PDA chromatograms of B3954, B3955, and B3976 with B3640 indicated that the principal contaminant in these amino acid derivatives was the parent compound B3640.

This method was also used to compare extracted and unextracted samples of each agent to confirm their stability under the NaOH/DCM extraction procedure. Comparison of the chromatograms and spectra for each agent showed no differences for any of the compounds indicating their stability in the extraction process. (see Figure 3.17.)

Attempts were made to develop a method of purifying these compounds since absolute purity is a prerequisite for most pharmaceutical agents. Purification of rimino-phenazines by HPLC was unsuccessful due to the low saturation concentration of the reverse phase column. Addition of greater than 10 µg of drug to a semi-preparative column produced peak broadening which prevented resolution of the impurities present. It was then decided to develop a purification method based on conventional column chromatography.

3.14.3. Column chromatography of B4100 as a model for other rimino-phenazines

B4100 was used to develop a general method for purification since it was already relatively pure and represented the substitution class most likely to supersede clofazimine. Initial attempts to increase the polarity of the solvent mix on the column to elute pure B4100 were unsuccessful due to the limited solubility of B4100 in more polar solvents such as methanol and, particularly, ethyl acetate. This problem was overcome by adding 5 ml of 5 M NaOH to a 40:60 (v/v) mix of methanol and DCM, which reduced the charge on the phenazine and hence, made elution faster and easier. The eluted pure compound was removed from the solvent on a rotary evaporator. TLC and HPLC analysis indicated that this method purified B4100 (99.5% by HPLC) and this was further verified by NMR spectroscopy (see section 3.14.4.). A 20 cm silica column was capable of purifying approximately 600 mg of B4100 at a time.

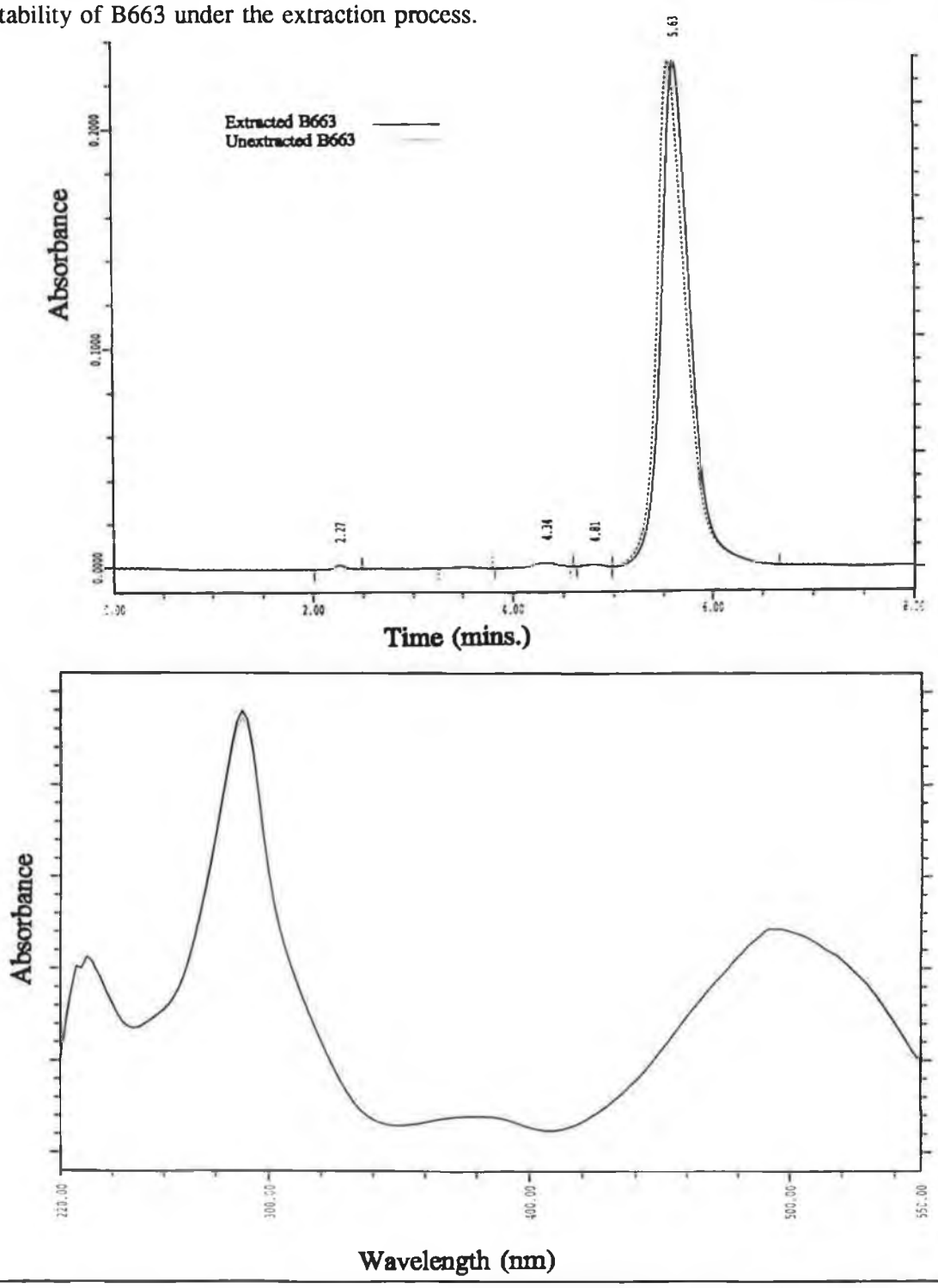
Purification using an alumina column was also investigated. Initially mixes of 50 to 90 % (v/v) ethyl acetate in DCM were investigated since alumina is partially soluble in methanol. This proved unsuccessful because increasing the solvent polarity with ethyl acetate also reduced the solvent's capacity to dissolve B4100. Addition of 20 ml of methanol to the head of the

Table 3.14.

A comparison of the purity of the rimino-phenazine compounds used in this investigation as assessed by measuring the peak areas of the peaks produced by HPLC analysis. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml/min, with absorbance monitored at 285 nm. The peaks were integrated by the HPLC software to give the peak area value. Purity varies among the synthesised derivatives from 99.0 % with B3976 to 46 % with B749.

Rimino-phenazine	**% Purity**
clofazimine (p)	99.8
clofazimine (s)	98.1
B4090	88.9
B4100	93.1
B4103	82.4
B4154	64.1
B3640	97.7
B3954	84.5
B3955	98.4
B3976	99.0
B749	46.0

Figure 3.17. (a) A comparison of the chromatograms and (b) spectra of an identical concentration of extracted and unextracted clofazimine. The HPLC conditions were a Bondclone C_{18} column, with a mobile phase of 40 % (v/v) THF, 60 % (v/v) of 1 % (v/v) acetic acid and 2.5 mM hexane sulfonic acid, flowing at 1.5 ml /min, with PDA detection. The chromatogram and spectrum of the extracted sample have been moved slightly to make them visible. The identical nature of spectrum and chromatogram indicate the stability of B663 under the extraction process.



alumina column eluted the retained pure B4100 band. The added methanol caused the eluted B4100 to be contaminated with dissolved alumina. This was removed by mixing the column eluent with a mix of water, NaOH, Methanol and DCM (200:2:200:600 respective volumes) in a separating funnel, the purified B4100 extracting into the DCM layer which was removed and evaporated. Although capable of purifying B4100, this method was more laborious than using a silica column.

3.14.4. NMR spectra of rimino-phenazines

Representative spectra for rimino-phenazines could not be found in the chemical literature so pure clofazimine (p) was initially analysed on a 400 MHz spectrometer and the spectra interpreted with the help of Dr. Paraic James and by comparison with spectra of constituent groups found in the literature (Pochert, 1974). Spectra of B4090 and B4100 were interpreted by comparison with the clofazimine spectrum. All spectra were consistent with the chemical structures of each agent. Figure 3.18. shows the spectra obtained for B663 (p) and B4090. The spectrum of the initial B4100 and silica purified B4100 as shown in Figure 3.19. are very similar as might be expected. However, the purified B4100 spectrum was better resolved and the small doublet at 2.9 ppm caused by an unidentified impurity was removed.

3.14.5. IR spectra of rimino-phenazines

The spectra of the synthesised clofazimine (s) and pharmaceutical grade clofazimine (p) were initially compared with the reference spectrum in the British Pharmacopoeia, (1980). As would be expected, the spectrum of clofazimine (p) was exactly identical. The clofazimine supplied by Dr. O'Sullivan, clofazimine (s), was also identical except for an additional peak at 1680 cm^{-1}, further confirming the presence of impurity (probably the C=N bond of the parent compound B628). The spectra produced by rimino-phenazine compounds is complex due to the number of atoms present and is therefore difficult to assign on an atomic basis. Impurity peaks are also difficult to pick out, and may be totally invisible due interferences from the main compound, as was the case with B4100 and its purified form. Figures 3.20.(i) - (iv). show the spectra of several of the compounds analysed. Certain features are evident in each which are consistent with their assigned molecular structures.

A broad band is evident in all spectra centred around 3500 cm^{-1} due to N-H stretches. A stronger band in this region is associated with the piperidine substituted compounds. This

Figure 3.18. 400 MHz spectra of (a) clofazimine (p), and, (b) B4090. The peak at 0.0 ppm in the B4090 spectrum is due to a calibrating standard of T.M.S. The peaks from 6 - 8 ppm are due to aromatic hydrogens on the phenazine and phenolic rings. The peak at 5.2 ppm is due to the anilino N-H, with a second peak appearing at this chemical shift in the B4090 spectrum due to the pyridine N-H. The peaks 3.7 and 2.9 ppm as well as other small peaks in the aromatic region are due to impurities. The other peaks from 1 - 3.5 represent absorbances from methyl and C-H hydrogens present in both compounds.

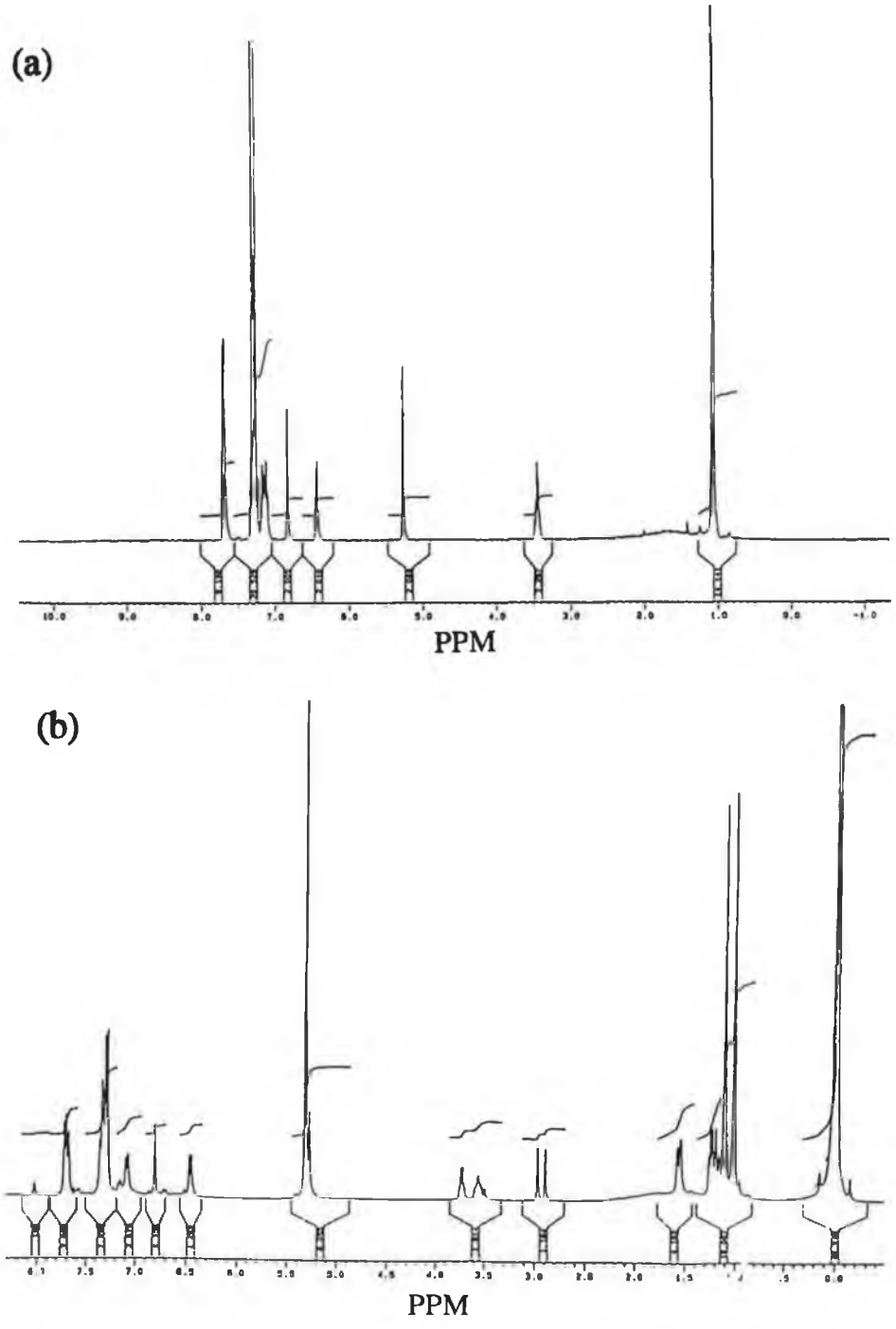


Figure 3.19. A comparison of the 400 MHz spectra of B4100, (a) before, and, (b) after, silica purification. Impurities in B4100 are indicated by small baseline peaks especially evident at 2.9 ppm and the poor resolution of the peak shapes. These peaks are absent and the peaks better resolved in the purified form of B4100.

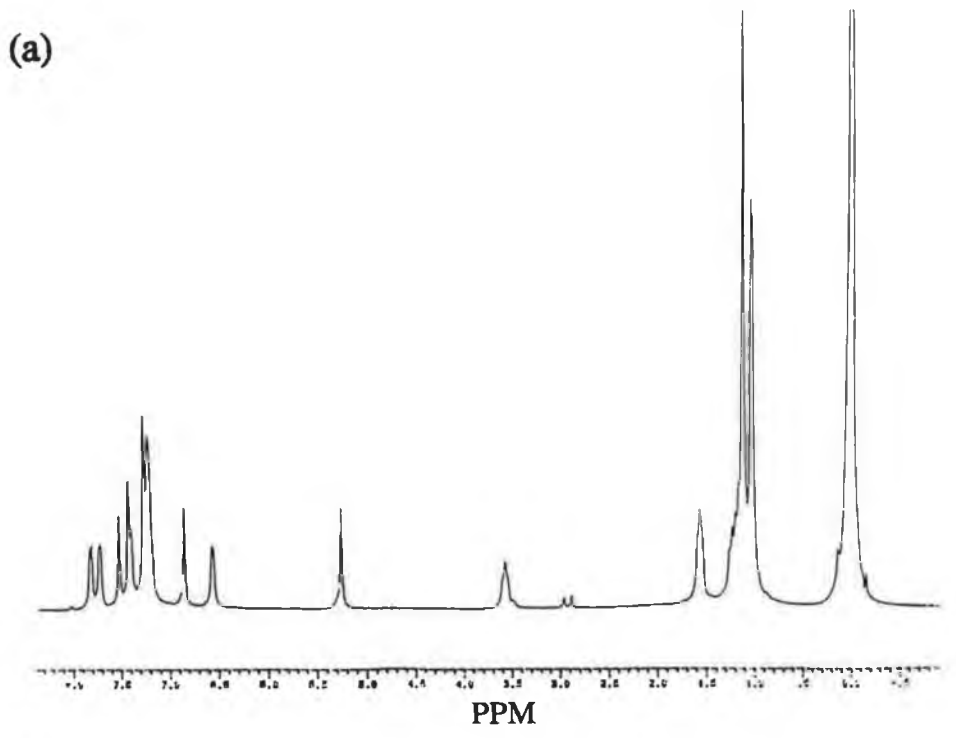


Figure 3.20.(i). IR spectra of, (a) clofazimine (p), and, (b) clofazimine (s), prepared in KBr discs, in the range 4,500 - 400 cm^{-1}. (See text for identification of the significant bands).

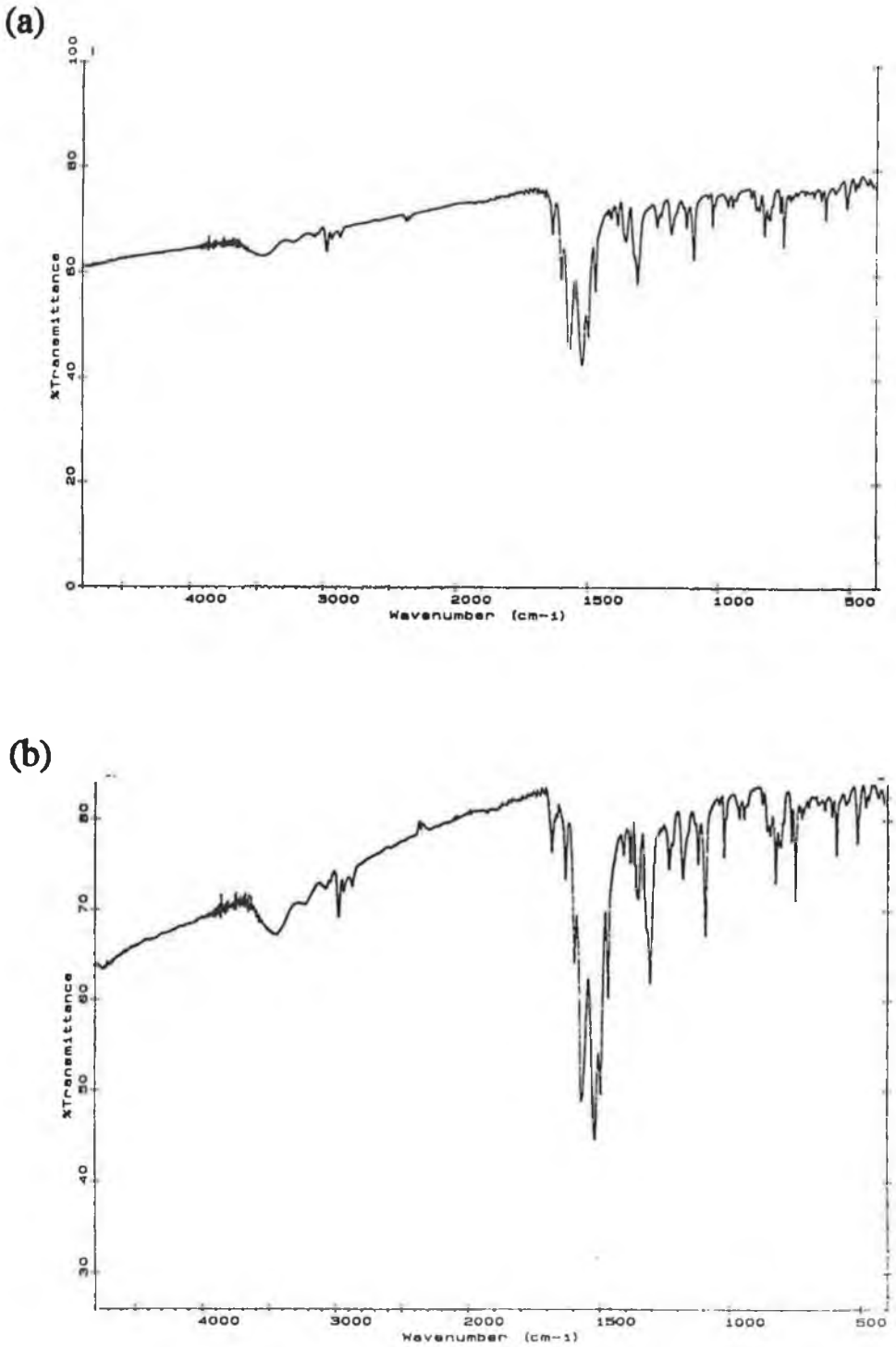
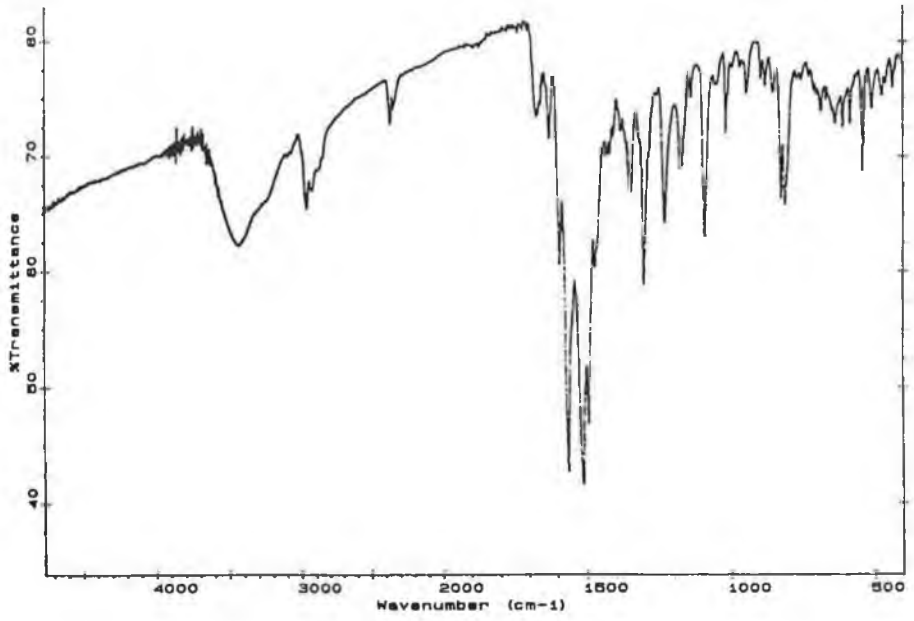


Figure 3.20.(ii). IR spectra of, (a) B4090, and, (b) B4100 prepared in KBr discs, in the range 4,500 - 400 cm^{-1}. (See text for identification of the significant bands).

(a)



(b)

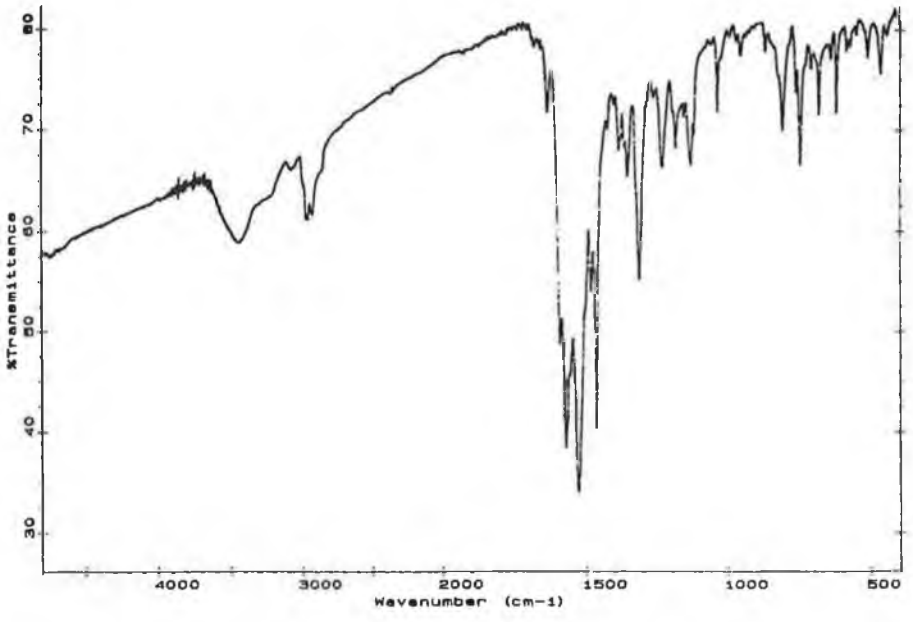


Figure 3.20.(iii). IR spectra of, (a) B4154, and, (b) B4103, prepared in KBr discs, in the range 3,700 - 400 cm^{-1}. (See text for identification of the significant bands).

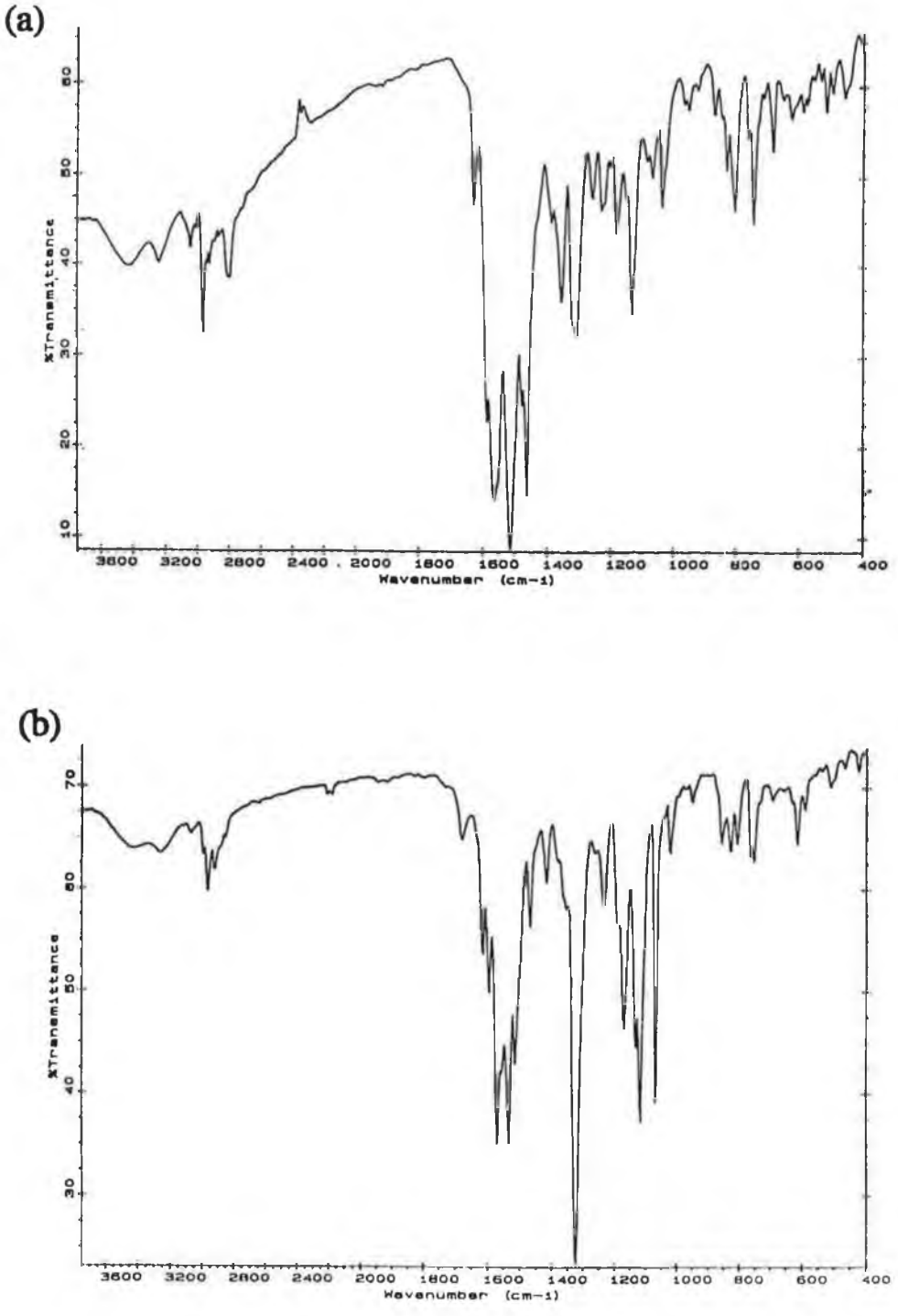
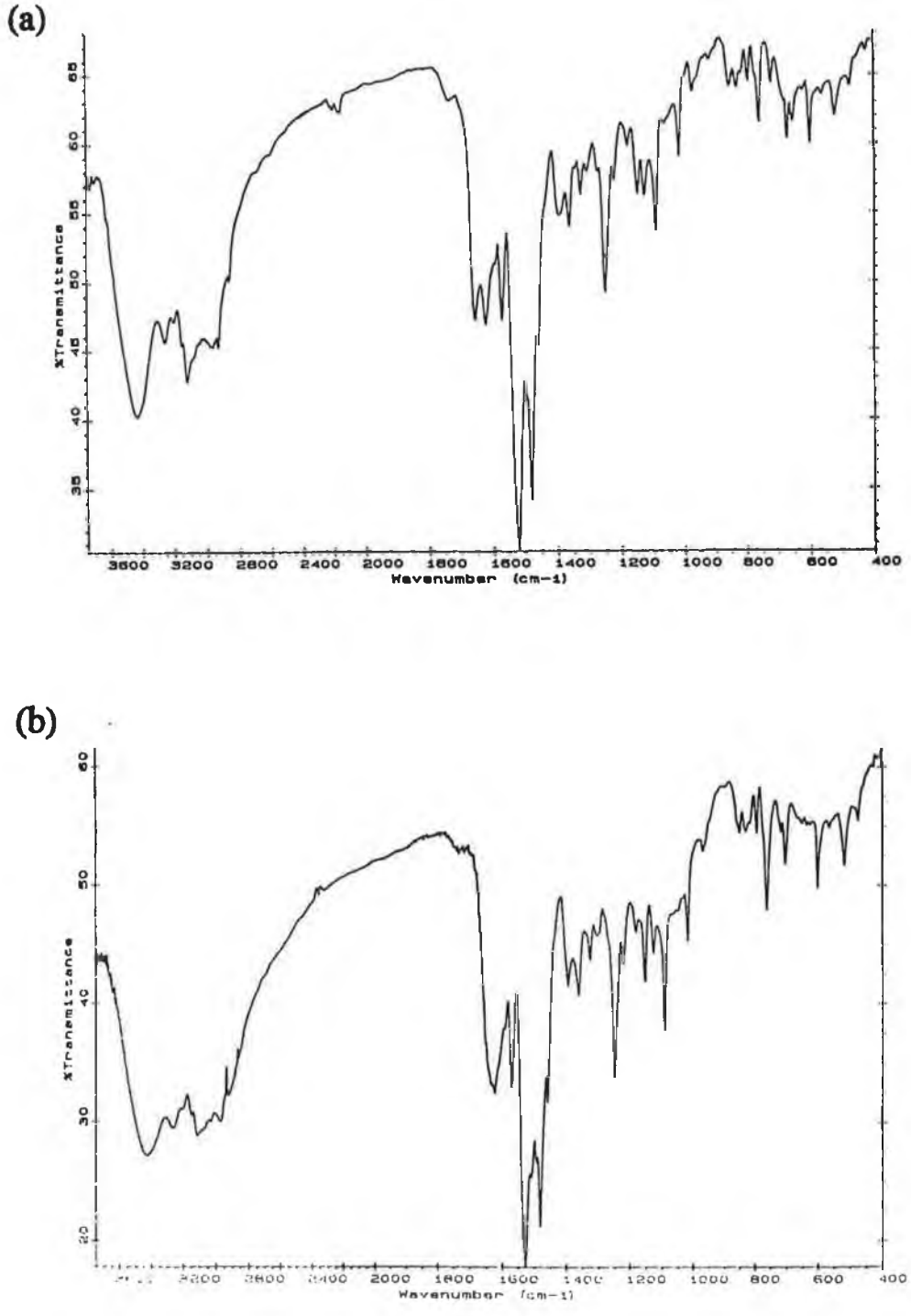


Figure 3.20.(iv). IR spectra of, (a) B3954, and, (b) B3955 prepared in KBr discs, in the range 3,700 - 400 cm^{-1}. (See text for identification of the significant bands).



band is even more intense in the amino acid substituted compounds due to the absorption of the amine stretches present. The NH_2 group also causes the strong band around 1550 cm^{-1} evident in the two examples. Small peaks due to aromatic C-H stretches are evident around 3060 cm^{-1} in all spectra. CH_3 and CH_2 vibrations are also evident in the peaks from 2970 - 2870 cm^{-1}. Aromatic ring stretches, C=C, cause the most noticeable bands from 1600-1450 cm^{-1} except in the amino acid substituted analogues where the NH_2 absorbance and amide carbonyl C=O absorbance at 1620 cm^{-1} cause them to be partially obscured. The distinctive spectrum of B4103 is due to the CF_3 stretch at 1320 cm^{-1} with C-F stretches causing strong absorbances at 1150 - 1050 cm^{-1}. A strong band at 1090 cm^{-1} is seen on all the mono-chlorinated derivatives due to the C-Cl stretch whilst the dichloro analogues (B4100 and B4154) have bands at 1030 and 1120 cm^{-1}. C-H stretches in the phenazine ring give rise to several bands in the region 900-680 cm^{-1}.

3.15. SIMPLE EXTRACTION OF PHENAZINES USING DCM

The method described extracts any of the tested phenazines into dichloromethane (DCM) by mixing with sodium hydroxide. Under strongly alkaline conditions basic compounds such as phenazines become less polar, increasing their hydrophobic affinity. Due to the inherent polarity of the lone pair of electrons in the nitrogen molecule, many rimino-phenazines are not fully soluble in completely non-polar organic solvents , such as hexane, and organic solvents with a partial polar character such as chloroform and DCM are ideal organic extractants. DCM is particularly useful due to its volatility and low boiling point (40 ^{0}C) which make evaporation for reconstitution into HPLC solvents particularly easy and rapid.

Tissue samples must be homogenised first to disaggregate the structural components of tissue and allow complete equilibration of phenazines between the biological matrix containing them. Serum samples only need to be mixed with the extraction mixture since clofazimine exists in serum as part of a liquid phase mixed lipid micelle (β-lipoprotein) (Conalty and Jina, 1971). Complete equilibration after mixing is further assured by placing the extraction mixture in a sonicating bath, which has the effect of emulsifying the organic phase into the aqueous phase promoting rapid equilibration. The two phases are clarified and separated from each other by centrifugation, which draws the heavier DCM phase to the bottom of the mixing tube and precipitates heavy biological particles as a ring at the aqueous interface. Removing only part of the organic phase reduces contamination by the aqueous phase which could cause problems for later HPLC analysis.

3.15.1. Fat and faecal extraction

Extraction of phenazines from fat deposits poses difficulties for a simple organic extraction since lipids also tend to co-extract with the clofazimine into the organic phase. Although these interfering compounds are soluble in the THF used to reconstitute samples for HPLC analysis, they have a very high affinity for the stationary phase of the HPLC column. They produce interfering peaks and ultimately a net change in the physicochemical-properties of the stationary phase, which is difficult to remove. This problem is largely overcome by heating the extracted phenazine with alcoholic sodium hydroxide converting the fats present to water soluble soaps which are removed by washing the organic phase with water.

Extraction of faecal samples also causes problems due to the presence of interfering compounds. After extraction of blank faecal samples, the organic layer has a pale yellow-green colour and these compounds produce peaks which co-elute in the time range of many of the phenazines with HPLC, reducing their limit of accurate quantification. The colour and spectrum of these interfering substances combined with their origin indicates that they are most likely a mixture of faecal porphyrins (breakdown products of heme) with bile salts also possibly being present. The net effect of these interfering compounds is to reduce the limit of quantification of faecal phenazine levels to approximately 100 ng/g of dry faeces. Although this interference from compounds in the faeces could not be overcome, phenazines levels above this limit of detection could be accurately measured in these samples.

3.15.2. Advantages/disadvantages of the DCM extraction system

Extraction with DCM is superior to previous extraction systems because :- it can be used to extract rimino-phenazines stably from all biological samples, its simplicity, allowing large volumes of samples to be extracted, its compatibility with a range of downstream analytical methods, especially HPLC, and flexibly, allowing extraction of a large variety of rimino-phenazines. The most significant drawback of alkaline DCM extraction is the utilisation of a chlorinated organic solvent whose use is regulated under the Montreal Agreement on chlorinated chemicals and the ozone layer.

3.16. DEVELOPMENT OF HPLC METHOD FOR RIMINO-PHENAZINES

Initially it was decided that a reversed-phase HPLC method would be the most suitable for accurately and sensitively measuring the diverse range of rimino-phenazines used in this project. Simple spectrophotometric methods are less sensitive, with associated problems due to co-extracting impurities. Gas chromatography (GC) is a more sensitive method for compound measurement, but, rimino-phenazines have boiling points above 200 ^{0}C and often decompose at around this temperature making them unsuitable for analysis by GC.

The highly hydrophobic nature of rimino-phenazines gives them a particularly high affinity for the RP stationary matrix and at neutral or alkaline pH this affinity is high enough to preclude analysis with conventional HPLC solvents due to the time necessary for elution. As the pH of the mobile phase decreases, the phenazine molecule becomes more charged reducing affinity for the stationary phase. Three organic modifiers are most appropriate for RP HPLC, methanol, acetonitrile and THF, in order of increasing organic solvability. Conventional RP

columns are limited to mobile phases with a pH of greater than 2 units and generally operated above a pH of 2.5 due to the acid lability of the silanol-C_{18} bond. In practice, THF is the most suitable organic modifier for rimino-phenazine analysis, because of these practical limitations, and the need for a relatively high water content to carry the pH modifier.

3.17. HPLC CONDITIONS FOR ANALYSIS OF RIMINO-PHENAZINES

An optimal HPLC method should give a reproducible sharp peak for the analyte resolved from interfering peaks in as short a time as possible. Method development initially began based on a 40:60 THF - 0.5 % (v/v) acetic acid mobile phase as described by Gidoh and Tsutsumi, (1981) using a μBondapak C_{18} column. This system gave broad peaks for clofazimine with very long and variable retention times and was, therefore, unsuitable for analysis.

Although the stationary phase in an RP column is generally regarded as being wholly hydrophobic, the synthetic methods used to derivatise and render the silica hydrophobic with long carbon chains never fully derivatise all the free silanol groups. As a result, a certain portion of the column possesses free silanol groups and the column is said to be incompletely end-capped. Under acidic conditions, the free silanol groups become negatively charged and can cause a separate form of interaction with suitable polar molecular species. Chromatography of hydrophobic compounds displaying basic characteristic can be problematic because these compounds become positively charged in acidic pH and are attracted both to the hydrophobic and free silanol groups. Therefore the interaction of the basic hydrophobic compound becomes dependent on equilibrium with the hydrophobic and charged species. The net result is poorly resolved chromatographic peaks with variable retention time. This phenomena was thought to be one of the contributing factors to the initially poor chromatography of rimino-phenazines.

One solution to this problem is to include a hydrophobic ion-pairing reagent in the mobile phase. 1-Hexane sulfonic acid was chosen as ion-pairing agent since the 6 carbon chain gives it a strongly hydrophobic character giving strong affinity for hydrophobic compounds, whilst the sulfonic acid moiety is sufficiently charged in any RP HPLC mobile phase to be useful. Hexane sulfonic acid also has no significant absorbance at 285 nm and so does not interfere with phenazine measurement at this wavelength. It is thought that negatively charged sulfonic acid groups can charge couple with the positive groups induced in the phenazine compound by the pH of its environment. The hydrophobic part of the molecule is attracted both to the hydrophobic phenazine and the hydrophobic stationary phase. Whether the hexane sulfonic acid initially adsorbs onto the stationary phase producing an adsorbed ion-exchange

stationary phase, or ion pairs to the charged analyte in the mobile phase which then interacts as a neutral species is uncertain (Bidlingmeyer, 1992), but the net result is that interaction with the free silanol groups is prevented. Addition of 2.5 mM hexane sulfonic acid markedly reduced the peak width and reduced retention time (r_t), but the r_t still remained variable over the course of several sample injections.

As outlined in section 3.13.3., the continuing variance in r_t was ultimately traced to the evaporation of THF from the open mobile phase reservoir. Due its volatility, THF was constantly evaporating and thus reducing the hydrophobicity of the mobile phase over time. This problem was overcome by sealing the column eluent into the mobile phase reservoir bottle. Once the headspace in the bottle was saturated with THF, the constitution, and therefore, the properties, of the mobile phase remained constant, eliminitating the variability in r_t.

Using the modern computer controlled HPLC system with autosampler produced by Beckman also meant that once calibrated, the machine could automatically analyse and measure up to 96 samples. By using a photo-diode array (PDA) detector instead of a single wavelength U.V. detector, this system could also be used to visualise the elution spectra of the rimino-phenazines.

3.17.1. Internal standards in the HPLC analysis of phenazines

The retention time of each phenazine is individual due to differences in the side chain. The combination of the extraction and HPLC methods allowed the complete extraction of phenazines over a broad concentration range and the use of certain phenazines as internal standards (I.S.) for the analysis of other members of the group.

The use of an I.S. is important in the quantification of these agents because the volatile nature of the organic extractants used can cause variability due variable evaporation between samples and difficulties with accurate measurement of the small volumes of solvents used. Expression of the chromatographic results as the peak height ratio (PHR) of analyte to I.S. controls for these extraction and sampling errors. Any inaccuracy will affect both phenazines present by the same factor, with the overall ratio of one to the other remaining constant for a given concentration.

3.17.2. Methods of improving HPLC limits of quantification

The dilution factor associated with sampling 1.1 ml from the 2 ml of extracting DCM and loading 20 µl from the 60 µl reconstituted means that only 18.3 % of the phenazine present is being used for measurement. This was used for convenience and to allow complete automation of the HPLC analysis process. However, if greater sensitivity were required, removal of almost all the extraction DCM together with injection of all the reconstituted sample could bring the limit of quantification down to approximately 2 ng/ml. The sensitivity could be further improved by using larger sample volumes.

3.17.3. Accuracy and precision of the HPLC method

The low % RSD for inter- and intra- day analysis show the power of this method for measuring a broad range of rimino-phenazine concentrations. In combination with the full recovery of phenazines from a variety of biological samples, this method is the most suitable yet developed for a complete analysis of different synthetic phenazines in a variety of matrices.

3.17.4. Suitability of HPLC analysis with other anti-leprosy drugs

The results outlined in section 3.13.7. indicate that neither dapsone nor rifampicin interfere directly with the HPLC analysis of clofazimine. Unfortunately, the retention time of rifampicin could interfere with the B4100 I.S. that was used in project for clofazimine analysis, since it possesses a strong UV absorbance and has a similar retention time. However, this problem could be overcome by using the agent B3954 as an I.S. because it has a longer retention time than these three compounds. The method could be readily used for the analysis of clofazimine levels in subjects, receiving concurrent anti-leprosy therapies, with this modification.

3.18. CHEMICAL ANALYSIS OF PHENAZINES

Analysis of the phenazines used in this programme by TLC and HPLC indicated that all the phenazines tested, except the Ciba-Geigy sample, contained impurities. TLC gave a quick visual estimate of the proportional level of impurity present. The similarity of absorbance among the impurities meant that HPLC could be used to quantify the purity of each compound. These results indicate that trials of some of these phenazines would necessitate the purification of the agents, since impurities could potentially produce side effect or reduce the efficacy of the drug.

HPLC is not suitable as a method for this purification due to column saturation even at proportionately low concentrations. This is probably due to the type of interaction between the phenazines and the stationary phase of the column. However, these compounds could be purified using silica chromatography. A silica column is capable of purifying much larger amounts of phenazine. Although alumina chromatography was also capable of purifying B4100, this method was more laborious than using a silica column. The purification of these compounds could be assessed by TLC, HPLC and NMR. Besides verifying the success of the purification methods, NMR is useful in confirming the chemical structure of phenazine compounds. IR spectroscopy is also useful in confirming the chemical structure of the compounds tested, especially because it is a quick and simple technique, but is unsuitable in this case for investigation of purity.

3.19. SUMMARY

A simple liquid-liquid extraction system was developed which could quantitatively and stably extract all the rimino-phenazines tested from a variety of biological matrices. Coupled to this extraction system, a sensitive, accurate and precise reversed phase HPLC method was developed and validated for the analysis of these agents. The purity of these agents was also investigated using a number of chemical techniques, and a procedure developed for purifying these compounds.

CHAPTER 4

PRODUCTION AND CHARACTERISATION OF ANTISERA TO RIMINO-PHENAZINES

4.1. INTRODUCTION

This chapter describes the production of polyclonal antisera to rimino-phenazines. This antiserum was produced in rabbits, using drug-protein conjugates, and used to develop antibody based assays. The production and characterisation of the conjugates and the resultant antisera are described.

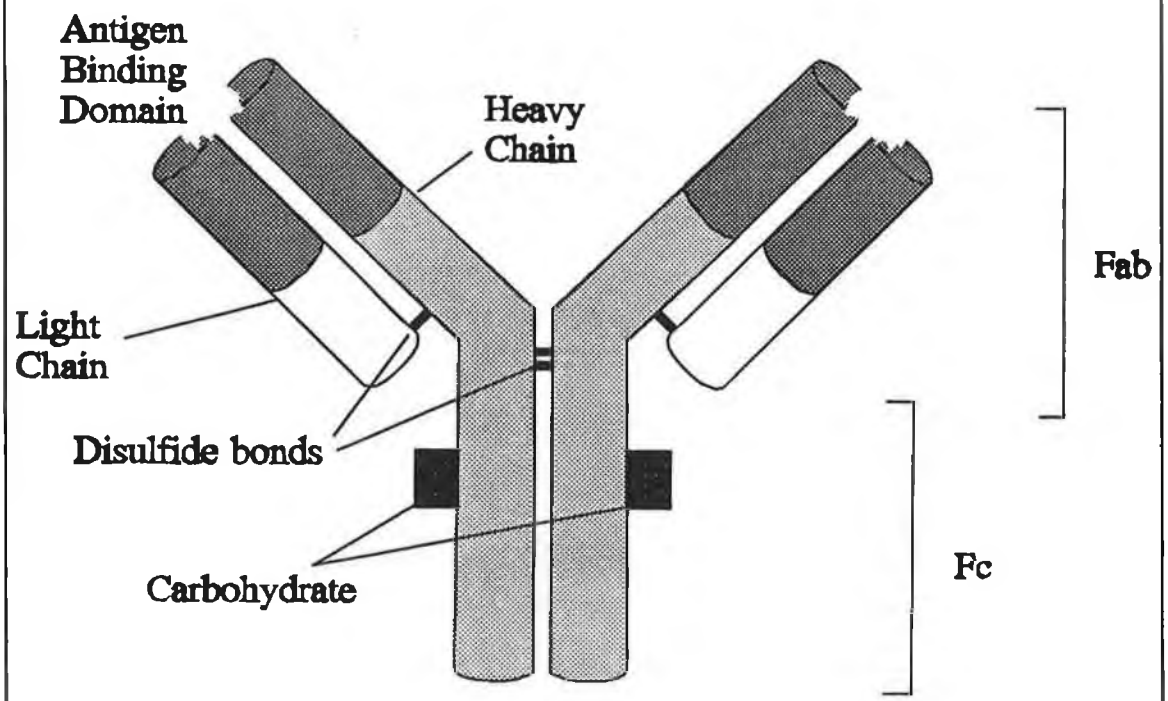
4.2. ANTIBODY PRODUCTION

4.2.1. Properties of antibodies

Antibodies are large molecular weight proteins produced by the plasma cells of the immune system and their principal function is the recognition and targeting of immune effector responses, to control perceived threats to the organism. Five major groups of antibody, IgA, IgD, IgE, IgG and IgM, have been described in most mammals (including man), each with slightly different characteristics and function. IgG immunoglobulin is widely distributed throughout the whole body and is normally the main class of antibody of interest in immune-based assays. As shown in Figure 4.1, it consists of two heavy and two light globular polypeptide chains linked together by disulfide bonds. Although in reality the resultant three dimensional structure is complex, it is commonly simplified to the Y-shaped structure shown in this figure. The heavy and light chains have respective molecular weights of 50 KDa and 25 KDa. The heavy chains are linked to each other by inter-chain disulfide bonds in the hinge region, and covalently bound to the each light chain by a single disulfide bond. Functionally two major element are evident, the recognition site [F(ab)] present on both arms, and, the effector binding [Fc] region (Roitt, 1980; Tijssen, 1985; Seabrook and Atkinson, 1991) . These parts of the basic antibody structure are illustrated in Figure 4.1

The primary function of the IgG molecule is the recognition of foreign substances (antigens) present in the body and the targeting of cellular and physiochemical arms of the immune system to attempt to destroy these compounds. Substances recognised as foreign include the external components of microorganisms visible to the immune system, foreign secretions of these organisms and biomolecules adulterated by (bio-)chemical action. The specificity of recognition of IgG molecules is produced on each arm by the interaction of variable regions in the heavy and light chain. The combination of the two variable regions with each other produces a distinctive three dimensional antigen binding domain (idiotype). The

Figure 4.1. A diagrammatic representation of the principle structural elements in an IgG molecule. Each molecular unit consists of two identical heavy and light polypeptide chains linked together by disulfide bonds. The F(ab) portion comprises a light chain and the complimentary part of the heavy chain, and contains the antigen binding domain. The F(c) region constitutes the remaining parts of the two heavy chains, and is responsible for binding other effector elements of the immune system.



idiotype is capable of specific and high affinity binding to selective antigenic elements (determinants), also known as epitopes, by a combination of an adaptive flexible 3-D structure corresponding to the antigenic determinant and possession of regions capable of complementary non-covalent bonding to that epitope. The idiotype of a given antibody molecule is fixed but tremendous variability is possible among all of the antibodies produced by different cells due to hypervariabile recombination of the genetic sequences responsible for the amino acid sequences in the idiotype of the heavy and light chain (Seabrook and Atkinson, 1991). This variability in recognition allows a high degree of selectivity in binding and is capable of differentiating between individual atoms in a particular compound (Landsteiner, 1947) up to different macromolecular structures of the same biomolecule. In antibodies, nature has provided an extremely precise and accurate method of selectively recognising particular agents, and we can manipulate this selectivity for the identification and measurement of substances of interest.

4.2.2. Use of antibodies for selective measurement

The selectivity of an antibody makes it potentially very useful for the identification and measurement of small amounts of biological agents. However, two difficulties must first be overcome to allow antibodies to be useful in an assay. Firstly, there is a need to produce an antibody against the substance in question of sufficient selectivity and in suitable volume for the requirements of the assay programme. Secondly, a sensitive format for measuring the specific interaction of the antibody with the analyte of interest must also be developed.

4.2.3. General antibody production methods

Three main options are available for the production of antibodies. These are polyclonal antibody production in animals, monoclonal antibody production in cultured cells and production of antibodies by recombinant genetic methods in eukaryotic and prokaryotic cells. The subsequent paragraphs will focus mainly on polyclonal methods, since these are most relevant to the work contained in this chapter.

4.2.3a.Polyclonal antibody production

When exposed to a perceived threat from a foreign substance two main avenues of attack are available to the body. If the substance has a low molecular weight, the body will generally try to convert and/or remove the substance by a complex series of physiochemical reactions which collectively form the metabolic and elimination systems of the body (Hurn and Chantler,

1980). For larger molecular weight compounds, generally above 10 KDa, and cells or viruses, the immune system will try to target and destroy the substance or invader with a variety of toxic agents and enzymes. Polyclonal antibody production involves turning the immune system against the antigen in question.

The antigen must, therefore, be injected into the body of a suitable animal in a formulation capable of eliciting an immune response. Antibodies are present in all mammals, so potentially any mammal can be used as an antibody producer, but in practice the commonly used animals are rabbits, goats, sheep and donkeys. The choice of animal is generally dependent on the volume of antibody required and a knowledge of the species antigenicity of the antigen (Catty and Raykundalia, 1988).

The antigen of interest is generally injected sub-cutaneously with an adjuvant to boost the immune response. For a novel antigen the immune system initially produces IgM and less specific antibodies. With repeated immunisation the response becomes primarily IgG and specificity and affinity of the antibody increases (Burrin and Newman, 1991). A process of selection and stimulation of antibody-producing B cells to multiply (clonal expansion) gradually selects the most effective antibody and concentrates on its production with levels of lower affinity antibodies declining (Tijssen, 1985). Since only larger molecular weight compounds, generally in excess of 10 KDa, are antigenic, the immune system will generally target many different epitopes of the antigen and the stereochemical complexity of an individual epitope may be such that several different high affinity antibody fits are produced. Therefore, the antiserum will contain IgG produced from the clonal expansion of several different cells and is said to be polyclonal.

4.2.3b. Monoclonal and recombinant genetic production of antibodies

The polyclonal nature of animal produced sera may be problematic especially where a single defined interaction of antibody and antigen are required. This difficulty can be overcome by artificially mass producing cells from an original cell producing the required (monoclonal) antibody. This is generally accomplished using B cells harvested from an immunised mouse. These cells are rendered immortal by fusion with an immortal mouse myeloma cell line and the hybrid stably producing the required antibody is selected and continuously grown in culture (Kohler and Milstein, 1976). Although this technique is well established, difficulties in production are still common and production costs are high (Tijssen, 1985).

Developments in manipulation of genetic information (DNA and RNA) have allowed the identification, selection and expression of the basic DNA sequence responsible for an antibody molecule. Incorporation of this information into prokaryotic and eukaryotic cells has allowed the engineering and production of antibodies and antibody fragments (Borrebaeck, 1992). This revolutionary advance allows many of the traditional problems of antibody production, such as species differences, purity and volume, to be overcome allowing developments in antibody-based measurement and antibody-based disease treatment (Seabrook and Atkinson, 1991).

4.2.4. Production of antibodies against low molecular weight compounds.

Despite the molecular level of sensitivity demonstrable by the immune system, only compounds of higher molecular weight are inherently antigenic and smaller compounds do not elicit an immune response on their own. However, this can be overcome by chemically linking the agent of interest to a suitable carrier so that the resultant total molecular weight is above the antigenic weight threshold. This process is responsible for the drug hypersensitivity reactions which can sometimes accompany treatment with drugs, which are either directly reactive, or, are rendered reactive, towards larger molecules in the body. Allergy to penicillin is an example of such a response. (Tijssen, 1985). Molecules which can be recognised by the immune response, but are too small to be antigenic on their own are termed haptens. Generally molecules of interest must be chemically linked to the carrier in a defined manner to ensure an adequate antibody response.

4.2.4a. Choice of carrier

Although any large molecular weight molecule antigenic to the animal could be employed as a carrier for use in immunisations, one of five types of protein are conventionally used. This is principally due to cost and reactivity considerations (Tijssen, 1985). Synthetic polypeptides such as poly L-lysine have been used since they are cheap and readily reactive, although their poor antigenicity limits their application (Erhlanger, 1980). Fibrinogen has also been used especially where its high carbohydrate content (3 %) can be advantageous for the chemistry of carrier coupling (Findlay, 1987). Foreign albumins such as bovine serum albumin (BSA) are available in large quantities, are well characterised and generally produce soluble conjugates (Erhlanger, 1980) although cross reactivity among albumins can present problems in particular applications. Thyroglobulin is a large molecular weight protein which is particularly suitable for immunisation due to its limited solubility, especially after conjugation, which is desirable for an immunogenic carrier. The final common carrier protein is keyhole limpet

haemocyanin (KLH), a large oxygen transport protein in the keyhole limpet (a mollusc). In addition to a high antigenicity, KLH possesses inherent adjuvant properties boosting the overall immune response (Hörnquist and Lycke 1993; Burrin and Newman, 1991).

When choosing the optimum carrier some factors are particularly important. The cost of the carrier should not be prohibitive for the amount of carrier necessary. The selected carrier should be inherently foreign to the organism, or the response will be small if any (Campbell, 1984; Burrin and Newman, 1991); a more insoluble conjugate is preferable, since it will remain at the site of immunisation boosting the local response. The carrier should possess suitable reactive groups to allow chemical linkage with the molecule of interest.

4.2.4b. Chemical linkage of hapten to the carrier protein

The chemical linkage of the compound to the carrier is also critical. The constituent amino acids of the carrier protein provide many reactive groups in the form of amino and carboxyl groups, although the structural arrangement of the whole protein can obscure a percentage of these available groups (Tijssen, 1985). Thiol groups present in sulphur-containing amino acids can also provide suitable reactive sites although their relative content is limited in most proteins.

Generally, the most critical determinant in the choice of conjugation linkage is the chemical nature of the molecule . The chemical method of linkage chosen should not affect the structure of the molecule or group of interest on the molecule, structurally, chemically or sterically. A single reaction site should be available so as not to elicit the production of antibodies to different antigens. The result of the chemical reaction should be a stable homogenous product and for an optimal response the chemical reaction should orientate the molecule with a suitable epitope to the exterior as far removed from the carrier as possible and with an optimal density of hapten on the carrier(Landsteiner, 1947; Burrin and Newman, 1991). Some chemical manipulation and derivatisation of the hapten molecule may also be necessary to introduce a reactive group in a suitable location, or, to protect potentially reactive components. Heterobifunctional chemistries, where the reaction can only link a group on the hapten to the carrier, produce much more efficient conjugates since hapten-hapten and carrier-carrier bonds are minimised.

Prevalent reaction chemistries include :- EDC-NHS coupling of amino to carboxyl groups, diisocyanate coupling to amine groups, peroxidation (particularly for sugar groups), diazo

coupling of amines to aromatic groups and aldehyde conversion to a schiff base (Erhlanger, 1980).

When introduced into an animal the immune system begins to process the injected antigen, by digestion by antigen presenting cells which break up the conjugate and express distinct epitopes to the immune system as part of a complex with MHC II proteins. With a typical hyperimmunisation schedule, a suitable level (titre) of antibody should be produced after approximately 3 months (Burrin and Newman, 1991).

4.2.5. Purification of serum

Once a suitable titre of antibody is generated in an animal, the crude antibody can be obtained by collection of the serum. Generally, the antibody is further purified from this serum, since the heterogenous nature of serum can cause problems in many assay systems. Many different purification schemes have been developed to cope with the different purity characteristics necessary for particular applications. A common initial clean up procedure for neat antiserum is to precipitate the IgG present by salting-out with ammonium sulfate, leaving contaminating proteins such as albumin in the supernatant (Tijssen, 1985). Further purification may be necessary since the purified IgG, resulting from non-specific purification methods, such as salting-out, will contain a mix of antibodies some directed against irrelevant epitopes. This is particularly true for antisera directed against haptens, where the majority of the specific IgG produced is directed against epitopes on the carrier. Even when antisera is produced against large molecular weight proteins, only approximately 20 % of the antibody harvested will be directed against the protein (Catty, 1988). Isolation of the selective antibody of interest is accomplished using an immunosorbent.

4.2.5a. Immunosorbent purification

An immunosorbent is a matrix containing the epitope of interest. Common matrices include stable sugar polymers such as agarose, dextrose or cellulose, glass beads, or, highly cross-linked acrylamide (Tijssen, 1985; Dean *et al.*, 1985). The choice of matrix is important for the separation and is usually a balance, of low antibody binding, optimal flow characteristics, physical and chemical stability for the binding and elution conditions, and ease of hapten coupling (Hebell, 1992; Dean *et al.*, 1985). These matrices can often be purchased in an activated form which will chemically bind to the hapten when it is added. A stable surface of hapten bound to matrix is then produced. When impure immunoglobulin is added to a column

of the matrix, the anti-hapten antibody binds to the bound hapten and the remaining IgG is in free solution. This free antibody fraction can be eluted by buffer flowing through and specific antibody remains bound to the column.

The specific antibody of interest is eluted by changing the buffer conditions to inhibit hapten-antibody interaction. Such changes include high salt low pH buffers, such as glycine pH 2.5, or reducing buffers, 6M guanidium hydrochloride, for example, which change the tertiary protein structure of the antibody and reduce the non-covalent hapten-antibody interaction. These conditions are potentially harmful to the antibody and once eluted it must be returned to physiological conditions as soon as possible to minimise the damage caused. In spite of the harsh conditions used, highest affinity antibodies can often remain bound to the immunosorbent and cannot be eluted.

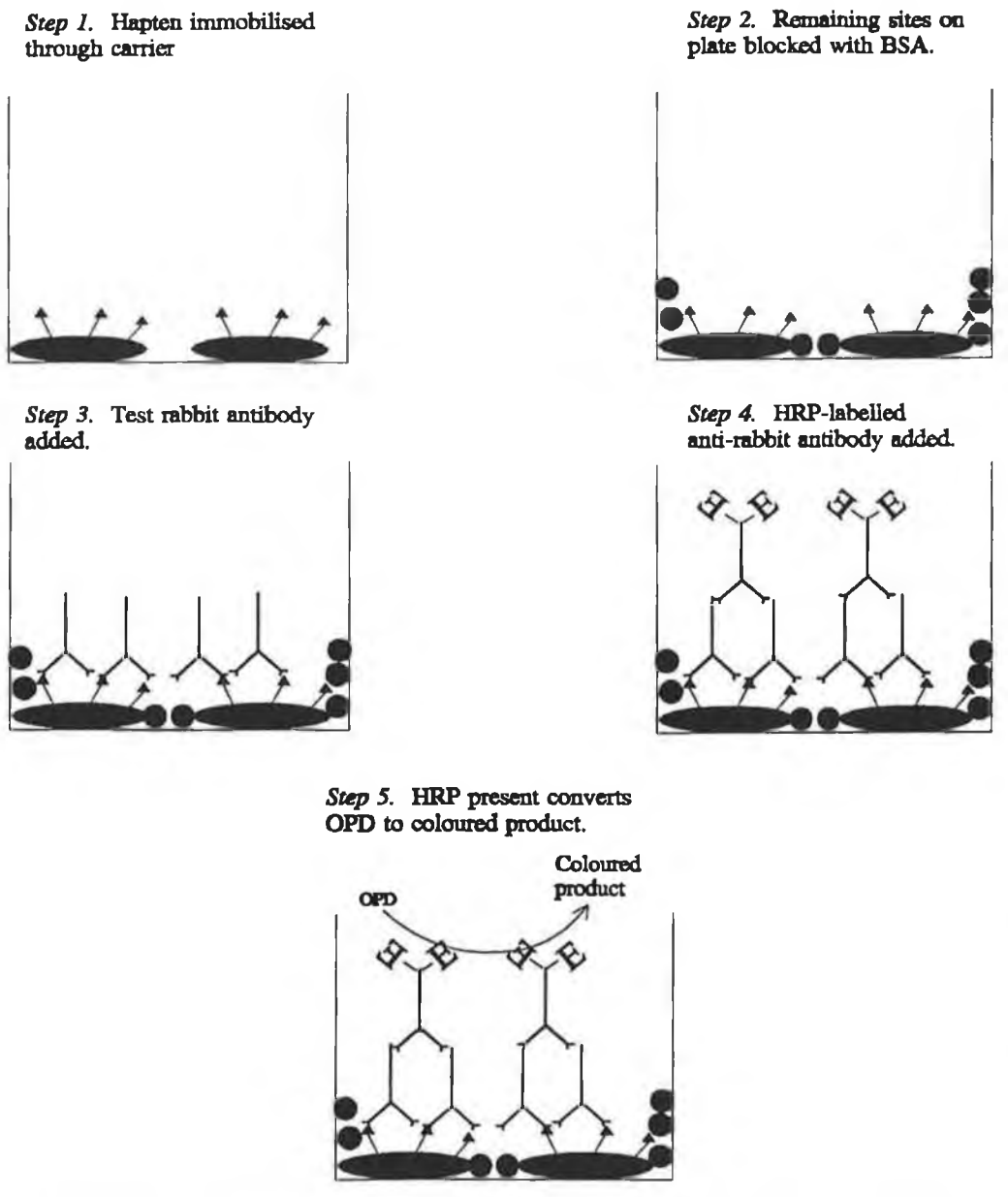
4.2.6. Measurement of antibody-hapten interaction

4.2.6a. ELISA

Many antibody based formats are available to quantify the antibody-hapten interaction. One of the most prevalently used is the Enzyme-Linked ImmunoSorbent Assay (ELISA), based on 96 well plastic plates. Many different arrangements of this format are possible but the principle can be illustrated by a description of a direct ELISA similar to that which was used in the project to verify the presence of anti-phenazine antibodies.

Figure 4.2 shows a diagrammatic representation of this form of ELISA. The ELISA plate consists of clear polystyrene plastic which is treated with gamma radiation to increase its stickiness towards proteins. In step 1, the antigen of interest, in this case a hapten linked to a protein, sticks to the activated surface. Non-covalent interactions, of the hydrophobic type are the main interactions responsible for this adherence (Tijssen, 1985; Parsons, 1981). Loosely bound protein is removed by washing, usually with a detergent, and what remains is firmly, though not chemically, bound to the plastic surface. In step 2 a solution of BSA protein is added to block the plate, that is, to occupy any of the plastic surface which is free and could potentially bind the antibodies added in later steps. The excess protein is removed by a further series of detergent washes, leaving a completely blocked surface. In step 3, test rabbit antibody is added to the plate and allowed to react with the hapten. If the antibody has a high affinity for the hapten, it binds to it, and the unbound fraction is removed by washing. In step 4, a second antibody is used to detect and quantify the binding of the test rabbit antibody. This

Figure 4.2. The principle steps in a direct ELISA to demonstrate the production of anti-hapten antibodies. The details of each step are described in the text.



second antibody consists of an antibody directed against rabbit IgG which has an enzyme, horse radish peroxidase (HRP), chemically linked to it. This labelled antibody binds to the bound test rabbit IgG and any excess is removed by washing. In step 5, a solution of o-phenylenediamine, a substrate of HRP, is added to the wells and is converted into a coloured product by the action of the enzyme. The amount of colour produced is quantified by a plate reader which measures the absorbance of each well at a specified wavelength. The absorbance produced is proportional to the amount of HRP present which is in turn proportional to the amount of rabbit IgG bound. In order to determine suitable levels of antibody to be used, since the ELISA plate surface is saturable, a number of dilutions of rabbit IgG are made. A comparison of the minimum dilutions of rabbit IgG from different sources, or, from the same source on different occasions, can then be made by comparing the minimum dilution of these different samples which gives a response above background.

The immobilisation of molecules of small molecular weight on to the ELISA plate can be problematic. The large molecular size of proteins provides many opportunities for non-covalent bonding to the plate, resulting in a stable adherence of these substances. Smaller haptens do not possess sufficient numbers of binding groups and tend to be easily removed from the plastic (Tijssen, 1985). One method to overcome this difficulty is to produce a protein conjugate of the hapten. The hapten is then bound to the plate through the protein. This conjugate must be prepared with a different protein, to that used for immunisation, and preferably with a different chemistry, to prevent cross-reaction of the rabbit antibody fraction directed against the immunogen carrier with the carrier used to coat the plate (Briand *et al.*, 1985).

Recent developments in ELISA plate technology have produced surfaces with reactive groups which can covalently bind small molecular weight compounds. Such plates include the NUNC Covalink plate with an amino group on the surface of the plastic which can be rendered reactive by chemical treatment (Yonezawa *et al.*, 1993; Rasmussen, 1990). The amine binding plate produced by Costar has a succinylated plastic surface rendering it directly reactive towards amino groups. Such plates make the measurement of small molecular weight compounds easier, since there is no need for the production of a second conjugate, and generally increase accuracy, precision and sensitivity of the immunoassay (Søndergård-Andersen *et al.*, 1990). Direct coupling to the ELISA plate has also been shown to advantageous for larger antigens, increasing the stability of the attachment with associated increases in sensitivity and reproducibility (Wood and Gadow, 1983; Rubin *et al.*, 1980).

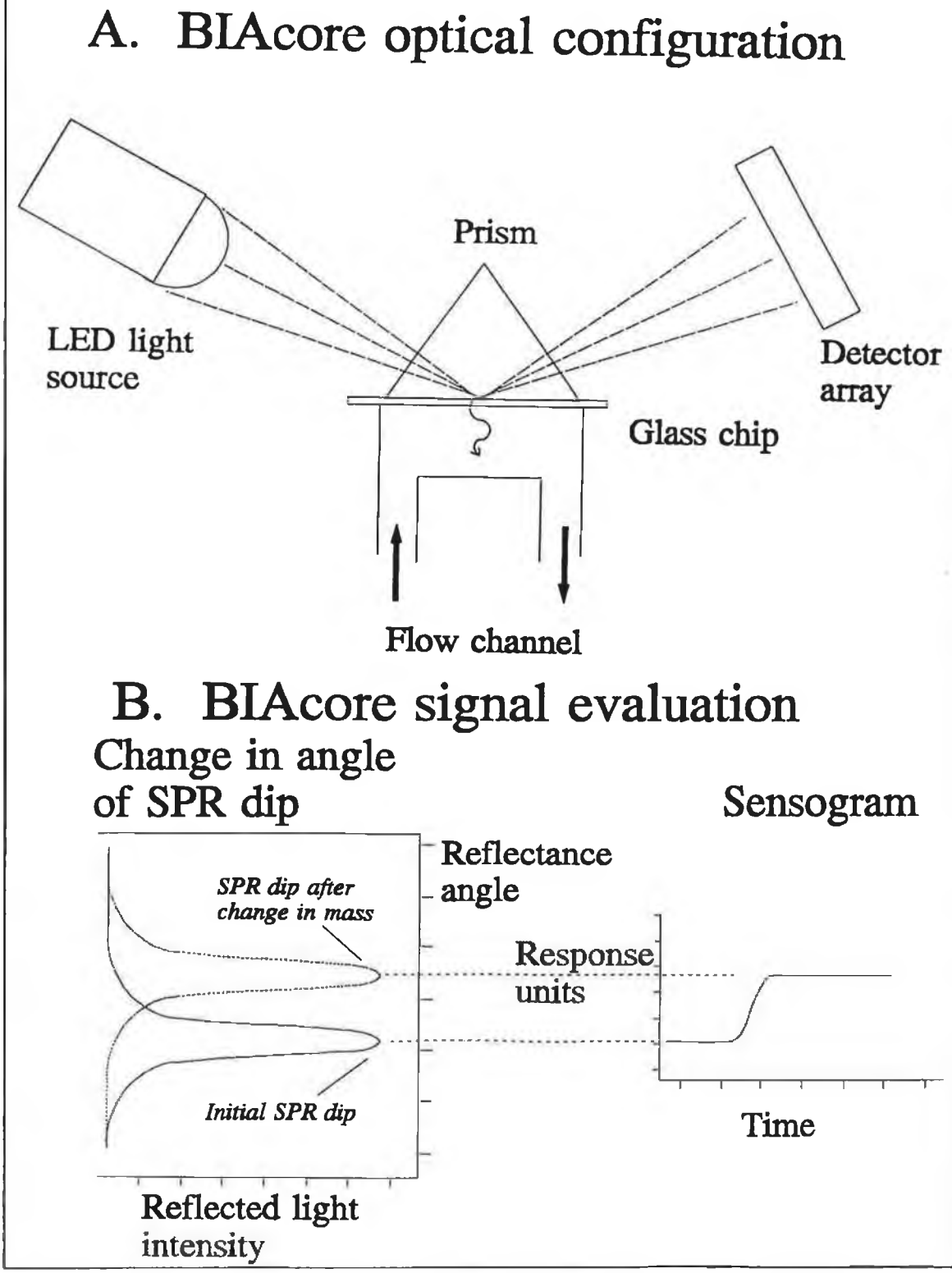
Constant developments are being made in technologies for measuring antigen-antibody interactions. A recently developed system for quantification of these interactions in real-time is the BIAcore, produced by Pharmacia.

4.2.6b. BIAcore™ analysis

The BIAcore is a machine which measures the interaction of molecules based on the principle of surface plasmon resonance, and is particularly applicable to studies of antigen-antibody interaction (Jonsson and Malmqvist, 1992; Fägerstam *et al.*, 1992). The principle of the system is illustrated in Figure 4.3. It consists of a glass chip coupled, to an optical interface on one side, and small flow channels on the other . The interface is comprised of a prism which is coupled to the glass slide. Monochromatic light, incident from the LED source, enters one side of the prism at an angle less than the critical angle of refraction and is therefore reflected back out, to be detected by a series of photodiode detectors. Although the light is totally internally reflected, an electromagnetic component of that light, called the evanescent wave, penetrates approximately one wavelength into the medium on the flow side of the chip. Coating of this side of the chip with a thin gold surface causes a resonant interaction of the electromagnetic wave with the electrons of the gold, producing a reflectance minimum in the reflected light. This phenomena is termed surface plasmon resonance (SPR). A view of the reflectance intensity versus the incident angle shows a dip in intensity at a characteristic angle, as shown in the figure. The angle at which the SPR dip appears is very sensitive to changes in the net refractive index beside the gold surface, which is in turn changed by variation in the mass present on this side. A thin matrix of carboxylated dextran is bonded to the gold film to allow the covalent linkage of molecules to the chip. Any changes in the mass present in this matrix produce a shift in the dip angle, and it is these changes in angle which are converted to response units by a computer coupled to the detector array. The carboxymethyl dextran chains provide a convenient matrix for the immobilisation of biomolecules, such as antigens, and the non-covalent interaction of antibodies with these increases the net local mass producing a change in the signal.

This system is very powerful for the characterisation of biomolecular interactions, particularly those of an antigen-antibody nature, and allows the visualisation of these interactions in real time. In this project the system was used merely to indicate the specific binding of a purified antibody to a phenazine antigen, but other routine applications which have been validated include, kinetics of antibody-antigen interaction, epitope analysis and concentration measurement (Fägerstam *et al.*, 1992; Pellequer and Regenmortel 1993).

Figure 4.3. The optical configuration of the BIAcore. Monochromatic light produced by the LED is refracted in a prism and the reflected light monitored by a detector array. Changes in mass on the chip surface cause a shift in the reflection angle due to SPR. In 4.3 B the change in this angle is evaluated by the computer system to produce a sensogram.



4.3. PRODUCTION OF PHENAZINE CONJUGATES

Phenazine compounds are difficult to conjugate to proteins due to their aqueous insolubility and lack of suitable reactive groups. To overcome these problems reactions of rimino-phenazines containing amino acid substituted side chains with proteins were investigated. The two compounds chosen, B3955 and B3976, contain the amino acids glycine and phenylalanine, respectively, substituted into the rimino-piperidine group of B3640 through their carboxyl group. This provides a free amino group for reaction with the carrier protein and an increase in aqueous solubility.

Initially two conjugates were prepared, by reacting thyroglobulin (THYR) and bovine serum albumin (BSA) with B3976 using glutaraldehyde. This was performed in conjunction with Ms. Patricia McCormack, D.C.U.. A third conjugate of B3955 linked to KLH by an EDC-NHS reaction was also prepared for immunising the rabbits when anti-phenazine titre started to fall. After reaction the conjugates were dialysed to remove unreacted starting compounds and it was necessary to characterise these conjugates to verify the success of the reaction.

4.3.1. Phenazine conjugate characterisation

Characterisation of drug-protein conjugates requires determination of the number of drug molecules incorporated. With many molecules, such as peptides, this estimation is difficult since there are no clear physicochemical differences between the carrier and the bound hapten. The visible absorbance of phenazines makes characterisation relatively straightforward since this is not present in the carrier or linker molecules. Several methods of characterisation are available and those used were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), TLC, HPLC, PDA-HPLC and size exclusion chromatography (SEC). It was noted with the glutaraldehyde conjugates that they became insoluble after freeze drying. Conjugate characterisation was performed on samples after dialysis.

4.4 CONJUGATE CHARACTERISATION

4.4.1. SDS-PAGE analysis of conjugates

The gel used did not distinguish any molecular weight difference between the BSA conjugates, BSA-glutaraldehyde control, BSA and a characterised BSA-7-hydroxy-coumarin conjugate sample provided by Dr. Denise Egan, D.C.U. Red bands of low molecular weight corresponding to phenazine were evident at the bottom of lanes from phenazine conjugates. Samples were also prepared in sample buffer without boiling, and electrophoresed, to investigate if the boiling caused this apparent instability. No difference was noted between boiled and un-boiled samples.

The results from the electrophoresis were therefore ambiguous since they failed to indicate any evidence of conjugation in these conjugates but also were unable to show conjugation in a characterised conjugate.

4.4.2. TLC analysis of conjugates

In a solvent system consisting of 80:20 (v/v) methanol/water, B3976 separated clearly from the protein which remained at the origin. With the BSA and THYR conjugates two red spots were visible. One migrated the same distance as the drug alone, while the other remained at the origin. The presence of the proteins with this band at the origin was confirmed by staining with iodine vapour. With controls of BSA and unreacted drug no B3976 spot was seen at the origin.

4.4.3. HPLC analysis of conjugates

Conjugate samples were analysed by HPLC using a size exclusion column as described in section 2.14.3. of the methods chapter. This type of column separates proteins on the basis of their molecular weight by a size dependent interaction with pores in the column particles.

Under the conditions used, the peaks produced by BSA and a control of BSA treated only with glutaraldehyde gave very similar peak shapes with the same retention time of 16

minutes. Conjugation with B3976 produced a chromatogram with this BSA peak and an additional peak at 14 mins (Figure 4.4.(i)). As Figures 4.4.(i) and (ii) show, the shape and relative height of the peak at 14 mins. was altered by varying the reaction conditions. The shorter retention time of this peak indicates that it was produced by a group of high molecular weight reaction products representing various intermolecular crosslinking of BSA. The strong absorbance of B3976 at the monitoring wavelength (280 nm) is responsible for the difference in the relative peak height of these higher molecular weight products.

By monitoring the column eluent with a photodiode array detector, instead of a conventional single wavelength detector, the complete spectrum of these peaks can be monitored. Figures 4.5.(i) and (ii) show the typical contour plot of the absorbance intensity versus wavelength and time for BSA and THYR, respectively, treated with glutaraldehyde showing strong U.V. absorbance for each protein. The production of a conjugate for both proteins is evident in Figures 4.6.(i) and (ii), by the coelution of B3976 with the protein, indicated by the visible absorbance. The shorter retention times of the conjugate contours also indicate that high molecular weight products have resulted from the conjugation reactions. An example of the photodiode spectrum at discrete times in the chromatogram is shown in Figure 4.7.

4.4.4. SEC analysis of conjugates.

To estimate the average molecular ratios of drug bound to protein, conjugates were analysed by SEC. With the gels used, the conjugates are eluted in the void volume of the column while unbound phenazine elutes much later. By monitoring the absorbance of the eluted fractions at 488 nm the mass of bound phenazine was calculated with appropriate standards. The protein content of each fraction was measured using the BCA assay. Table 4.1 shows the average molar ratio of drug bound to protein. As this table shows, alteration of the glutaraldehyde reaction conditions has a significant effect on the ratio of drug bound. A two step reaction produces a higher relative ratio of drug bound than a one step reaction. The effect of variations in reaction conditions were not investigated with the KLH conjugate.

4.5. METHODS TO MONITOR PRODUCTION OF ANTI-PHENAZINE ANTIBODY

The insoluble THYR conjugate was chosen to immunise two rabbits as described in section 2.16.1. of the method section. To monitor the production of antibodies in these animals, ELISA-based assays were developed. A form of direct assay was initially developed to measure antibody production. The BSA conjugate became insoluble when freeze dried and, hence, was

Figure 4.4.(i) The HPLC chromatograms of (A) BSA-glutaraldehyde control and (B) BSA-B3976 conjugate. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring at 280 nm with a flow rate of 0.5 ml/min. The BSA control, with 0.1 % (v/v) glutaraldehyde, gives a single peak with the same retention time as BSA alone (16 mins.) The production of a higher molecular weight conjugate is indicated in (B) by a peak at 14 mins.

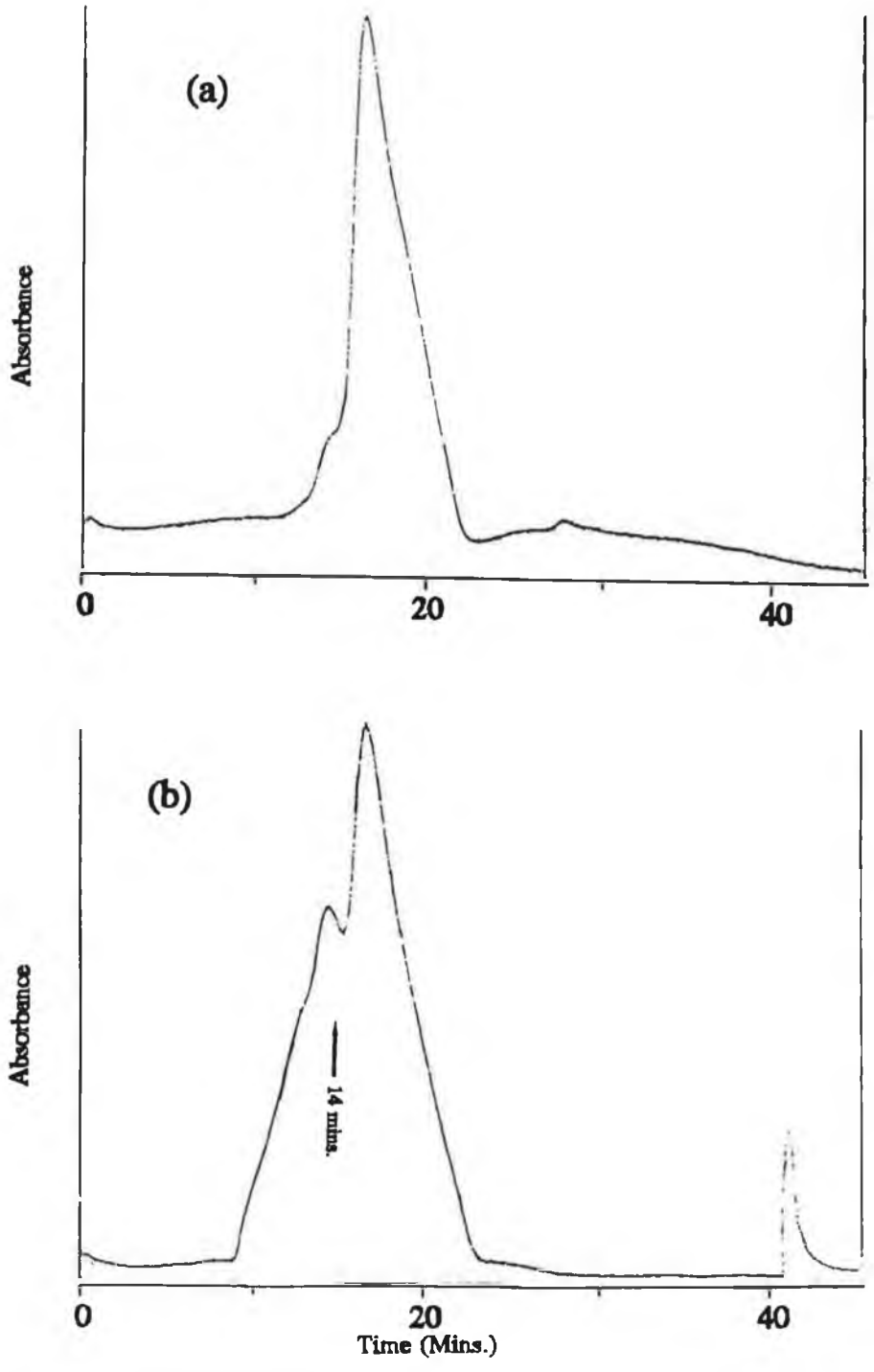


Figure 4.4.(ii) HPLC chromatograms of BSA-B3976 conjugates produced under different reaction conditions. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring at 280 nm with a flow rate of 0.5 ml/min. The chromatogram in (a) shows the conjugate produced by a one step reaction using 0.07 % (v/v) glutaraldehyde, while (b) shows the conjugate resulting from a two step reaction. The different reaction conditions produce differences in the height and shape of the conjugate peak, indicating a combination of different ratios of higher molecular weight proteins, and, different drug conjugation ratios.

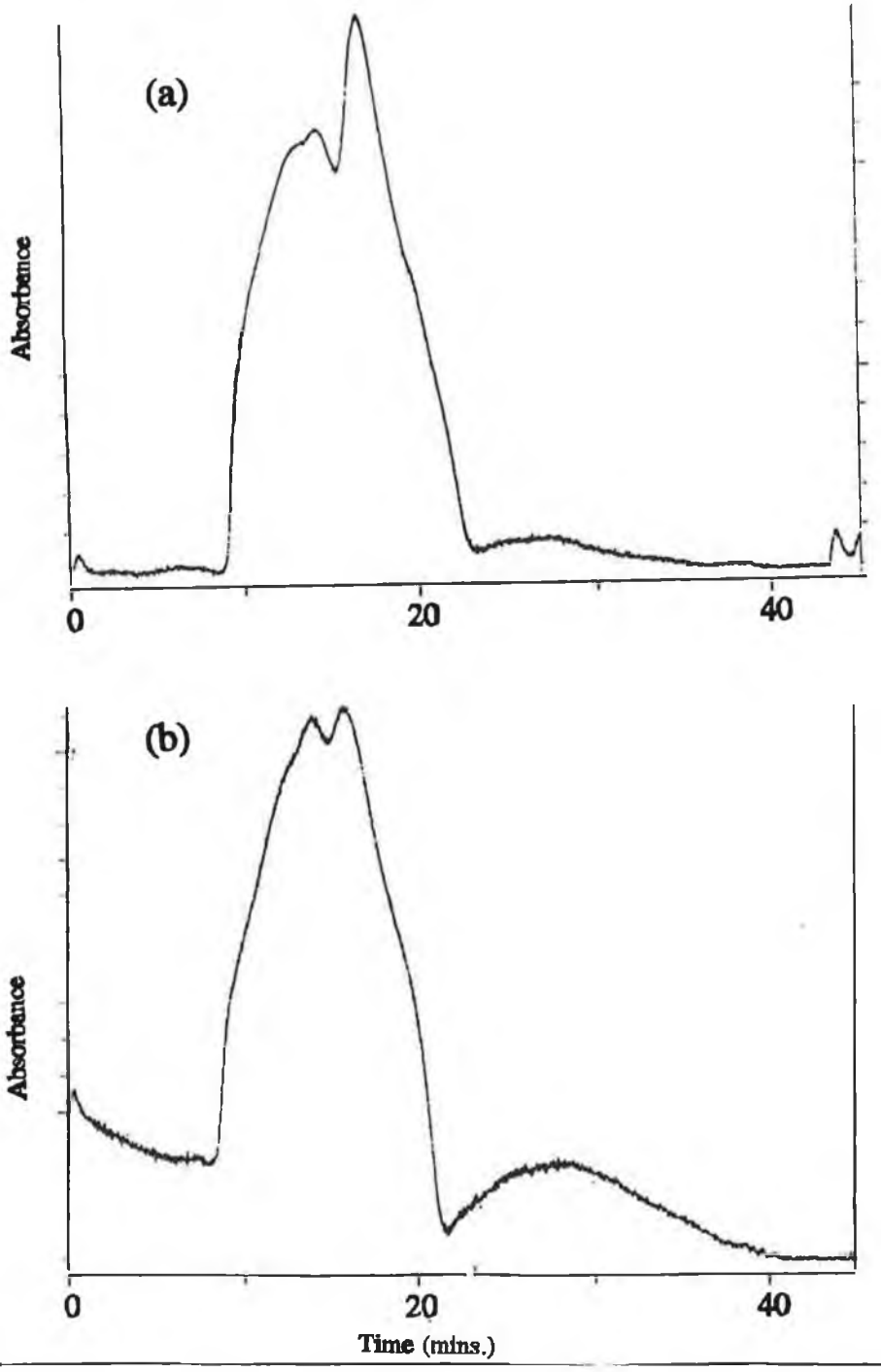


Figure 4.5.(i) A photodiode HPLC chromatogram showing the spectral contour pattern of the control BSA-glutaraldehyde reaction. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring from 250 to 550 nm with a PDA detector, at a flow rate of 0.5 ml/min. The BSA protein has a strong U.V. absorbance.

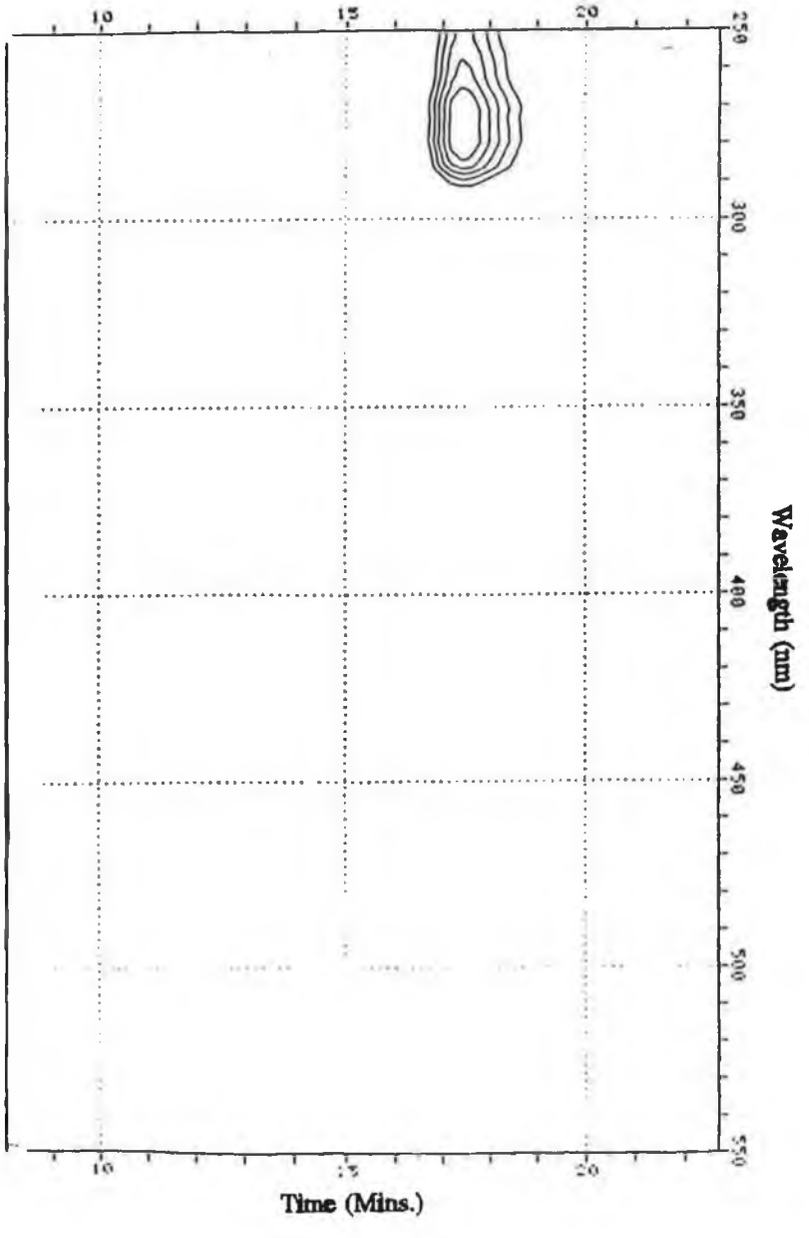


Figure 4.5.(ii) A photodiode HPLC chromatogram showing the spectral contour pattern of the control THYR-glutaraldehyde reaction. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring from 250 to 550 nm with a PDA detector, at a flow rate of 0.5 ml/min. The THYR protein has a strong U.V. absorbance with a shorter retention time than BSA due to its higher molecular weight.

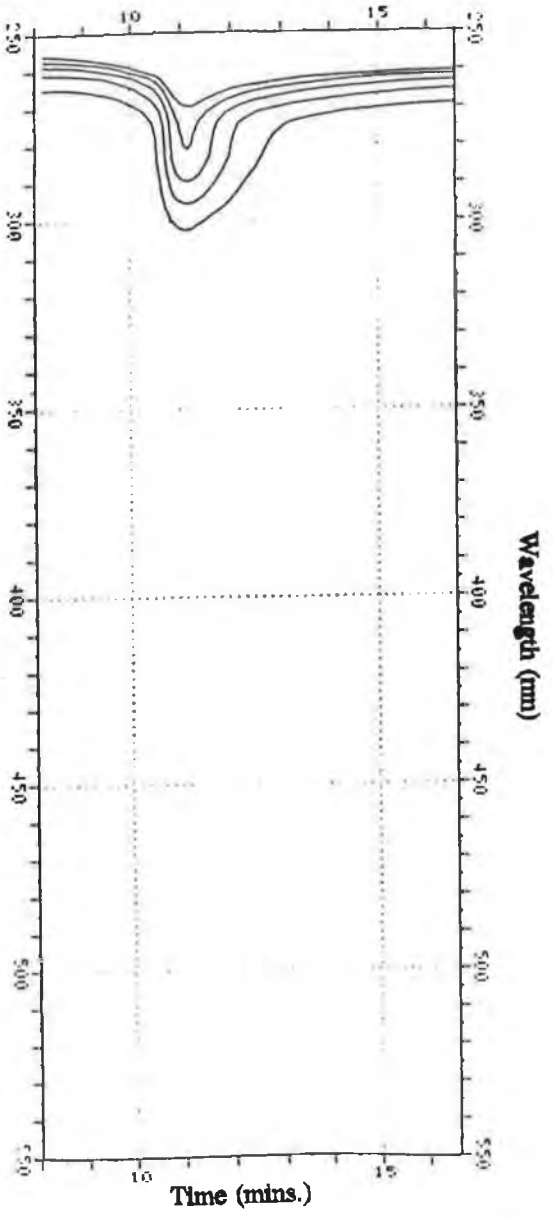


Figure 4.6.(i) A photodiode HPLC chromatogram showing the spectral contour pattern of BSA-B3976 conjugate. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring from 250 to 550 nm with a PDA detector, at a flow rate of 0.5 ml/min. The BSA protein has a strong U.V. absorbance, with a broad retention time due to glutaraldehyde-induced polymerisation of the protein. The presence of a visible absorbance (around 500 nm), eluting at the same time as the protein, indicates the chemical linkage of B3976 to the protein.

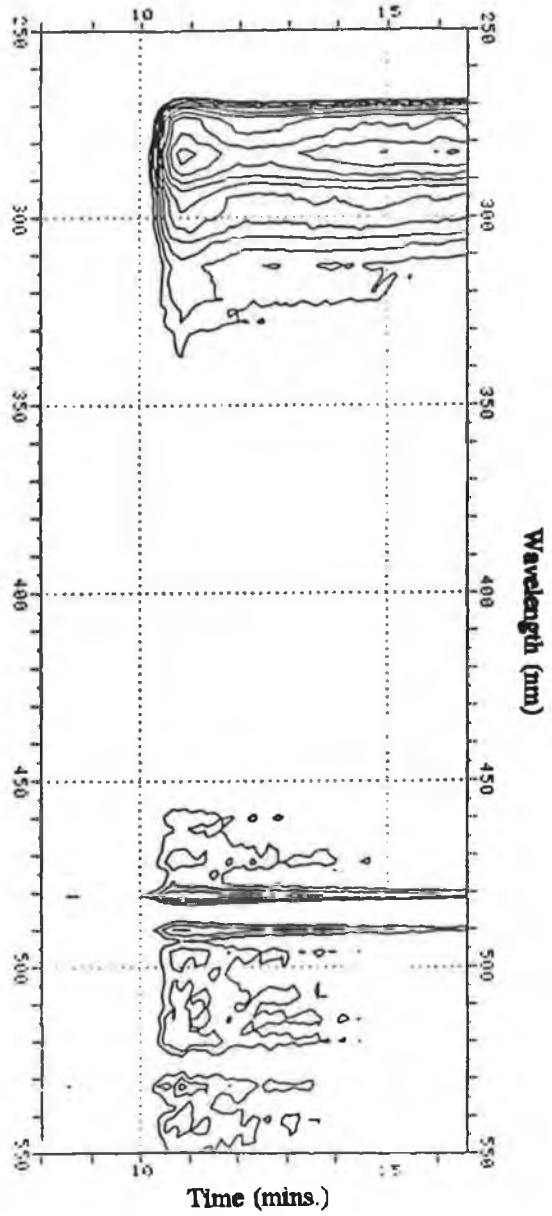


Figure 4.6.(ii) A photodiode HPLC chromatogram showing the spectral contour pattern of a THYR-B3976 conjugate. The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring from 250 to 550 nm with a PDA detector, at a flow rate of 0.5 ml/min. The THYR protein has a strong U.V. absorbance, but a visible absorbance (around 500 nm), indicating the chemical linkage of B3976 to the protein, is also present.

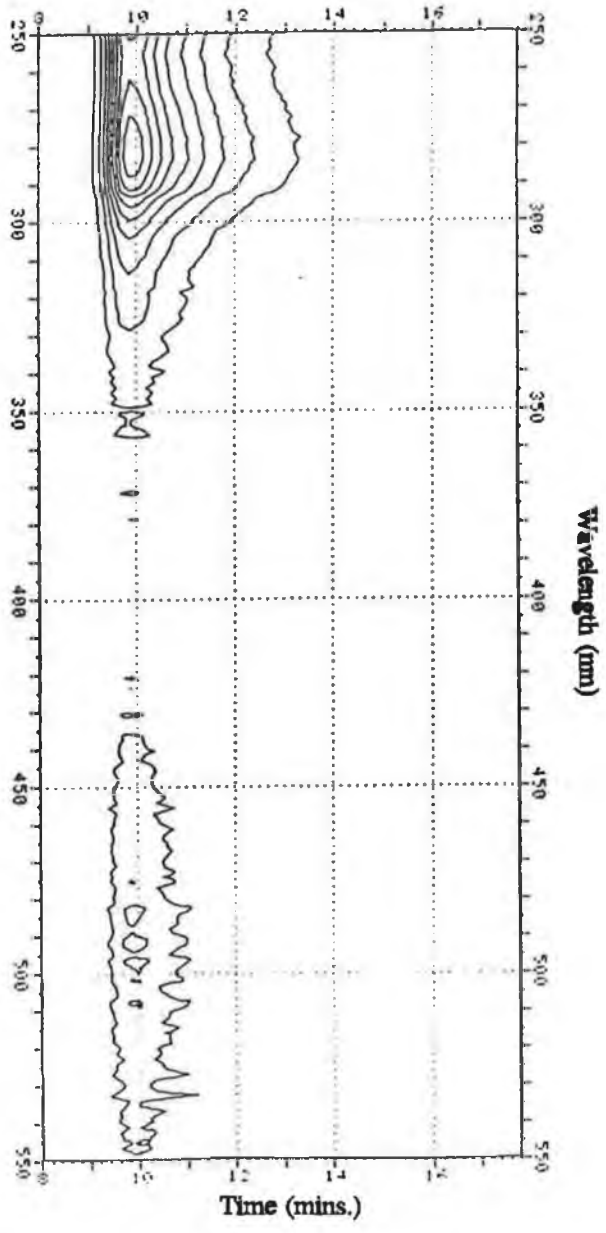


Figure 4.7. Photodiode spectra from the HPLC photodiode chromatogram of the THYR-B3976 conjugate shown in figure 4.6(ii). The proteins were analysed on a Protein Pak SW 300 column, with a mobile phase of 0.1 M phosphate buffer, pH 7.0, monitoring from 250 to 550 nm with a PDA detector, at a flow rate of 0.5 ml/min. A scan at 8 mins. shows the baseline spectrum of the mobile phase before elution of the conjugate. At 9.9 minutes the spectrum shows the strong U.V. absorbance of the protein and B3976 present in the conjugate. The presence of both spectra at the same time confirms the covalent linkage of B3976 to the protein. At 12 mins. the absorbance spectrum is less intense, since there is less protein present. However, B3976 and protein are still evident.

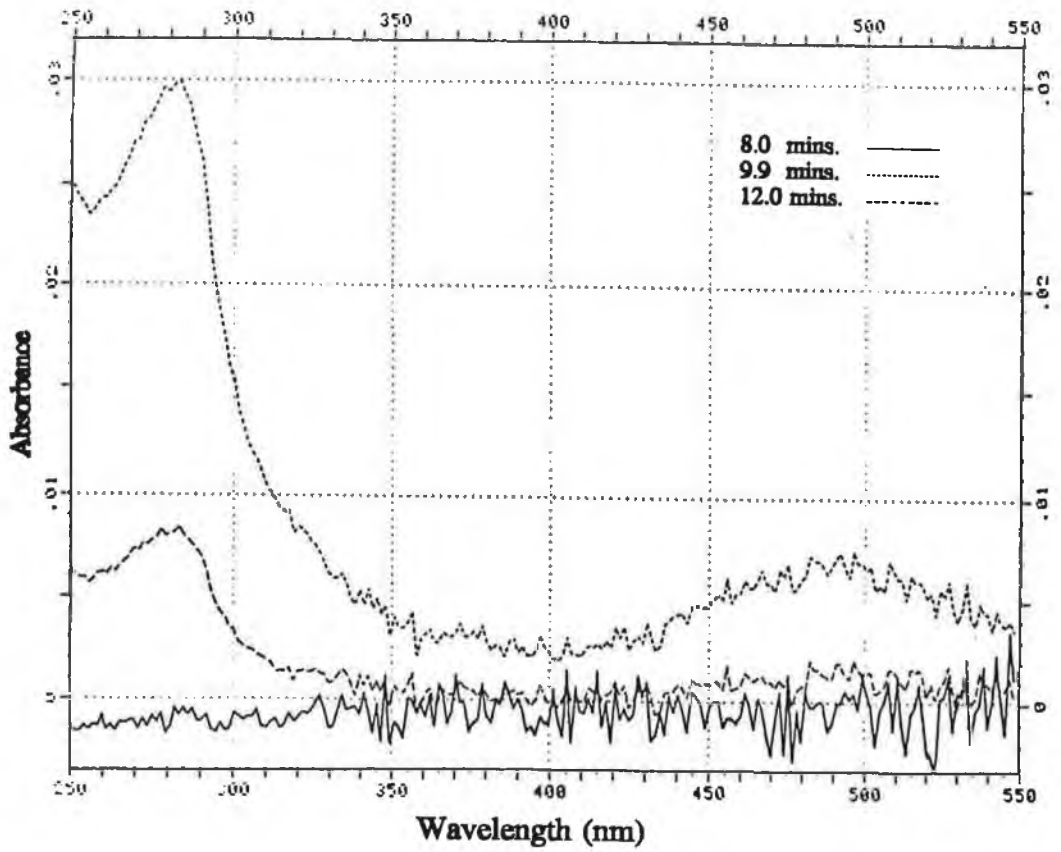


Table 4.1. A comparison of the drug-protein ratios of BSA, THYR and KLH conjugates. One step reactions produced conjugates with a higher drug to protein ratio than two step reactions. As evident with the thyroglobulin results, increasing the concentration of glutaraldehyde or B3976 in the reaction also increased the drug to protein ratio.

Protein	% Glutaraldehyde	Reaction drug-protein ratio	Conjugate drug-protein ratio
BSA	0.07	30	15
	0.13	30	20
Two step	----	--	10
THYR	0.08	100	74
	0.01	100	89
	0.01	150	100
Two step	----	---	49
KLH	EDC/NHS	733	990

unsuitable for immobilisation of B3976. This was overcome by coating the ELISA plate with BSA and then conjugating B3976, and in later experiments, B3955, to the protein surface using glutaraldehyde.

4.5.1. Optimisation of *in situ* conjugate method

4.5.1a.Glutaraldehyde concentration

Various aqueous concentrations of glutaraldehyde, from 50 % (v/v) to 0.5 % (v/v), were used to immobilise a concentration of 5 mg/ml B3976 to coated BSA. The titre of antibody investigated using serum from each rabbit after 3 immunisations. Controls with no BSA, gave a background response. This indicated the necessity for BSA to mediate the binding of B3976. Interestingly, controls with no glutaraldehyde gave a greater response than that seen with high concentrations of glutaraldehyde indicating some immobilisation of this agent was occurring without conjugation. Figure 4.8 shows the response found with these different concentrations of glutaraldehyde. Improved sensitivity was also detected with plates where a two step immobilisation was employed, as shown in Figure 4.9. The BSA coated on the plate was activated with glutaraldehyde, washed and drug added instead of reacting B3976 with glutaraldehyde and BSA directly. In the two step assay, a concentration of 0.1 % (v/v) glutaraldehyde was shown to give the highest response. Using the micro BSA assay, the amount of BSA immobilised by a one step and two step glutaraldehyde procedures was assessed. As Table 4.2 shows, both methods immobilised a significantly greater amount of protein to the wells, than was bound with no glutaraldehyde. Further assays showed that the optimal concentration of glutaraldehyde was 0.1 % (v/v) in the activation stage and immobilisation with glutaraldehyde in 0.1 M PBS gave a superior response than glutaraldehyde in water. However, a significant antibody response was seen with controls where no drug was immobilised. This indicated that a part of the antisera was binding to the glutaraldehyde surface. In addition, washing the immobilised drug initially with solutions containing the detergent, Tween 20, was necessary for reproducible responses with all assay formats.

4.5.1b. Phenazine concentration

Using the direct two step method, the effect of varying the concentration of B3976 and B3955 was investigated. With this assay, immobilisation with a concentration of 0.5 mg/ml of B3976 was shown to give the highest response with various antibody dilutions (Figure 4.10). However, comparison between responses seen with various dilutions of B3955and B3976 showed

Figure 4.8. A plot of the absorbance obtained following antibody binding, as a function of glutaraldehyde concentration used in a single step immobilisation of B3976 and quantified by direct ELISA. A concentration of 0.5 % (v/v) glutaraldehyde gave the highest response of the concentrations used in this experiment.

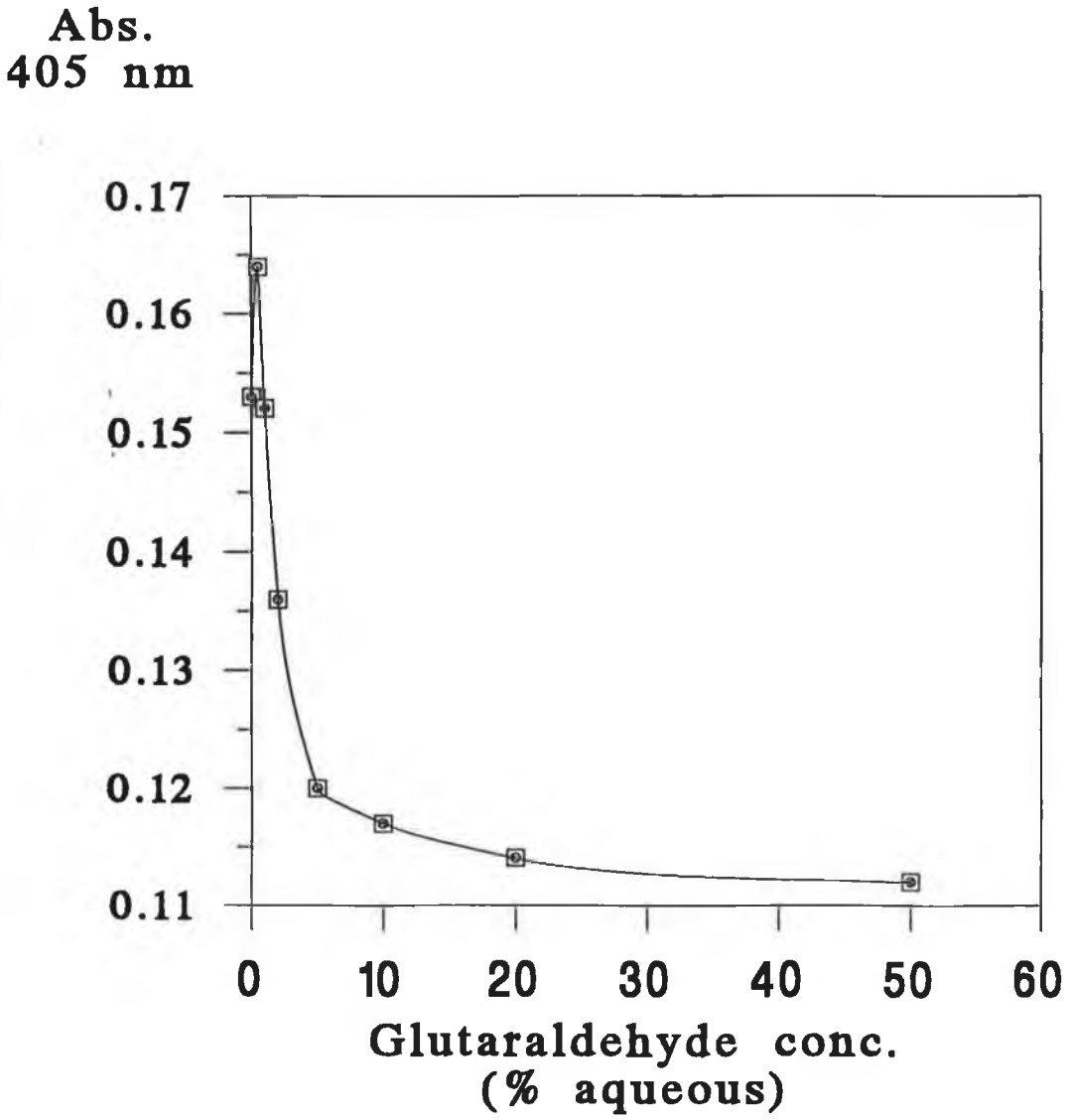


Figure 4.9. A plot of the absorbance obtained following antibody binding, as a function of glutaraldehyde concentration in one step and two step immobilisations of B3976 and quantified by direct ELISA. The two step assay gave a superior response with a lower relative background response compared to the one step format. For the two step assay a concentration of 0.1 % (v/v) gave the highest response of the concentrations used in this experiment.

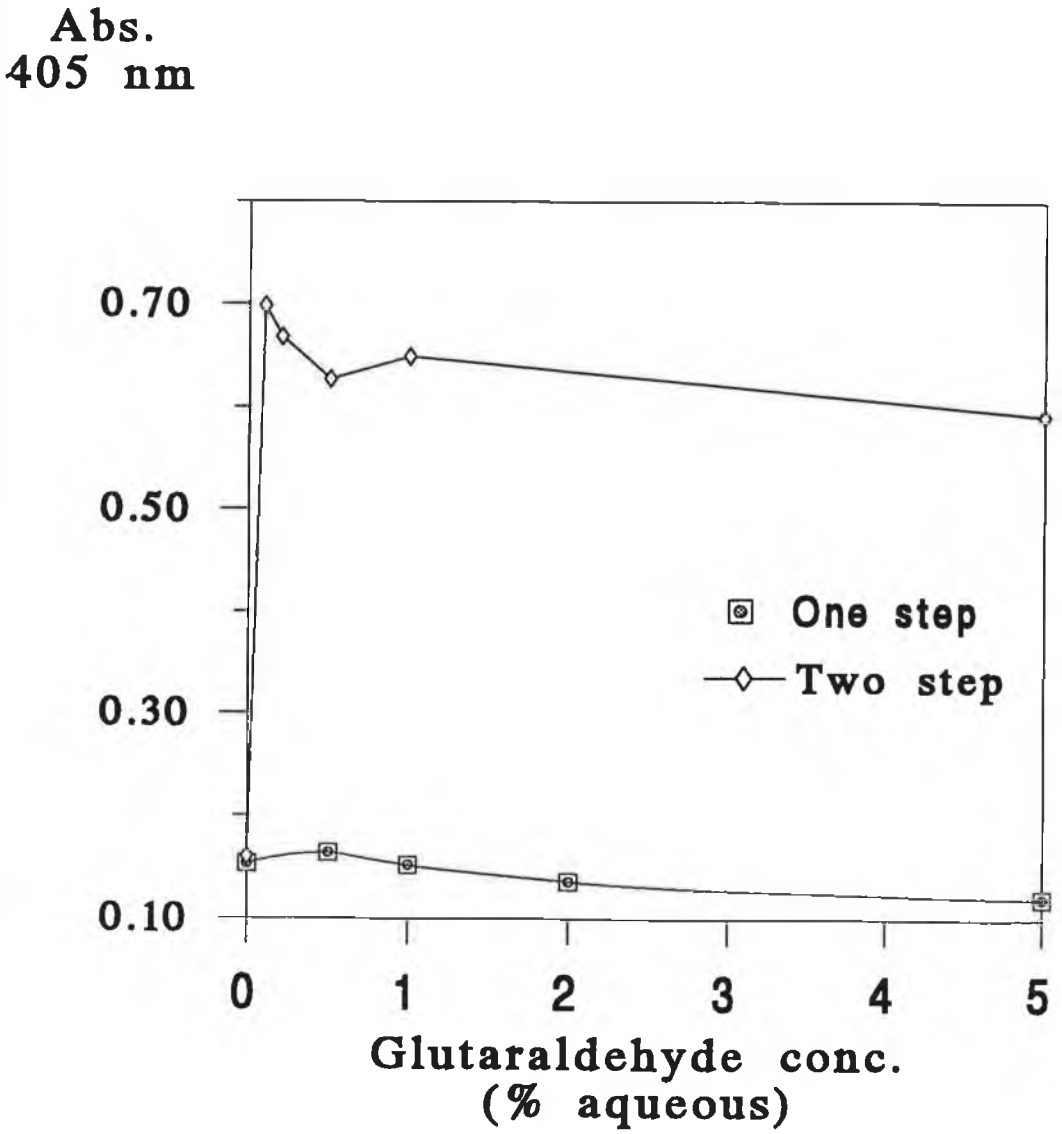
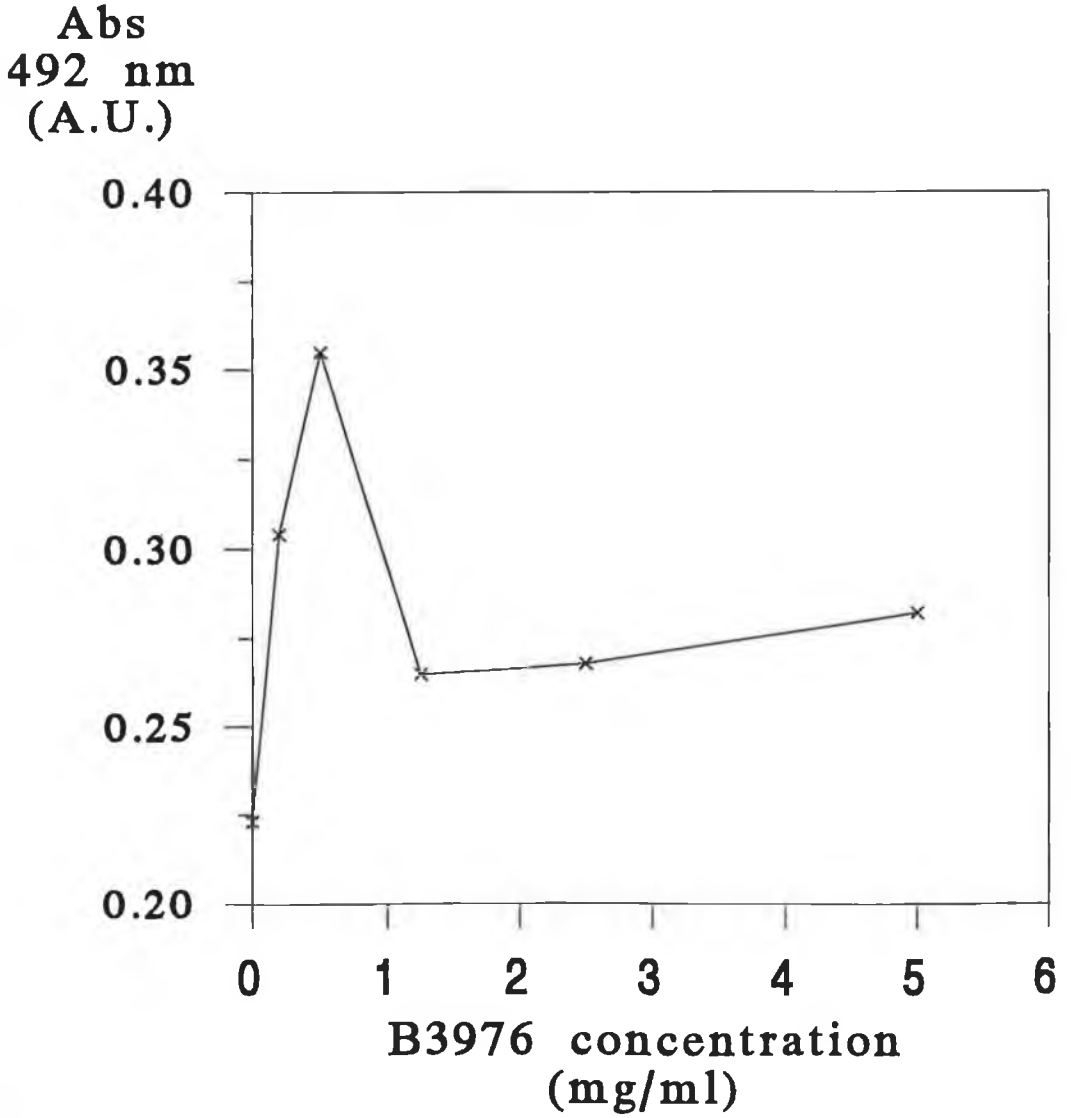


Table 4.2. A comparison of the amount of BSA bound to the wells of an ELISA plate after washing. Increasing volume of BSA from 25 to 50 µl doubles the amount of protein present. Addition of 0.1 % (v/v) glutaraldehyde also further increases the amount of BSA bound.

Sample	Mass of BSA immobilised (ug)	RSD (%)
50 µl 2 % (v/v) BSA in PBS	0.46	17.5
25 µl 2 % (v/v) BSA in PBS	0.20	15.7
25 µl 0.1 % (v/v) glutaraldehyde + 25 µl 2 % (v/v) BSA in PBS	0.85	10.7

Figure 4.10. A plot of the absorbance obtained following antibody binding, as a function of B3976 concentration in a two step immobilisation and quantified by direct ELISA. A concentration of 0.5 mg/ml B3976 gave the highest response of the concentrations used in this experiment. Concentrations above this maximum cause a reduction in the response seen. The mean values of duplicate results are shown.



that higher absorbances were seen with B3955, with an optimal response in the range 0.2 - 0.5 mg/ml (Figure 4.11). During experiments to try to block the immobilisation of these phenazines by adding dilutions of lysine, it was noticed that certain concentrations of lysine actually augmented the response seen with a direct ELISA. The expected response was that lysine would reduce the antibody binding in a concentration dependent manner, by reducing the amount of hapten bound through competition. As Figure 4.12 shows, addition of 25 µl of 0.1 mg/ml lysine to 25 µl of the drug concentration was optimal for this response.

4.5.1c. Effect of pH on direct glutaraldehyde based assays assay

The effect of pH of the coated BSA, anti-phenazine sera and HRP-labelled antibody was investigated. As Table 4.3 shows, there was little difference between the results at different pH. However, coating with BSA at pH 7.4 and both antibodies at pH 7.4 gave the highest overall response.

4.5.1d. Competitive assays

As mentioned in section 4.5.1a., a significant antibody response to glutaraldehyde was evident in control wells with glutaraldehyde alone. To ensure that the results seen were a measure of specific interaction of antibody and phenazine, competitive ELISAs were developed against B3976 and B3955. An inversely proportional response to the drug concentration was detected with both B3976 and B3955 at a concentration of 0.5 - 0.002 mg/ml. As shown in Figure 4.13, a log plot indicated each agent gave a linear response from 0.5 - 0.02 mg/ml. Various attempts to produce a competitive assay with phenazines in methanol or aqueous dilutions of THF or DMF proved to be unsuccessful.

To further verify that the antisera reacted with phenazines, B3955 was coupled to BSA on the surface of an ELISA plate using EDC/NHS. Using a concentration of 0.1 mg/ml B3955 in the conjugation procedure, a surface was produced which was successful both as a direct, Figure 4.14, and competitive ELISA, Figure 4.15, although, the response seen was quite small.

4.5.2. Change in antibody titre over time

The EDC/NHS based conjugation system was used in an assay format, as described in the previous section, to follow the specific anti-phenazine titre of antibody as the glutaraldehyde immunisations progressed. As shown with the response of the male rabbit in

Figure 4.11. A plot of the absorbance obtained following antibody binding, as a function of B3955 and B3976 concentrations immobilised by the two step method and quantified by direct ELISA. A concentration of 0.5 mg/ml was optimal for both agents, but a greater response was seen with B3955. The mean values of duplicate results for the two phenazines are shown.

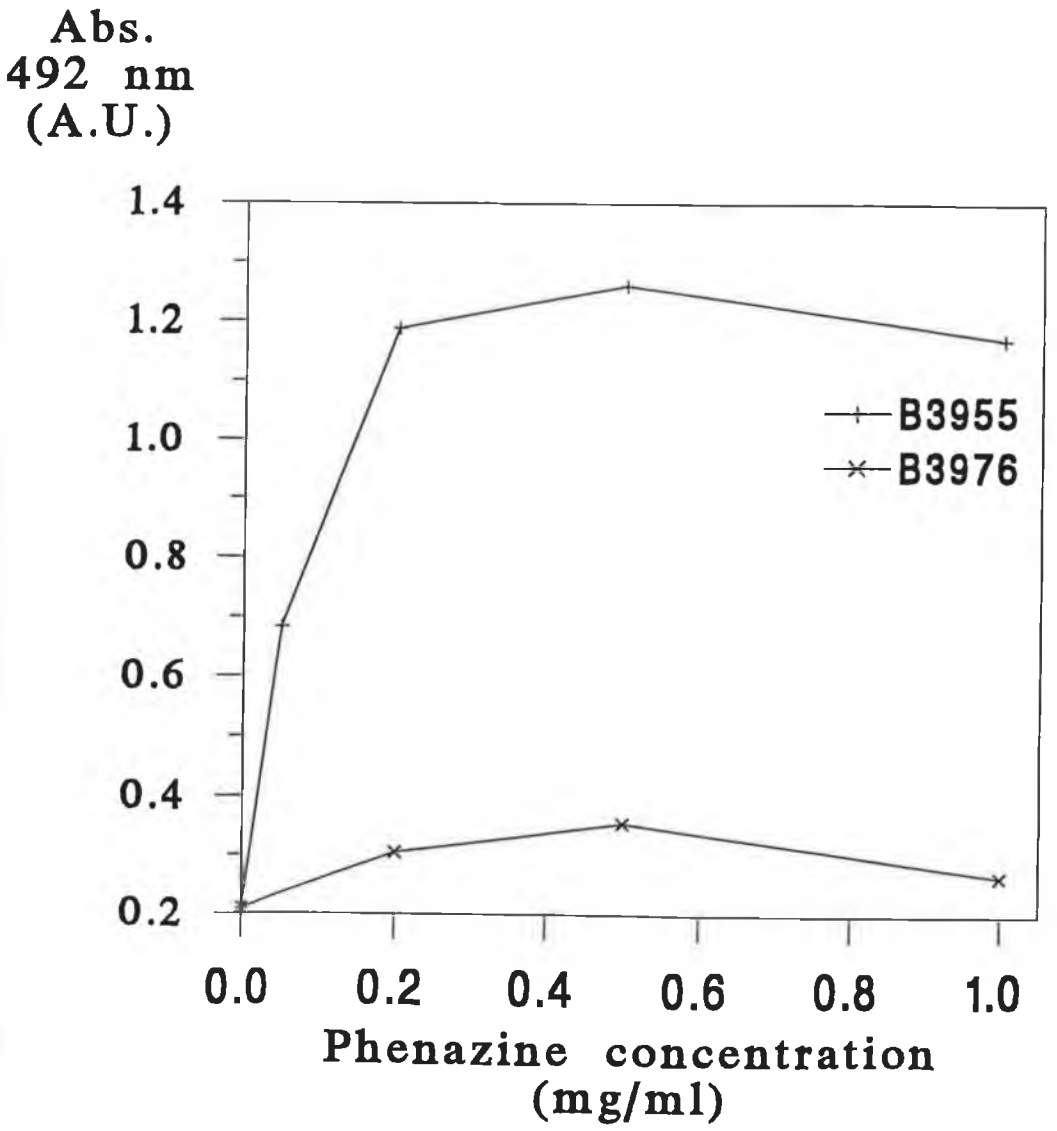


Figure 4.12. A plot of the absorbance obtained following antibody binding, as a function of lysine concentration in competition with 0.5 mg/ml of B3955 and B3976, used in a two step immobilisation of each phenazine and quantified by direct ELISA. A concentration of 100 ug/ml lysine gave the highest response in this experiment, with both agents. The mean values of duplicate results for the two phenazines are shown.

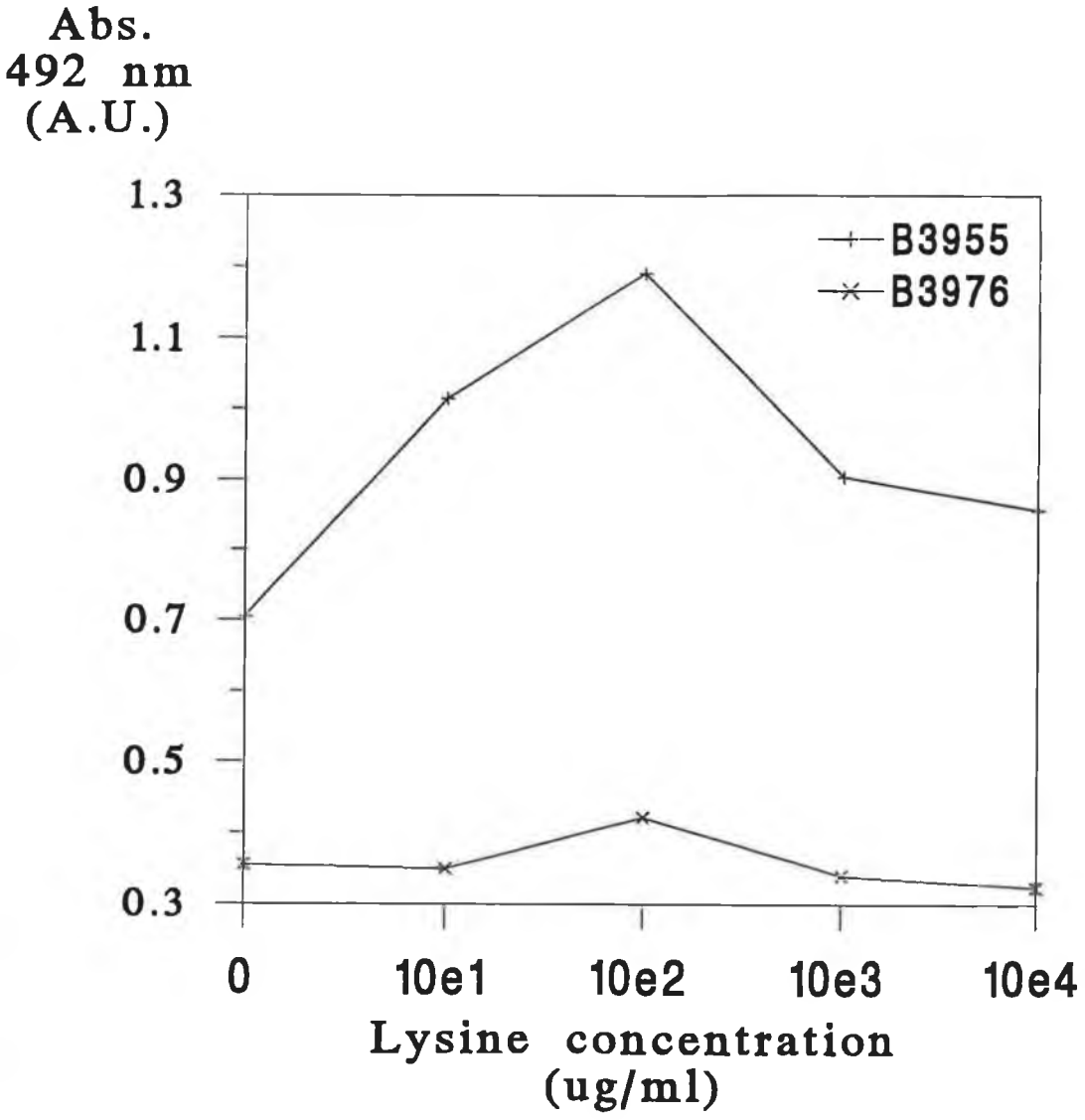


Table 4.3. A comparison of the antibody responses seen with the coating protein, rabbit anti-phenazine antibody, and anti-rabbit antibody at pH 7.4 or 9.6. The amount of variation is small, but the largest response is seen with all proteins in a pH 7.4 environment. The mean of duplicates results is shown.

Base pH	**7.4**				**9.6**			
Rabbit Ab. pH	**7.4**		**9.6**		**7.4**		**9.6**	
α-rabbit Ab. pH	**7.4**	**9.6**	**7.4**	**9.6**	**7.4**	**9.6**	**7.4**	**9.6**
Absorbance of 1/1000 rabbit diln. at 492 nm (A.U.)	0.312	0.283	0.287	0.285	0.293	0.261	0.268	0.255

Figure 4.13. A plot of the absorbance obtained following antibody binding, as a function of B3976 and B3955 concentration, used in a competitive ELISA. A concentration dependent response was seen from 0.5 - 0.002 mg/ml with a linear response from 0.5 - 0.02 mg/ml for both agents. The mean values of duplicate results for the two phenazines are shown.

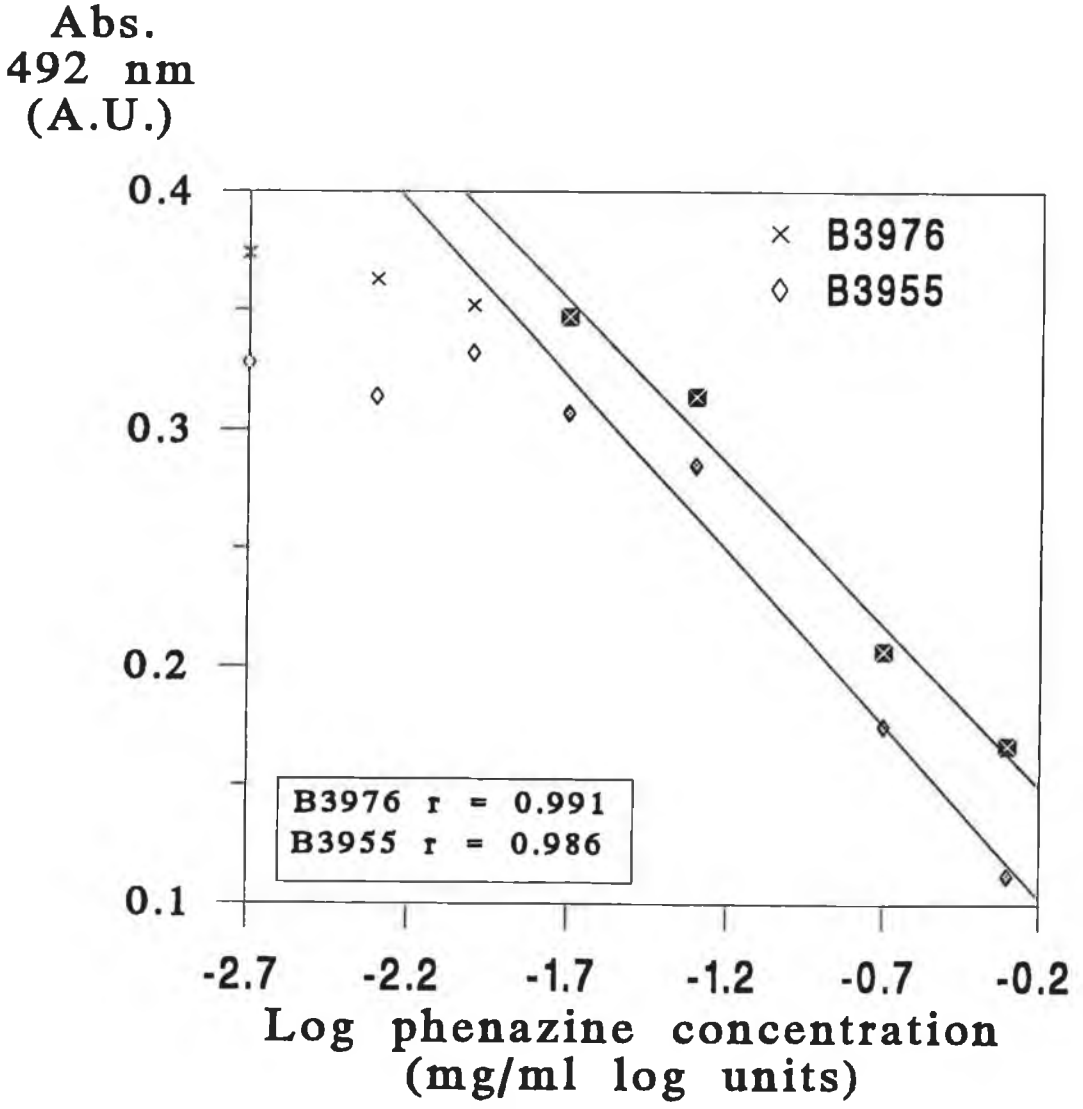


Figure 4.14. A plot of the absorbance obtained following antibody binding, as a function of B3955 concentration used in a single step immobilisation using EDC and quantified by direct ELISA. A concentration of 0.1 mg/ml B3955 gave the highest response of the concentrations used in this experiment. The mean values of duplicate results are shown.

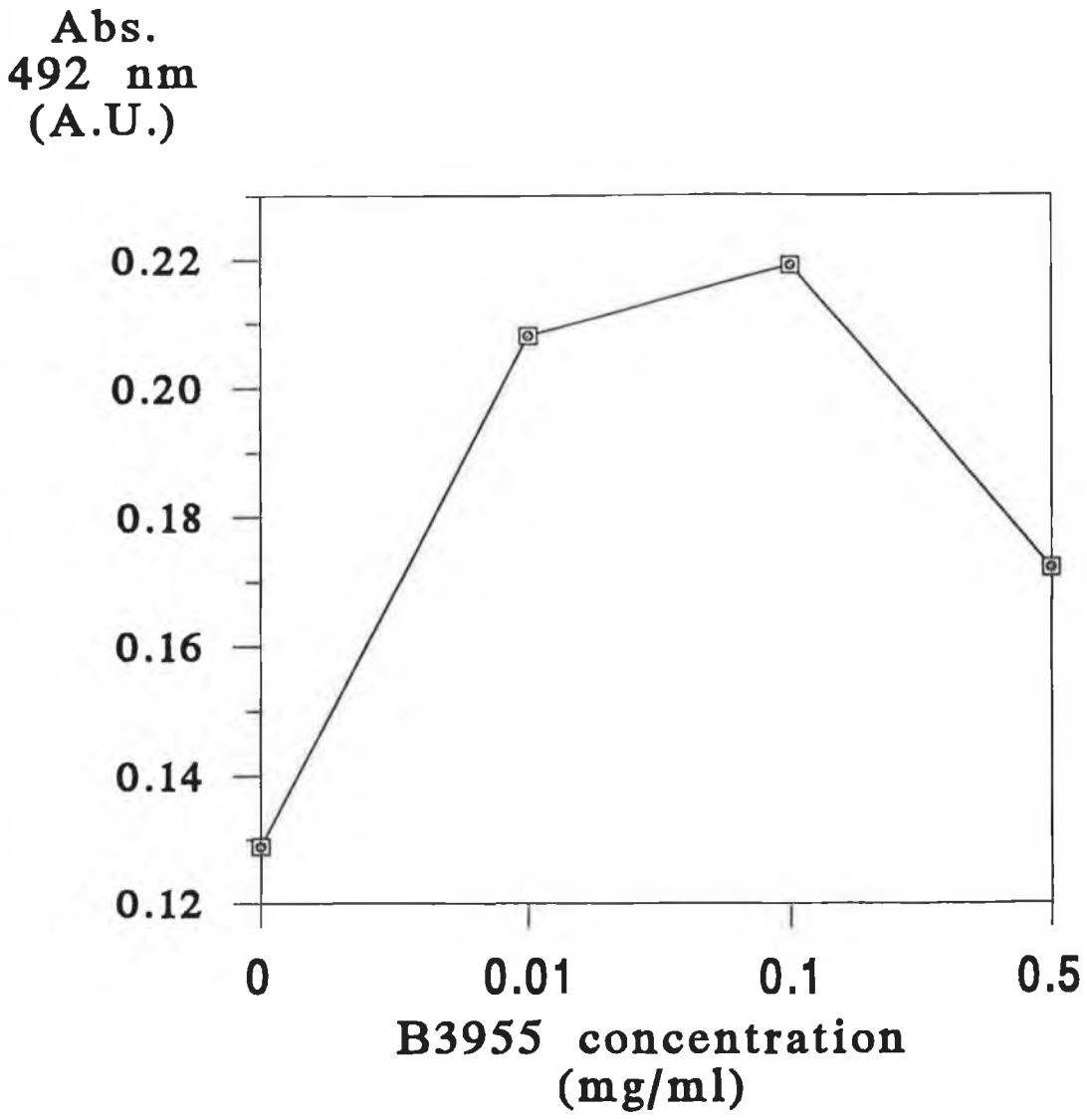


Figure 4.15. A plot of the absorbance obtained following antibody binding, as a function of B3955 concentration in a competitive assay using EDC immobilisation. A concentration dependent response was seen from 0.005 to 0.0001 mg/ml of B3955. The mean values of duplicate results are shown.

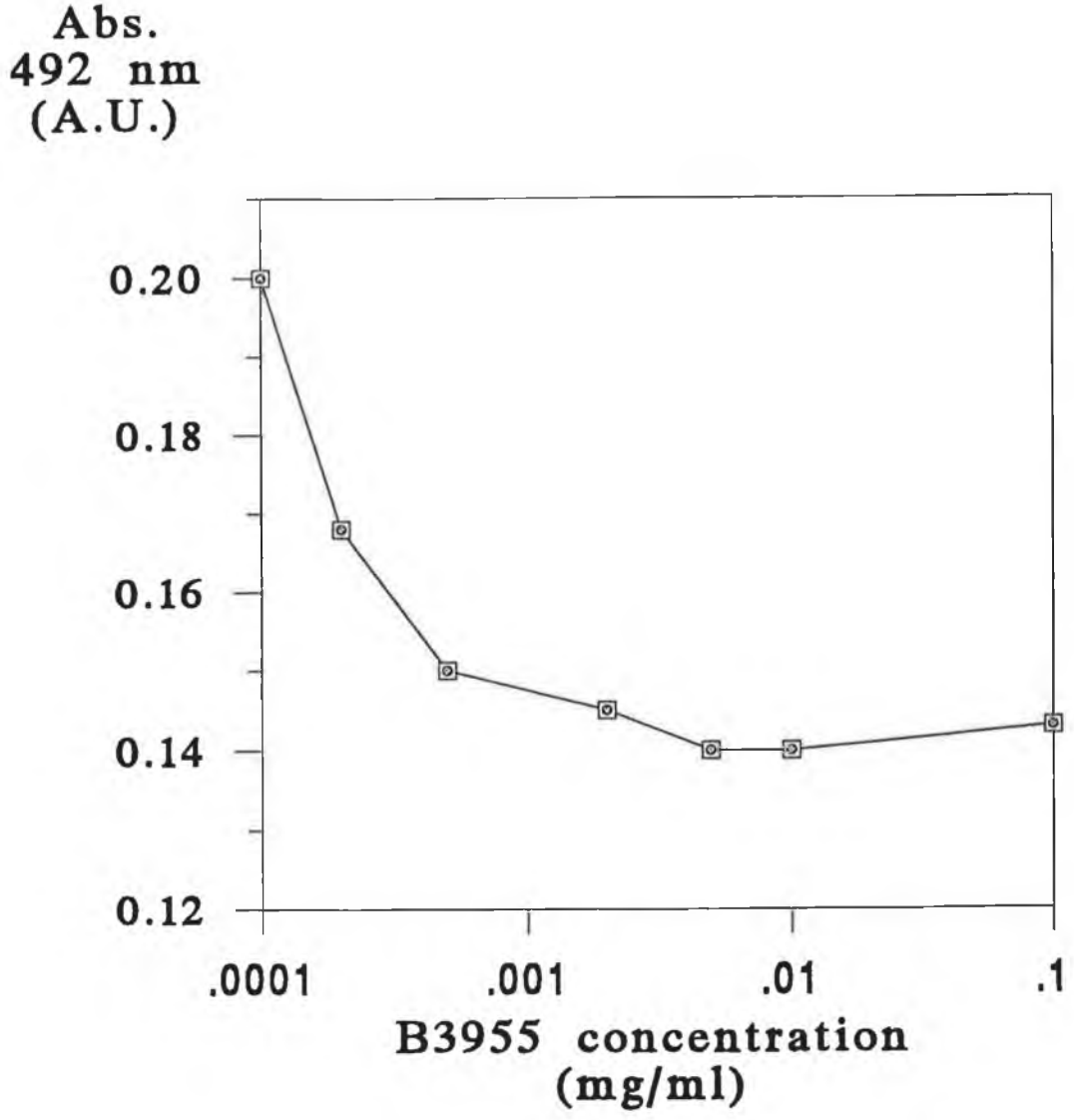


Figure 4.16, this response peaked after seven immunisations. The background response in glutaraldehyde increased over the same length of time, indicating that the relative response of the antisera had increased against glutaraldehyde to the detriment of the anti-phenazine response. The rabbits were switched to an immunisation with the KLH conjugate produced with EDC/NHS.

4.5.3. Other ELISA plate formats

In an effort to improve the response of the antibody in an assay system, and to further verify its affinity for phenazine agents, other plate formats were investigated. These were formats involving direct coupling of phenazines to derivatised plate surfaces (Covalink and Amine binding) and non-covalent attachment to silica-coated plates.

4.5.3a. Assays using covalink ELISA plate

This plate contains a secondary amine grafted onto the polystyrene surface (Yonezawa *et al.*, 1993; Rasmussen, 1990). Coupling of B3976 with the homobifunctional reagent, BS^3, was investigated. As shown in Figure 4.17, a concentration of 0.02 mg/ml of B3976 was found to give the greatest response in a direct assay.

4.5.3b. Assays using silica coated plate

The wells in this plate are coated with silica which creates a polar surface of large area for adsorption of polar molecules, with a very large surface area (Frey *et al.*, 1993). As shown in Figure 4.18, concentrations of B3976 and especially B3955 could be detected in a direct format. However, visually the amount of silica immobilised appeared to be very variable between wells producing a poor reproducibility. Competitive formats failed to show a reproducible response and no concentration dependent response was seen with the other organically soluble rimino-phenazines.

4.5.3c.Assays using amine binding plate

These plates have a N-oxysuccinimide surface to immobilise compounds possessing a free amine group. Direct immobilisation of various concentrations of B3955 and B3976 showed that a higher response was with B3955 (Figure 4.19). Optimal responses were seen across a broad immobilisation concentration range from 0.01 - 0.2 mg/ml of these phenazines and

Figure 4.16. A plot of the absorbance obtained following antibody binding, as a function of the number of immunisations. A 1/2500 dilution of antisera collected from the male rabbit after successive glutaraldehyde conjugate immunisations was used in each case. These results were performed by a direct ELISA, using an EDC immobilised B3955 surface. The antibody response was seen to vary with successive immunisations, and peak after seven immunisations.

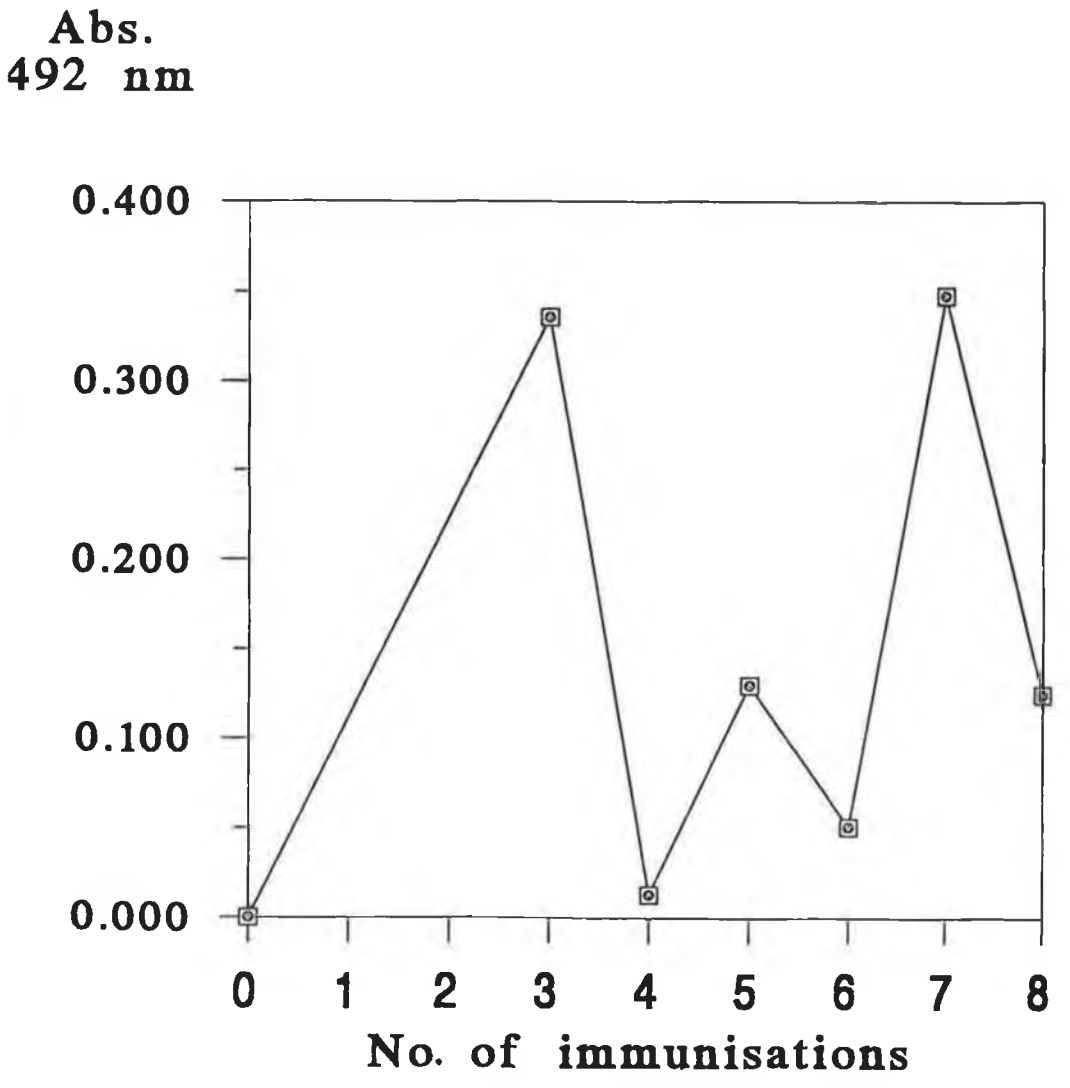


Figure 4.17. A plot of the absorbance obtained following antibody binding, as a function of B3955 concentration used in immobilisation to a Covalink ELISA plate and quantified by direct ELISA. A concentration of 0.02 mg/ml B3955 gave the optimal response for this format of plate.

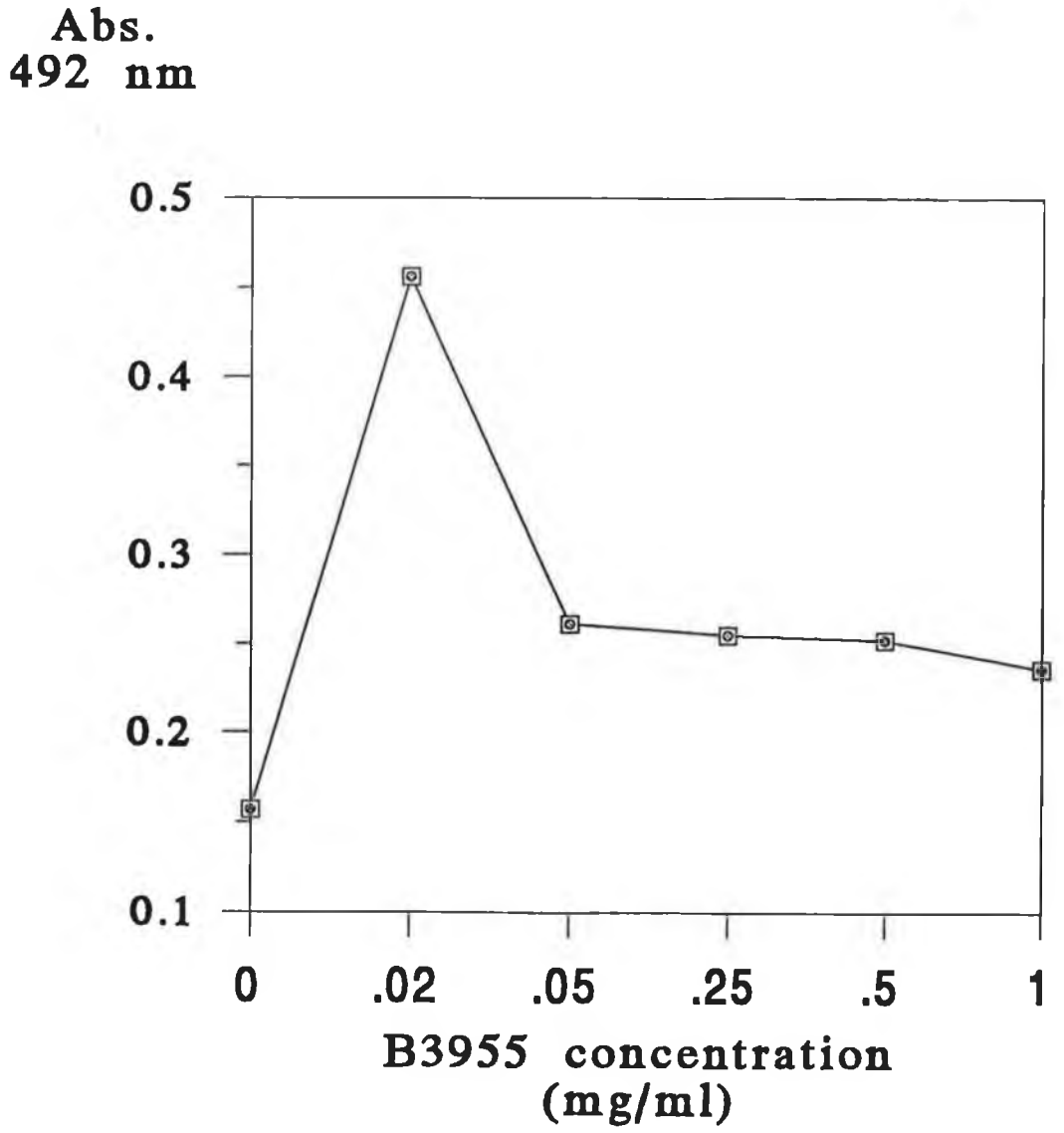


Figure 4.18. A plot of the absorbance obtained following antibody binding, as a function of B3955 and B3976 concentration used in a direct ELISA using silica immobilisation. A concentration of 0.5 mg/ml of each agent produced a maximal response for each agent, with the highest response seen with B3955. The mean values of duplicate results for the two phenazines are shown.

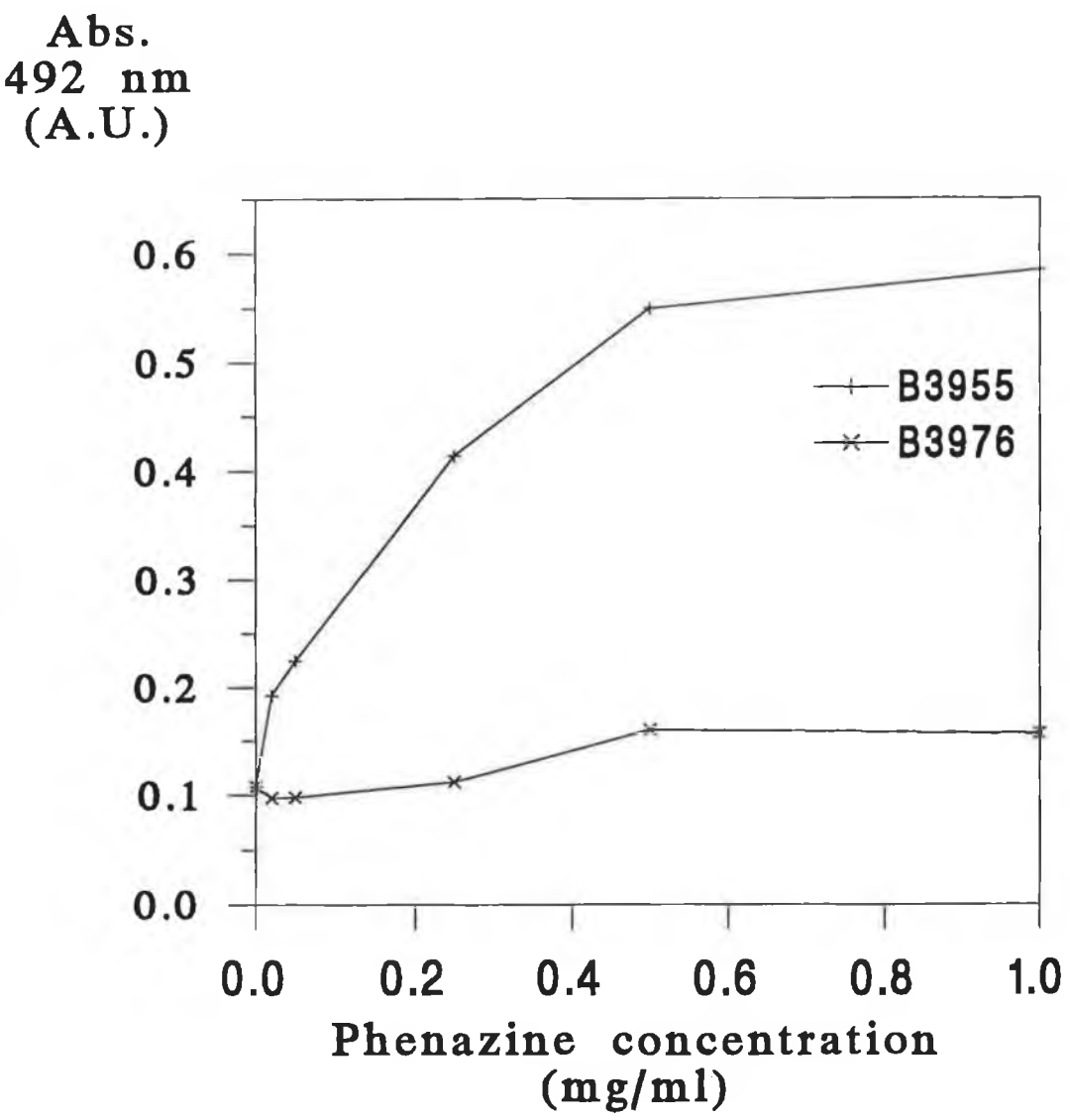
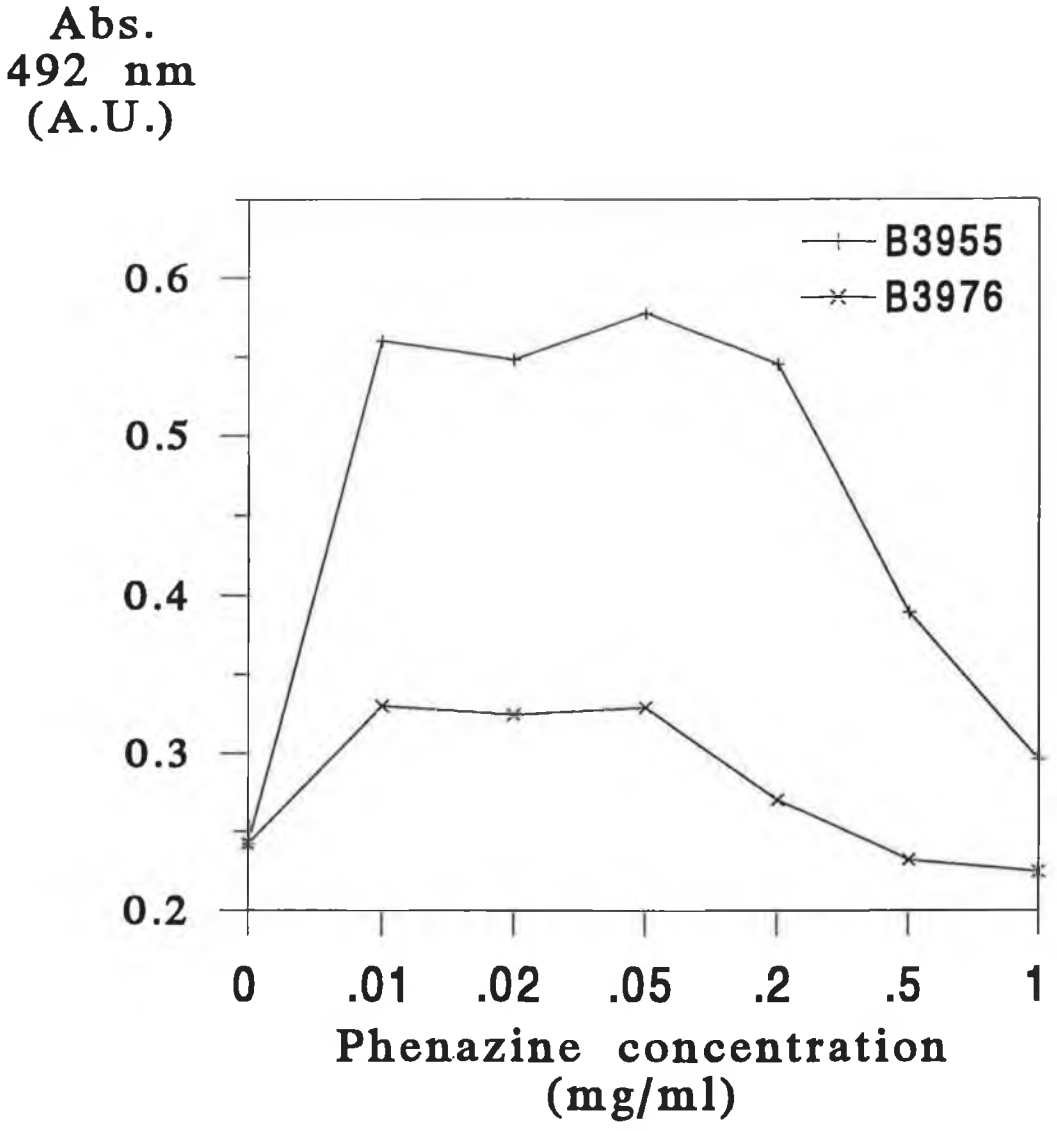


Figure 4.19. A plot of the absorbance obtained following antibody binding, as a function of B3955 and B3976 concentration used in an amine binding plate and quantified by direct ELISA. Optimal responses were seen across a broad concentration range of 0.01 -0.2 mg/ml for each agent, with B3955 giving a higher level of binding. The mean values of duplicate results for the two phenazines are shown.



concentrations in excess of these values had a reduced response for both agents. These plates also successfully coupled another amine-containing analogue, B3832. This analogue was made soluble in water by making aqueous dilutions from a stock concentration in DMF. However plates coated with either B3955 or B3832 by this method gave irreproducible results in a competitive format.

4.6. PURIFICATION OF ANTI-PHENAZINE ANTIBODY

After four immunisations with the KLH conjugate the anti-serum was purified and characterised using SEC-HPLC and BIAcore methods.

4.6.1. Production of a phenazine immunoaffinity column

An Emphase activated support medium was chosen as the matrix for the immunoaffinity column. This matrix consists of beads of a methylenebisacrylamide/azlactone copolymer. When water is added, the azlactone ring is hydrolysed and rendered reactive to nucleophiles, such as amine groups.

Initially attempts were made to couple aqueous B3955 to this support. However, when washed with either 1.5 M lysine or 0.01 M PBS, pH 7.4, the red colour was seen to elute from the column, until very little remained. Further chemical coupling of B3955 to this column was attempted using EDC and, subsequently, glutaraldehyde. In each case the colour remained even with PBS, but addition of the elution buffer (0.1 M glycine pH 2.5) leached the remaining colour from the column.

A more complex immobilisation scheme was developed using the high affinity interaction of biotin and avidin. Using the method described in section 2.17.1. an organically soluble amine containing rimino-phenazine, B3832, was coupled through a linker molecule to biotin. Avidin was stably coupled to the Emphase matrix and the biotin-B3832 conjugate added. After elution of the unbound B3832, a stable phenazine immunoaffinity column was produced.

4.6.1a. Coupling of Biotin to B3832

As described in section 2.17.1. of the methods, as excess of B3832 was mixed with biotin amidocaproate-N-hydroxysuccinide in an alkaline mix of DMF. A difference in the r_f

value of the major spot, before and after NHS reaction, on TLC plates using different solvent mixes suggested that a product had formed in the reaction. However, the difficulty in visualising the conversion of the biotin substituent with D.A.C.A. made this result ambiguous. Using HPLC a reaction product was seen. As Figure 4.20 shows, chromatographic analysis indicated that this compound was impure, producing 5 peaks. When reacted with the NHS-biotin compound a peak was produced at 3.8 mins. As shown in Figure 4.21, the presence of this peak is associated with a decrease in the size of peaks at 4.6 and 5.7 minutes, while the size of the peak at 3.05 mins. remains the same in both chromatograms. Integration of the area of all the peaks showed that there was an 18 % decrease in the relative area of the 4.6 and 5.7 min. peaks, with a 20 % increase in the peak area from 3.5 to 4.2 minutes. This indicates that the peak at 3.8 mins. represents the reaction of the two compounds with retention times of 4.6 and 5.7 mins. Spectral investigation of this peak also indicated that this reaction was with a side-chain substituent of a phenazine, since the chromatogram of the 3.8 min. peak exactly matched those at 4.6 and 5.7 mins. Comparison of chromatograms produced by treating B3832, NHS-biotin compound and the B3832-biotin conjugate with DACA, showed a peak at 1.9 mins due to this agent but failed to show any other reaction of the product at 3.8 mins.

4.6.1b. Immobilisation of avidin to emphase column

A BCA assay of the 15 ml of supernatant from the avidin immobilisation did not show any protein present indicating that most of the protein had bound. After addition of the B3832-biotin reaction mix the column was washed with DMF, PBS and glycine pH 2.5 until no more colour eluted. The red colour of the column remained throughout the purification of the antibody, indicating that no B3832 was leaching from the column.

4.6.2. Purification of antibody

The initial 7 ml of serum had a total protein content of 545 mg. After centrifugation through the 100 KDa molecular weight cut-off membrane, 443 mg of high molecular weight protein remained. This was loaded onto the column and eluted with glycine ,pH 2.5. 0.99 mg of purified IgG protein was eluted.

Analysing the eluted enriched fraction by HPLC showed a single peak with the same retention time as a purified rabbit IgG sample, 10.6 mins, as shown in Figure 4.22.

Figure 4.20. The HPLC chromatogram of B3832. The sample was prepared and analysed as described in section 2.10 of the methods. The presence of five major peaks indicates that this compound is not pure. Based on the reaction of this mixture with the biotin-NHS ester (figure 4.21), B3832 is most likely the peak at 4.6 or 5.7 minutes.

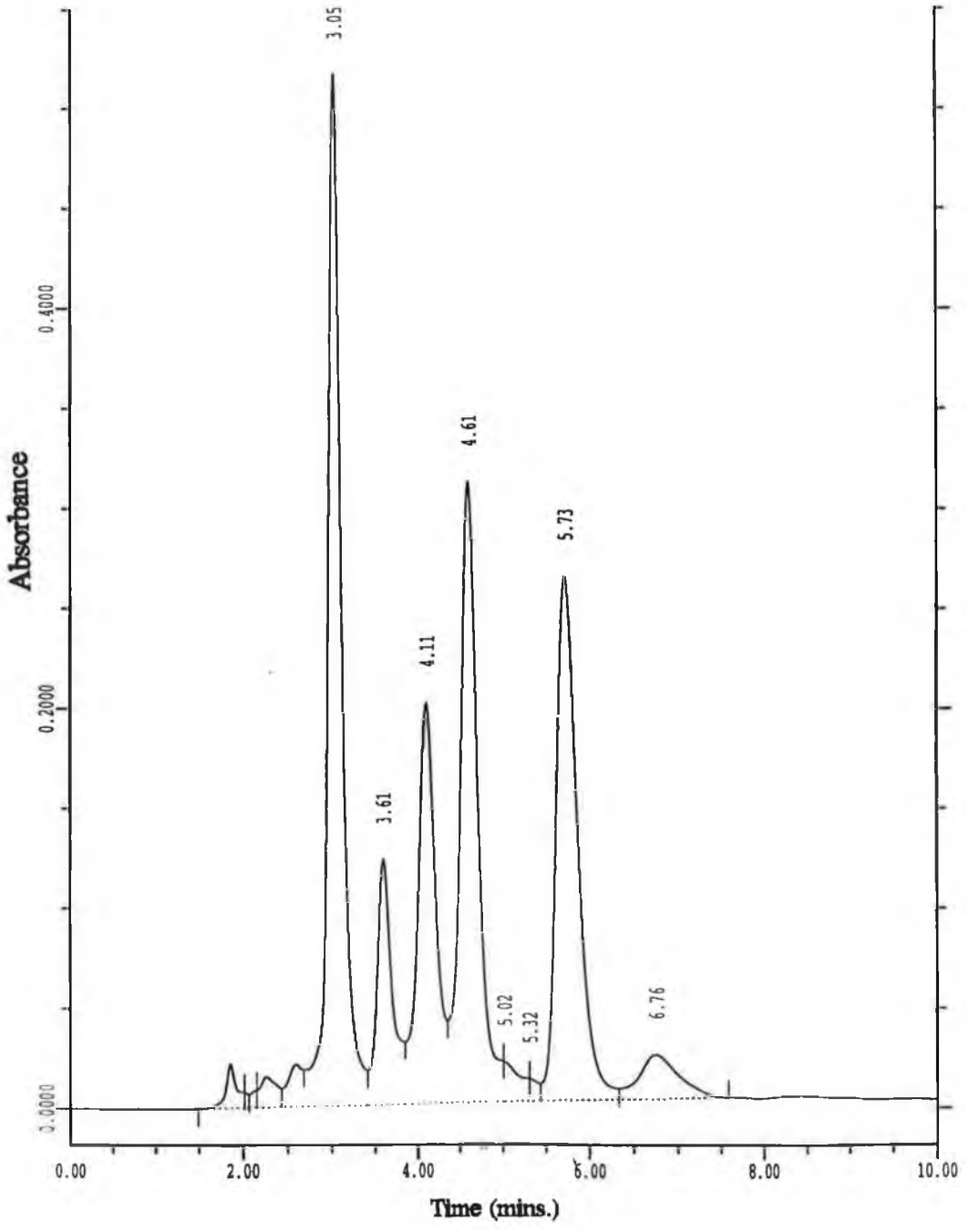


Figure 4.21. An overlaid comparison of the HPLC chromatograms of B3832 before and after reaction with the biotin-NHS ester. When reacted with this ester, a new peak at 3.8 mins. is produced. The area of this peak matches the reduction in the area of the peaks at 4.6 and 5.7 mins. suggesting that the compounds represented by these peaks react with the biotin ester.

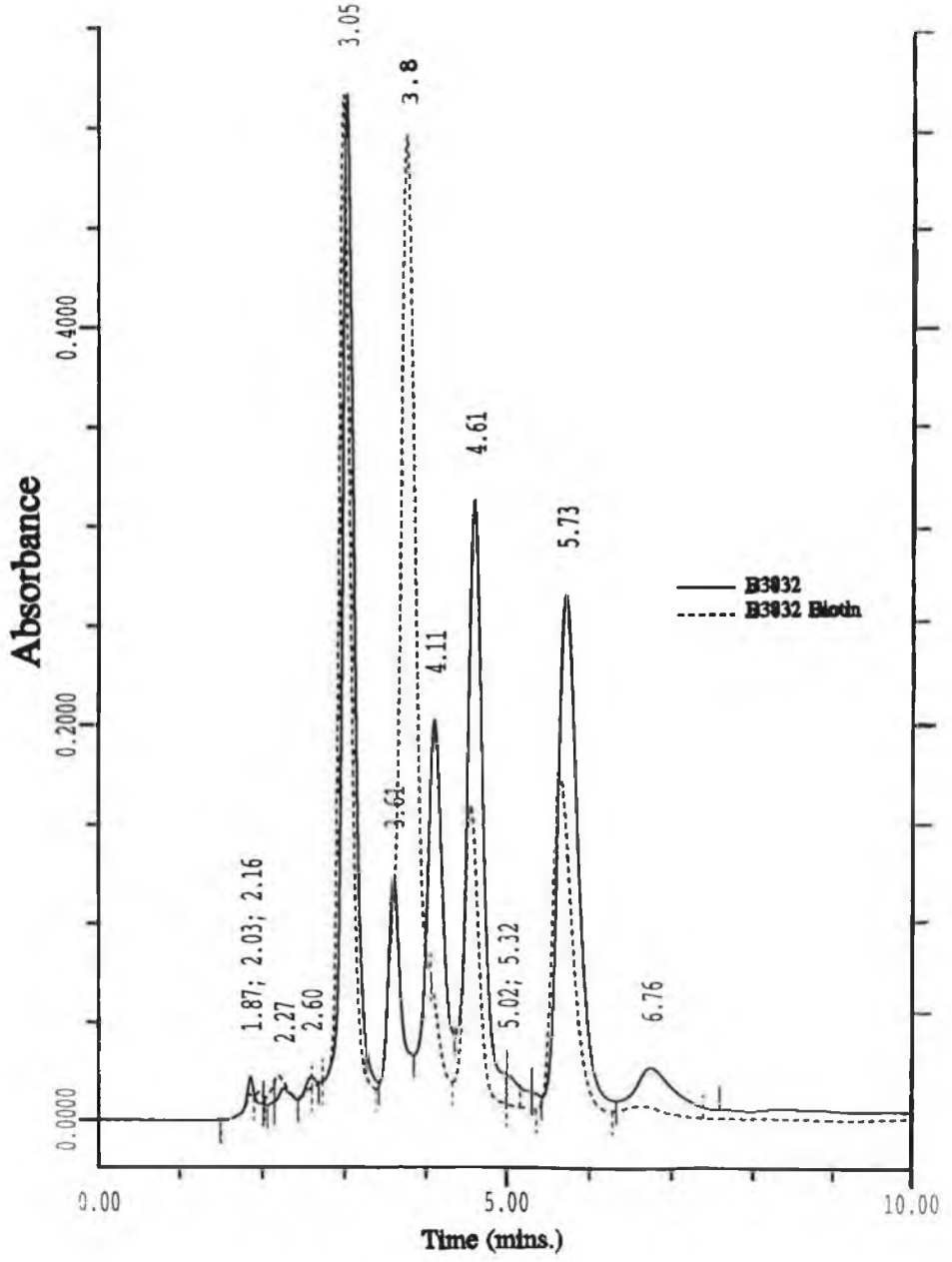
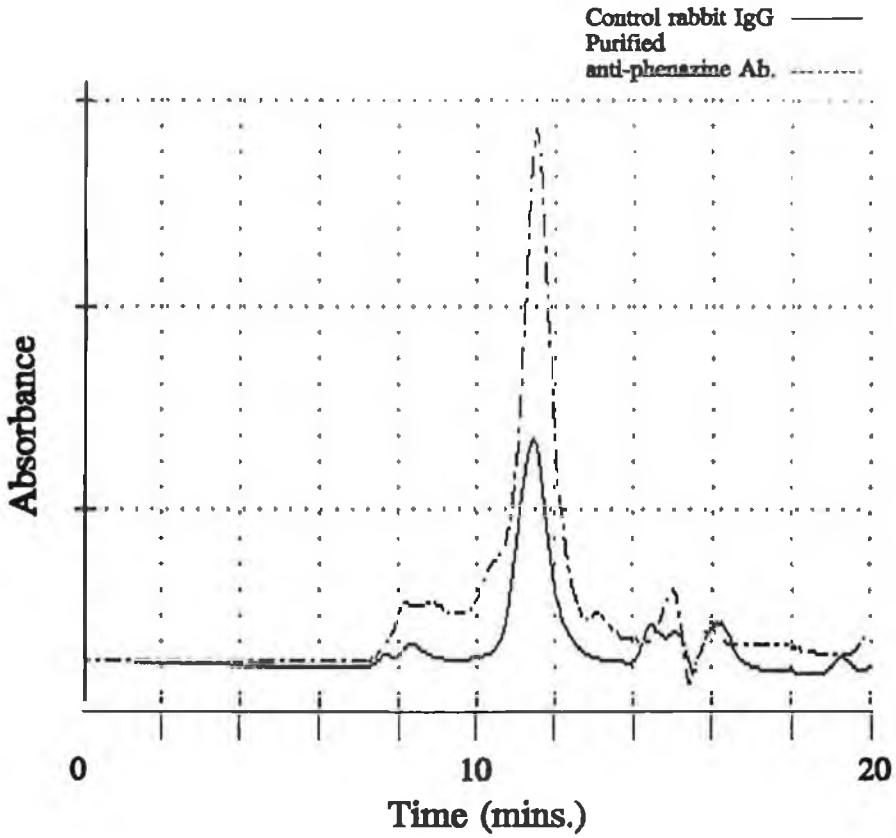


Figure 4.22. A comparison of the HPLC chromatograms of the purified rabbit anti-phenazine antibody and a control purified antibody. The samples were analysed as described in section 2.14.3. of the methods. This indicates that both proteins are of the same molecular weight.



The purified antibody could detect concentrations of B3955, B3976 and B3832 from 0.2 to 0.02 mg/ml immobilised by direct amine binding plate ELISA down to a dilution of 1 in 20,000 (Figure 4.23).

4.6.3. Biacore analysis

Initially, using a concentration of B3955 at 1 mg/ml 11,000 response units (RU) of B3955 were immobilised to the chip surface. Although this gave a 1500 RU response for the purified antibody, a large response of 330 RU was also seen with a control of another purified rabbit IgG. When the immobilising concentration of B3955 was brought down to 10 µg/ml a response of 2000 RU was seen. As Figure 4.24 shows, the immobilised B3955 bound 68 RU of control antibody, however, most of this was quickly eluted without a wash step. A subsequent addition of purified antibody gave a response of 606 RU, of which, even after 12 hours of continuous wash, 543 RU remained. Attempts at eluting this antibody with 0.05 M HCl and 0.1 M glycine pH 2.5 were unsuccessful and three washes with 20 % (v/v) acetonitrile in 0.01 M NaOH removed 184 RU of antibody. As a control, the other purified rabbit antibody was also immobilised to a chip surface and anti-phenazine antibody added. A small non-specific response was seen despite the immobilisation of a large mass of this control antibody. This indicates the selectivity of the antibody for the phenazine matrix alone.

Figure 4.23 A plot of the absorbance obtained following antibody binding, as a function of different dilutions of the purified antibody, for an immobilising concentration of 0.5 mg/ml B3955, B3976 and B3832 used in a direct amine binding plate ELISA. The maximum dilution of antibody to give a response was 1/20,000 of the stock antibody solution.

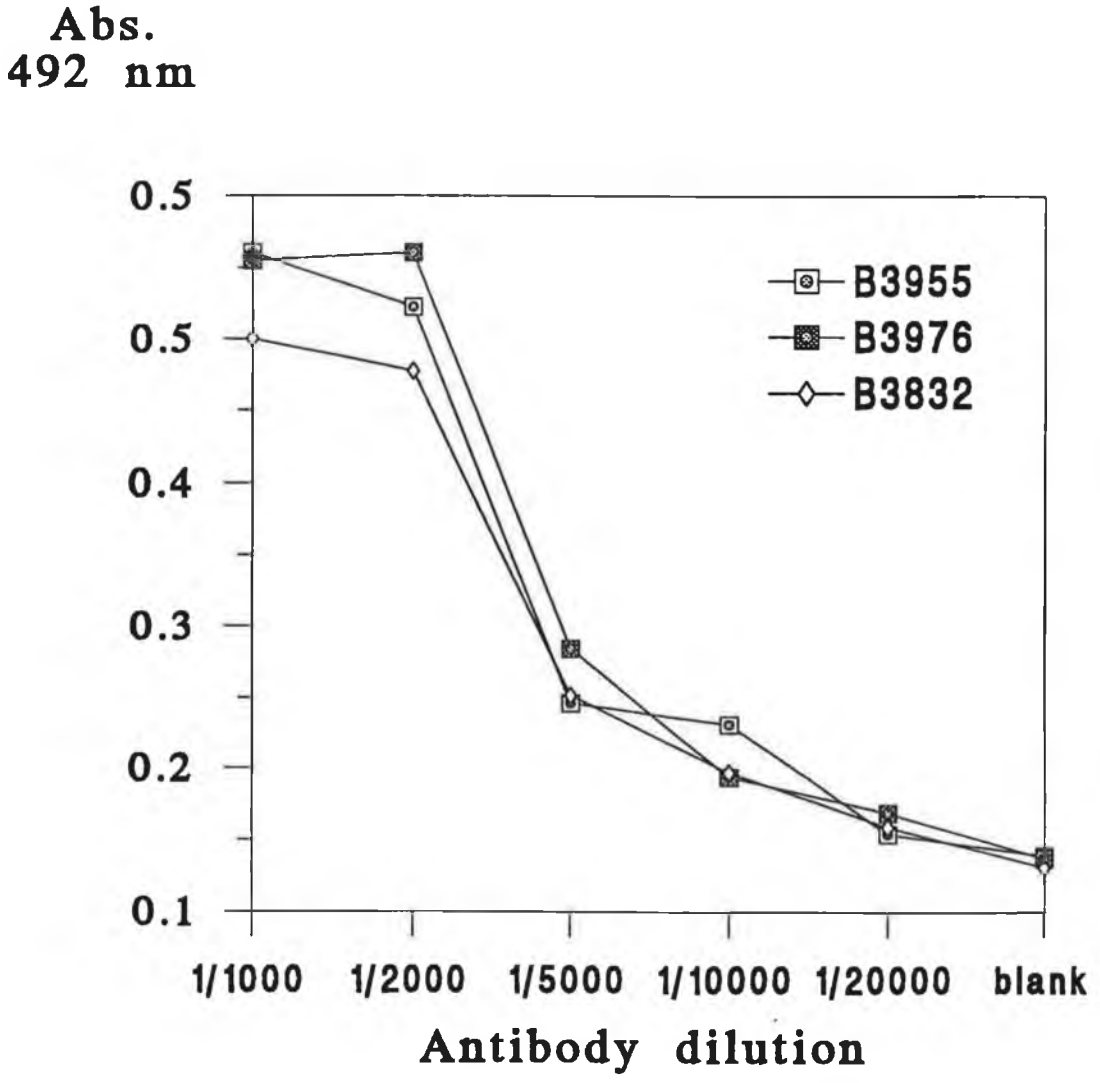
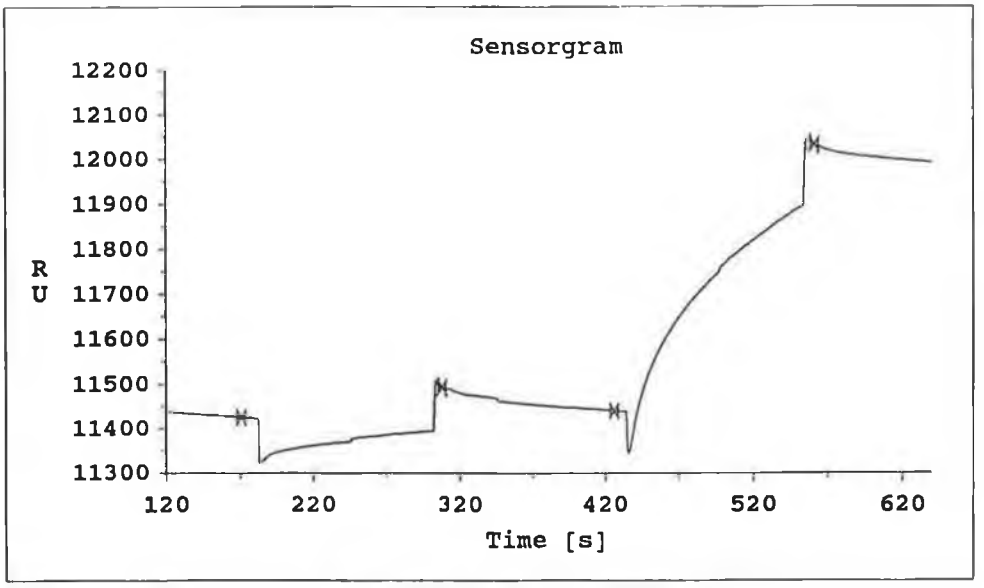


Figure 4.24. The BIAcore sensogram produced by the addition a control and, later, anti-phenazine antibody to a sensor chip surface immobilised with B3955 . The immobilisation method and analysis conditions are described in section 2.5.3 of the method chapter. The initial response was set as baseline. Addition of 20 ul of 0.2 mg/ml of the control antibody produced a response of 68 RU which went down to 15 RU after only 120 seconds. Addition of the same amount of anti-phenazine antibody produced a response of 606 RU. This response remained high even overnight and ultimately could not be brought back to baseline.



Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
170.50	5.00	11426	0.71451	-0.32259	0.49928	YES	0	baseline
307.50	5.00	11493	2.92182	-1.74483	0.42790	NO	68	control_ab
426.00	5.00	11441	0.55778	-0.26712	0.35727	NO	15	2nd_baseline
561.00	5.00	12032	3.60644	-2.14941	0.57874	NO	606	anti-clof

4.7. PRODUCTION AND CHARACTERISATION OF GLUTARALDEHYDE CONJUGATES

It is a general axiom of immunology that for a compound to be antigenic, it must have a molecular weight in excess of 40 KDa. Smaller compounds, such as peptides, may be weakly immunogenic down to a molecular weight of 2 KDA, but often produce low affinity antibodies (Catty, 1988; Catty and Raykundalia, 1988; Burrin and Newman, 1991; Hurn and Chantler, 1980). Therefore, it was necessary to produce a conjugate of a suitable phenazine with a protein before an anti-phenazine antibody could be raised.

The production of a phenazine protein conjugate was made possible by the synthesis of rimino-phenazines which are water-soluble with a reactive group which does not interfere with the phenazine ring. Compounds such as B3955 and B3976 are piperidyl-substituted rimino-phenazines which have been covalently linked to amino acids via their carboxyl groups. In one step, this produces a riminophenazine which is water soluble, allowing reaction with proteins. These phenazines also possess an amine group which is readily reactive towards proteins via a number of well described chemistries.

Thyroglobulin was chosen as a suitable protein carrier for the immunogen because of its availability, large size, abundance of reactive groups and limited solubility when conjugated. BSA was chosen as a screening conjugate carrier because of its cheap cost and availability, and general high solubility. Initially, a glutaraldehyde-based conjugation was chosen since it is routinely used for hapten-carrier linkage and reacts readily with many haptens and proteins.

Test mixes of glutaraldehyde with B3976 and BSA gave a protein which remained red coloured even after dialysis. This suggested that a conjugation reaction could take place using glutaraldehyde and aqueous B3976. This was important because the phenylalanine substitution in this compound increases its solubility in water, it rapidly precipitates in neutral or alkaline aqueous buffers. Alkaline reaction conditions are generally necessary for many of the common conjugation conditions.

In solution, glutaraldehyde exists as a mixture of monomer and polymers of various lengths which are in a complex equilibrium. At pH 3, low temperature and high concentration the monomeric and low number polymer forms predominate. As the pH and temperature

increase, and the glutaraldehyde concentration decreases, the equilibrium is driven to longer stable polymers until precipitation occurs. Glutaraldehyde reacts with free amino groups, especially the ε-amino group of lysine (Peters and Richards, 1977; Habeeb and Hiramoto, 1968). In the simple one step reaction, glutaraldehyde will therefore produce four main types of reaction with a drug-protein mixture. These are an intra-molecular crosslinking of lysine residues within the protein, inter-molecular linking of protein molecules to each other, linking of drug molecules to each other, and finally, linking drug molecules to the protein. Drug-protein conjugation is the desired reaction and therefore, the other reactions are inefficient and wasteful of reagents. In our research, this inefficiency was not critical, since enough B3976 was available. However, the need to conjugate limited amounts of expensive drugs or peptides can preclude using this method. The polymerisation of glutaraldehyde has the added advantage of introducing a linker between the drug and carrier which is very advantageous, both for a strong selective immune response, and for antibody screening (Exley *et al.*, 1971; Lindner *et al.*, 1972).

Conjugation via a two step reaction was investigated as a more efficient alternative and also because it has been shown that these type of conjugates are superior as immunogens (Zegers *et al.*, 1990). Dialysis of the protein against glutaraldehyde introduces a reactive surface which can bind the free drug, with remaining reactive groups capped with the more reactive free lysine. Characterisation of both conjugates by SEC indicated that the one step reaction bound a higher molar ratio of drug than the two step reaction. Although a high ratio of drug to protein is desirable for an efficient immunogen, total drug binding of available residues may not be optimal. Erhlanger, (1980) and Landsteiner, (1947) have suggested that conjugation of 10 drug molecules to BSA was best. Thyroglobulin and BSA contain 177 and 55 lysine residues, respectively and, of these, approximately 90 and 19 are accessible on the external surface of the protein (Schipper *et al.*, 1991). The conjugation ratio results indicate, that the one step method can bind drug to all the available lysine residues, and also, to other residues in the protein, especially with higher concentrations of glutaraldehyde and B3976. The two step reaction conjugates a smaller ratio of drug molecules indicating that only the available lysine residues are reacted.

When freeze dried, the conjugates became insoluble. The production of insoluble conjugates with glutaraldehyde is common, especially when it is present in higher relative concentrations (Avrameas and Ternynck, 1969). This is desirable for immunising conjugates since it localises the immunogen, boosting the APC response and concentrating other immune cells and mediators in the site of injection. However, this insolubility prevented the prepared BSA conjugate being used to screen antibodies in ELISA. The insolubility was probably due

to a combination of increased activation of residual free glutaraldehyde, and conversion of longer polymers present, to monomers, by the low temperature, and increased concentration as water was removed. The insolubility of the BSA conjugate could have been overcome by not freeze drying it; however, it was noticed that some of the conjugate precipitated when stored, and it was decided to produce the conjugate *in situ* with each ELISA plate.

High concentrations of glutaraldehyde in the one step immobilisation produced lower responses. This may indicate either excess cross-linking of the BSA, reducing the available residues for drug conjugation, or, binding of too much drug to the BSA surface. Other authors have also found that a concentration of 0.2 % (v/v) glutaraldehyde was optimal for conjugation reactions (Avrameas and Ternynck, 1969; Avrameas, 1969). The importance of an optimal drug density in the screening ELISA was seen by the increased response seen with the inclusion of lysine in the ELISA formats.

4.8. GLUTARALDEHYDE IMMOBILISATION OF PHENAZINES ON ELISA PLATES

Assessment of the amount of BSA bound with different immobilisation reactions showed that glutaraldehyde had a significant effect on the immobilisation of the protein. The augmentation in binding results from increasing the net molecular mass of BSA, through cross-linking, and also through, poorly characterised, covalent linkage of the polyglutaraldehyde with the plastic surface (Tijssen, 1985; Stanislawski *et al.*, 1980; Barret, 1977; Dobbins-Place and Schroeder, 1982). Although this had a minor effect in the assays described, this is very significant for assays involving proteins, especially those of relatively low molecular weight (Klasen *et al.*, 1982; Suter, 1982; Wood and Gadow, 1983).

The polymeric nature of glutaraldehyde, and its inherent foreignness, caused problems in the antibody production. All glutaraldehyde-based ELISAs had a high background due to the presence of an anti-glutaraldehyde component in the antisera. The development of an anti-linker response is common and has been noted by other workers using glutaraldehyde conjugates (Briand *et al.*, 1985). Although this response was difficult to quantify, since an anti-glutaraldehyde format was not developed, the constant increase in the response to the BSA-glutaraldehyde wells, and the reduction in the drug titre over time indicated that the anti-glutaraldehyde response was predominating at the expense of the anti-B3976 response. This was overcome by switching to another immunising conjugate.

4.9. PRODUCTION AND CHARACTERISATION OF EDC/NHS-KLH CONJUGATE

Keyhole limpet haemocyanin (KLH) is the oxygen transport protein in the keyhole limpet mollusc. It is one of the largest known protein molecules, with a molecular weight up to nine million Daltons (Bannister, 1981). KLH has some inherent advantages as an immunogen. Its large size provides a very large area and variety of conjugation sites, and makes it very antigenic. It is phylogenically very distant from mammalian proteins, again making it very antigenic, but also ensuring that it does not crossreact with sera against mammalian proteins (Burrin and Newman, 1991). It was decided to use a KLH conjugate with another amino acid-substituted phenazine, B3955, to provide sera which could be purified and characterised. Coupling with EDC/NHS was chosen since this is very different to the glutaraldehyde reaction and successful conjugates with other organically soluble drugs had been described in the literature (Grothaus *et al.*, 1993).

Characterisation of this EDC conjugate by SEC indicated that the approximate molar ratio of drug bound was 990. The exact ratio of binding is impossible to calculate because of the multi-subunit nature of KLH, but these calculations are based on an average molecular weight of 5 MDa assumed since the molecular weight range quoted by the supplier was 3 - 7.5 MDa. At first glance this ratio appears very large, but it is of similar proportion to the ratios quoted for THYR and BSA. EDC catalyses the formation of an amide bond between carboxylic acids and amines on its own. However, it has been reported that the addition of NHS improves yield by reducing hydrolysis of the *O*-acylurea intermediate (Staros *et al.*, 1986). The heterobifunctionality of this reaction provides a degree of selectivity, since B3955 only possesses a free amino group which must couple to a carboxyl group through EDC. This conjugate remained partially soluble even when freeze-dried, unlike the glutaraldehyde reaction.

4.10. CHARACTERISATION METHODS

4.10.1 Analysis of conjugates by SDS-PAGE

The form of SDS-PAGE used in this project proved to be unsuitable for the analysis of conjugates. The presence of a red band at the bottom of the gel, coupled with the lack of a red colouration associated with the protein band, which had the same migration distance as unmodified protein, initially suggested that the conjugation had been unsuccessful or unstable. Analysis of the conjugate by other methods indicated that a significant portion of B3976 remained non-covalently attached to the conjugate even after dialysis. The red band was

therefore due to unbound drug. The lack of red colouration in the protein band was probably due to too little being present to see the colouration. The lack of difference in migration time was due to the properties of the gel and similar problems have been reported by others (Briand *et al.*, 1985). An electrophoresis method for the analysis of conjugates was subsequently published by Kamps-Holtzapple *et al.*, (1993).

4.10.2. Analysis of conjugates by TLC

Proteins, such as BSA and THYR, are not usually analysed by TLC methods because the large number of polar groups they possess prevent them from migrating in TLC solvents. However, in the case of the phenazine conjugates produced, the free phenazine should be mobile on the silica plate, while any conjugated agent should remain with the protein. This system is therefore suitable as a qualitative method for assessment of conjugation.

The results indicated that a conjugate had been produced and also showed that simple spectrophotometric measurements of the dialysed conjugate would be useless for determination of the conjugation ratio, since, a large mass of unreacted drug remained bound to the protein even after dialysis.

4.10.3. Analysis of conjugates by HPLC

The conjugate samples were analysed by HPLC using a column which separates principally on the basis of molecular weight. The eluent was monitored at 280 nm since both drug and protein have a strong absorbance at this wavelength. Unfortunately, this precluded selective identification of the B3976 bound to protein, but did demonstrate the production of higher molecular weight conjugates by the presence of peaks with shorter retention times. Monitoring the eluent at 476 nm did not give any additional information. However, using a photodiode array detector the absorbance of the eluent could be monitored at all wavelengths simultaneously. As expected, THYR and BSA controls, which had been reacted with glutaraldehyde, give strong U.V. absorption on their own. However, with the conjugate samples, the association of the visible absorbance spectrum of B3976 with the protein absorbance clearly indicates direct linkage of the drug to protein. Controls of glutaraldehyde-treated protein are important in the PDA verification of such conjugates since, under certain circumstances, glutaraldehyde can produce coloured products when reacted with amino-acids (Peters and Richards, 1977). Although knowledge of the extinction coefficient of B3976 would allow the measurement of the amount of drug present at any given time, the strong U.V. absorbance of

this agent prevents estimation of the mass of associated protein present.

4.10.4. Analysis of conjugates by SEC

Separation of free drug from the conjugates produced by SEC allowed estimation of the amount of drug present. Coupling this measurement with protein determination of the same fractions by B.C.A. allowed a direct estimation of the ratio of each present. With KLH, the estimation has large associated errors due to variability in molecular weight of the units of protein present. All the estimations merely represent an average ratio of drug bound to protein, since no effort was made to separate the conjugate on the basis of molecular weight. The results also clearly show that the larger proteins bind a higher ratio of drug molecules due to the presence of more reactive groups per unit molecule.

4.11. CONJUGATE IMMUNISATION

No effort was made to purify the conjugates further since the reactive compounds had been inactivated and rimino-phenazines are not inherently toxic (see section 5.22.). As expected, the rabbit had started to produce an antibody after the third immunisation. Interestingly, the titre of the antibody neither increased consistently nor remained constant over time suggesting that the amount of antibody produced over a given period of time is affected by many variables. Possibly important factors include general state of health, of the animal, the presence of infections and changes in the sub-class of antibody being produced (Tijssen, 1985). Ultimately the anti-glutaraldehyde response was seen to predominate and a phenazine conjugate with KLH was used to produce sufficient anti-phenazine sera for purification and characterisation.

4.12. ELISA ASSAYS

4.12.1. Direct assay response

Initially, a direct assay was used to show that both rabbits produced a specific antibody response against B3976. However, BSA-glutaraldehyde control wells gave high responses. Therefore, it was necessary to investigate and verify antibody production by other methods, since the response seen could have been an artifact of glutaraldehyde binding. Modification of the method of immobilising B3976 using a two step procedure produced a significantly better response with lower relative background absorbances. Measurements of the ratio of drug-protein binding in the conjugates, suggests this may be due to the binding of B3976 to the exterior

surface of the protein matrix, in the two step method, where it is more accessible to antibody binding (Zegers *et al.*, 1990).

4.12.2. Competitive assay response

The production of an anti-phenazine antibody was proved using the competitive assay and assays not involving glutaraldehyde immobilisation of phenazines. In the competitive assay, the only variable is the amount of free phenazine, and if higher concentrations of free phenazine reduce the response seen, then an anti-phenazine antibody must be present. This is further supported by eliminating glutaraldehyde completely from the assay and using other methods of immobilisation. The establishment of a proportional response, between the amount of immobilised phenazine and the resultant amount of antibody binding, confirms the presence of an anti-phenazine antibody.

The results of the competitive assay indicate that the antibody produced had a poor affinity. The linear range of the assay (0.02 - 0.6 mg/ml) is both broad and insensitive. Higher affinity antibodies detect low concentrations of analyte, generally, in a narrow range, due to the strong association and low dissociation constants of antibody-analyte interaction.

4.12.3. Variation in antibody affinity for phenazines

B3976 and B3955 differ only in the amino acid substitution present. However, a greater antibody response was seen in all the direct assays formats with B3955, even though the antibody was raised against a B3976 conjugate. Both agents had a maximal response using similar reaction concentrations, which indicates that similar levels of drug are being immobilised in each case. The difference must therefore reflect a variation in the affinity of the antibody for the two agents. The glycine substituent in B3955 increases its aqueous solubility and, therefore, increases access to water soluble substances, including antibodies, when immobilised. Better molecular access would give the antibody a superior affinity for B3955, despite the identical nature of the epitope for B3955 and B3976.

4.12.4. Effect of spacial factors in response

Comparison of the responses seen with the immobilisation of B3976 and B3955 showed that both had an optimal reaction concentration of approximately 0.5 mg/ml. When lower concentrations of these phenazines were used, smaller responses were seen, indicating that the

level of drug immobilised was dependent on the amount available. Concentrations in excess of the 0.5 mg/ml value caused a reduction in the response seen. Results from the conjugation reactions suggest that excess free phenazine does not inhibit the binding of these agents to the protein. Therefore the reduction in response must represent a situation where excess phenazine immobilised inhibits the antibody-hapten interaction. Similar results were also noted with direct EDC, Covalink and amine-binding plate assays, and have been noted by other authors using different antigens on several plate formats (Frey *et al.*, 1993).

These results might be explained by three non-mutually exclusive possibilities. The first possibility is that an optimal spacing of phenazine molecules exists on the immobilised protein surface. A surface where the phenazine molecules are immobilised using concentrations of agent above 0.5 mg/ml in the reaction mixture produces a surface where there is little free space between each drug molecule. This could sterically hinder complete binding of the antibody present which would then be easily eluted in subsequent washing steps (Jefferis and Deverill, 1991; Stenberg and Nygren, 1988). This suggestion is supported by the increase in antibody binding seen when particular concentrations of lysine were included to compete with drug binding. This form of steric hinderance has been described for other immobilised haptens (Butler, 1980; Rotmans and Scheven 1984; Yonezawa *et al.*, 1993).

Secondly, saturation of the protein surface with phenazine might produce a "sticky" surface, where drug molecules stuck to each other and blocking proteins adhered non-specifically, preventing access to the antibody molecules. Evidence for this conclusion comes from the BIAcore, where excess immobilisation caused a large amount of binding from a control antibody. At lower immobilisation concentrations, less anti-phenazine antibody was seen to bind, but non-specific binding of the control antibody was almost eliminated. Although amino acid substitution renders B3976 and B3955 water soluble, these molecules are still very hydrophobic, and, in an aqueous environment, will tend to stick together and to other hydrophobic compounds, such as hydrophobic residues in proteins. Coupled with their polar characteristics, this makes these agents very sticky towards proteins.

The final possibility is that higher concentrations of free phenazine cause non-covalent adherence of these agents to the protein and plastic surfaces present. During the assay, antibody binds to this free component, but the washing steps overcome the non-covalent binding of the drug, and so the associated antibody is also lost during the washing steps. Evidence suggesting this possibility is shown in control wells where BSA and phenazine, but no glutaraldehyde were included. These wells were seen to bind antibody. The need for an initial wash with a Tween

solution which would remove much of this non-covalently bound fraction also supports this possibility. This necessity for a Tween wash in hapten-immobilised plates has been noted by other authors (Yonezawa *et al.*, 1993; Søndergård-Andersen *et al.*, 1990).

4.12.5. Responses to other phenazines

The failure to develop a concentration dependent response to the other phenazines in either a competitive format or with silica coated plates, is probably due to the aqueous insolubility of these compounds. When dissolved in methanol, these organically soluble compounds can be immobilised onto a silica surface. However their insolubility prevents interaction with the antibody in an aqueous environment. These substances can be introduced into an aqueous environment by initial solubilisation in DMF. This creates a DMF solvent sphere around the molecules which is further solvated by water molecules. However, this solvent sphere is relatively impenetrable to the, water solvated, antibody molecules, preventing antibody binding. Immunoassays have been developed against relatively aqueously insoluble compounds such as taxol using up to 20 % (v/v) methanol in antibody solutions (Grothaus *et al.*, 1993), but 20 % (v/v) methanol would not solubilise the organically soluble compounds investigated in this project. The difficulty in using stronger water soluble organic solvents is illustrated for example by the use of 20 % (v/v) dioxane in immunoaffinity elution buffers, where the solvent inhibits the antigen-antibody interaction, causing elution of the antibody from the column. The importance of the aqueous solubility of these compounds is further illustrated by the increased antibody affinity towards B3955 instead of B3976, which was discussed above.

4.13. ANTIBODY PURIFICATION

Conventionally, initial antibody purification is performed by salting out the antibody present using ammonium sulfate. However, this procedure is time consuming and can lead to significant losses of antibody. It was decided to investigate a more rapid clean-up approach, using ultrafiltration. This process separates the proteins present on the basis of molecular weight. Protein greater than the 100 KDa molecular cut-off are retained inside the membrane solvent reservoir. Smaller molecular weight substances and solvent pass through the pores under the influence of centrifugal forces. This both concentrates the high molecular weight fraction present, and, by washing through with buffer, removes contaminating proteins and small molecular weight compounds. IgG antibodies have a molecular weight of approximately 160 KDa and are therefore concentrated in the process. Unfortunately, other high molecular weight substances are also present, particularly lipoproteins. Methods are available to remove these

substances (Tijssen and Kurstak, 1974; Tijssen, 1985), since they can interfere in certain immunoassays, but it was decided to try to immunoaffinity purify the antibody without removing these contaminants. Immunoaffinity purification is necessary since the majority of the antibodies present are directed against other substances, including the carrier protein.

4.13.1. Use of the Emphase matrix

The Emphase matrix was chosen as a suitable immunoaffinity support for several reasons. The polymeric beads of polyacrylamide provide a very rigid support, allowing resistance to high pressures and flow rates. This means that large volumes of antibody can be purified quickly. This matrix is relatively resistant to harsh aqueous conditions and to organic solvents, important since immobilisation ultimately necessitated using organic solvents. The matrix is also very hydrophilic, reducing non-specific protein adsorption and helping to maintain hydrophobic compounds in solution phase (Hermanson *et al.*, 1992). With the first attempt at production the immobilisation probably failed since the coupling could not be carried out in a high salt, high pH buffer. Incorporation of a suitable organic solvent might be a feasible alternative for this type of immobilisation. However, the failure of this reaction was not evident until high salt/low pH buffers were used, which overcame the non-covalent interactions of B3955 with the column.

4.13.2. Coupling using avidin/biotin

It was decided to utilise the extremely high affinity of the avidin-biotin reaction to immobilise B3955. Avidin is a tetramer of a 15 KDa sub-unit protein possessing a single high affinity binding site for the vitamin, biotin. The affinity of this interaction is so strong that the association and dissociation constants are impossible to accurately measure, but dissociation constants of approximately 10^{-15} M have been calculated (Bayer and Wilchek, 1990). The binding is also insensitive to changes in pH, salt, addition of chaotrophic agents, or organic solvents (Bayer and Wilchek, 1990). This interaction can, therefore, bridge the difficulties of interactions in a mixed aqueous-organic system. The biotin caproate is particularly useful in this case. Biotin is water soluble and binds to a site inside the avidin molecule (Bayer and Wilchek, 1990). Addition of a NHS ester to the molecule renders it reactive towards nucleophiles, especially amino groups. The ester and the caproate linker make the whole molecule organically soluble, and the linker also overcomes the potential steric problems of the biotin-avidin interaction (Tijssen, 1985). The success of biotinylation reactions is generally monitored by TLC, but this did not provide a clear picture in this case. However, the polar nature of this

biotin analogue made the biotin-derivatised phenazine more polar allowing the reaction to be monitored by the phenazine HPLC method. The azlactone group coupled the avidin to the matrix and addition of the reaction immobilised the biotinyl-phenazine derivative. Successful production of an affinity matrix was indicated by the absence of colour elution even in high salt, low pH buffer.

The matrix was blocked with BSA since the immobilised phenazine could be anticipated to have a high non-specific affinity for proteins. The high molecular weight serum fraction was added to the column and non-specific proteins were eluted with PBS. The specific antibody present was then eluted using glycine, pH 2.5, with 10 % (v/v) dioxane. Glycine and tris buffers and the dioxane present with the antibody were removed using the ultrafiltration membranes and several washes. Comparison of the HPLC chromatographic profiles of this antibody with another purified antibody, indicated that this method produced IgG of similar purity. The activity of this antibody was demonstrated by direct ELISA and BIAcore analysis.

4.14. USEFULNESS OF ANTIBODY

The failure of the antibody to recognise the organically soluble phenazine, limits its use and application, since all therapeutically useful rimino-phenazines to date are exclusively organically soluble. In addition, as shown in chapter five, these organically soluble compounds do not exist in a free form in the blood or tissue, so an additional extraction method would also have to be used to measure these compounds, even if a suitable antibody measurement format, such as the silica coated plates, were developed. However, the work outline in this chapter is illustrative of the methods of producing antibodies against hydrophobic compounds and the difficulties involved. The antibody produced might also be suitable for the measurement of other naturally occurring phenazines such as those produced by several microorganisms, although this possibility was not investigated.

4.15. SUMMARY

Several conjugates of proteins with amino acid substituted rimino phenazines were produced using different methods. The conjugates were characterised and the approximate ratio of drug bound calculated. Two of these conjugates were used to develop an antibody in rabbits. This antibody was used in a number of plate formats and successfully recognised the more soluble rimino-phenazines B3955, B3976 and B3832, but could not bind organically soluble phenazine compounds. An immunoaffinity column was produced which utilised the strong

affinity of biotin for avidin to immobilise biotinylated B3955 to an avidin column. The antibody was purified using this column and characterised by HPLC and BIAcore studies.

CHAPTER 5

INVESTIGATIONS OF THE PHARMACOLOGY OF SELECTED RIMINO-PHENAZINES

5.1. INTRODUCTION

This chapter describes some investigations into the absorption, distribution, elimination and toxicity of a series of rimino-phenazine compounds. These agents were administered to animals and the drug levels in tissue, blood and faeces quantified. With selected agents, a preliminary investigation of their potential toxicity was made. The potential anti-malarial activity of two of these agents was also explored.

5.2. DISPOSITION ON PHENAZINE AGENTS

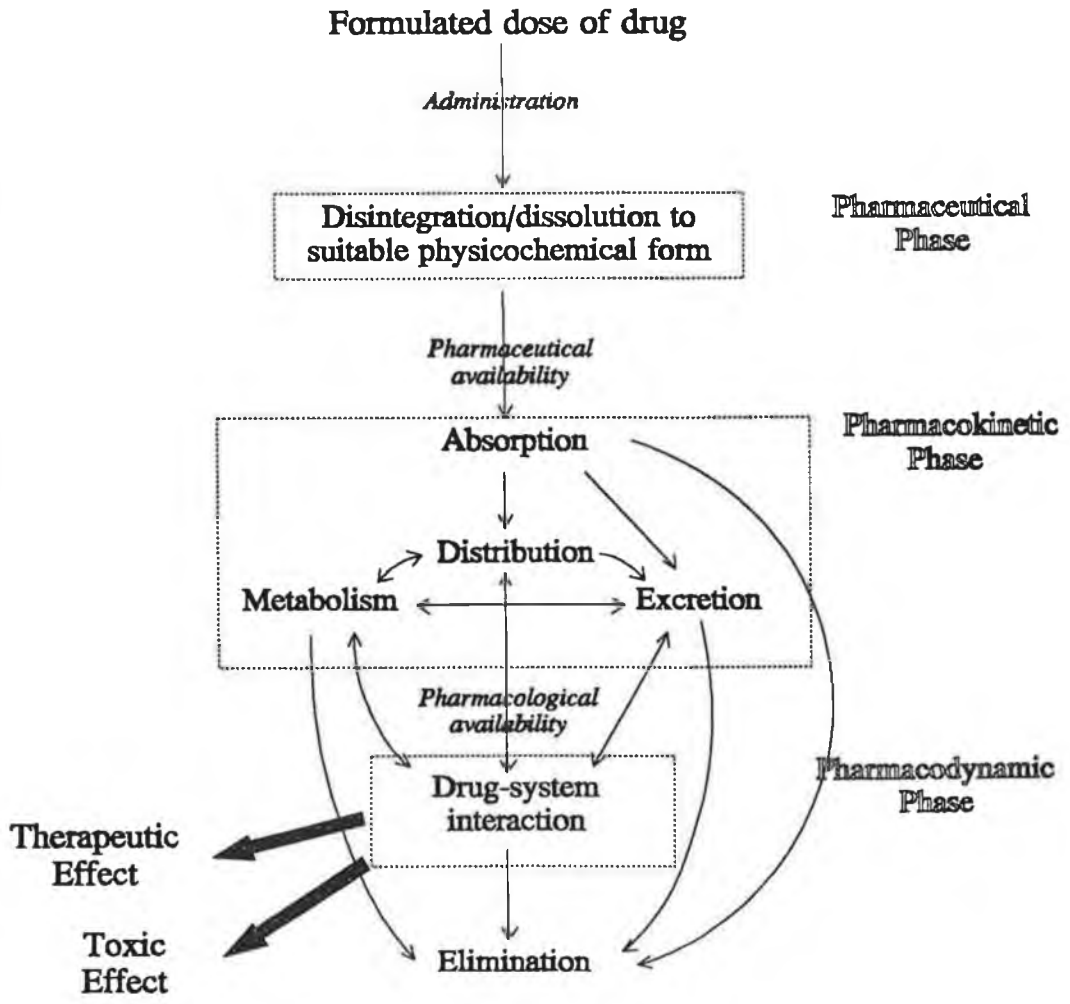
All the phenazines used in this project were initially supplied in the form of crystal powders. When synthesised, the conventional method of purification is recrystallisation, which involves reducing the organic solubility of the product liquor, until the phenazine precipitates. Less organically soluble impurites remain in solution, and the two are then readily separated by filtration. Crystallisation of these agents also renders these compounds stable in storage. When given to a subject in this form, the crystals must first dissolve to give the free drug, before absorption can occur. Larger crystals are more resistant to the solvation conditions in the gut, so overall absorption will be reduced or take longer. With the initial human trials of clofazimine only approximately 10 - 20 % of the administered dose was absorbed, but using a finer micronised crystal form the absorption was significantly increased (Barry *et al.*, 1959 and 1960; Barry, 1969; Vischer, 1969; Yawalker and Vischer, 1979).

The intrinsic chemical properties of phenazines, such as their lipophillicity and pKa, are also important for absorption and distribution. A tentative structure activity relationship has been established for many of the pharmacodynamic properties of phenazines including activity against different bacteria and cancer cells, and, stimulation of phospholipase A_2 (Barry, 1969; Barry *et al.*, 1970; Franzblau and O'Sullivan, 1988; Franzblau *et al.*, 1989; O'Sullivan *et al.*, 1992; Van Landingham *et al.*, 1993; Zeis *et al.*, 1987 and 1990; Savage *et al.*, 1989). However, the optimal compounds may be therapeutically inactive due to sub-optimal pharmacokinetics.

5.3. PHARMACOLOGICAL PROFILE AND DRUG DISPOSITION

Before any drug can produce its desired action, it must first pass through several processes, all of which will have an effect on its final pharmacogical profile. A general scheme for the profile of an orally administered drug, such as clofazimine, is outlined in Figure 5.1.

Figure 5.1. A flow diagram of the process which contribute to the pharmacoological profile of an orally administered drug. This process is normally divided into three phases. The pharmaceutical phase describes the processes taking place from the administration of an agent to release of the substance into the body fluids. The pharmacokinetic phase describes what the body does to the drug and the resultant effect on the body is encompassed in the pharmacodynamic phase.



The various processes involved are divided into three phases. In the pharmaceutical phase; the formulation of an agent is changed, from the stable production form, in which it is given, to the form by which it is absorbed. The pharmacokinetic phase encompasses the subsequent steps of absorption, distribution, metabolism and excretion. The pharmaceutical and kinetic phases describe what happens to a drug. This is also termed the drug disposition. The pharmacological effect, or what the drug does to the body, is evident in the pharmacodynamic phase, where an agents interacts with a specific system to produce the complete, therapeutic and toxic, effect seen. This effect is, therefore, a product of the interaction of all elements of each phase, and any variation in one may ultimately lead to a change in the whole pharmacological profile of the agent (Bowman and Rand, 1980; Taylor and Kennewell, 1993). The profile of a pharmaceutical agent is strictly unique to an individual, since it represents a product of biochemical variation. However, the physico-chemical properties of the drug are usually the most significant factor, allowing generalisations to be made about the profile of an agent in the population as a whole. Subsequent sections describe some of the elements important to the pharmacology of phenazines.

5.3.1. Important pharmaceutical phase considerations

Administration of drugs via the oral route is the most common and popular method of giving medications (Taylor and Kennewell, 1993). This popularity is due to ease and convenience of administration, both from a doctor and patient perspective, and the lack of pain or discomfort, commonly associated with other routes. Effective treatment of the particular condition for which the medication is given is dependent on the disintegration of the dose, stability in the gastro-intestinal tract (GIT), absorption into the systemic circulation and delivery to the site of action. Other factors which are particularly important include the variance in the physicochemical environment with different regions of the GIT, for example the acidity of the stomach, and the localisation of specific processes, such as fat absorption, to particular regions.

5.3.2. Pharmacokinetic phase

5.3.2a. Physicochemical factors affecting absorption

The principle barrier to the initial absorption of orally delivered drugs are the membranes of the cells lining the gut. Drugs can pass through this membrane by diffusion or by utilising a specific carrier transport mechanism (Bowman and Rand, 1980). Absorption via a carrier-mediated process is unusual, since these systems are very specific for natural compounds and

few drugs meet the structural requirements. Aqueous diffusion is generally limited to small water soluble compounds, such as ethanol. The most important absorption process for drugs is lipid diffusion. As a hurdle to absorption, the cell membranes behave like a lipid barrier. The most important parameter governing this diffusion is the permeability constant, which is closely related to the lipid/water partition coefficient of a drug. The lipid solubility of an agent is dependent on its degree of ionisation, which changes according to the pH environment of the drug (Foster, 1991). A low pH will tend to protonate acidic drugs, such as salicylates, which reduces ionisation and increases absorption (Bowman and Rand, 1980). Therefore, the greatest absorption per unit area for these compounds is in the stomach, where the environment is acidic. More alkaline agents need an alkaline environment to be un-ionised and are generally better absorbed in the near neutral environment of the small intestine. As the organic solubility of the agent increases, this behaviour tends to deviate from these rules (Taylor and Kennewell, 1993). Strongly hydrophobic agents may tend to remain in the cell membrane, due to their affinity for this environment, and these compounds will also have limited solubility. Phenazines, especially clofazimine, are examples of such agents (Morrison and Marley, 1976(a,b)).

Most orally administered drugs are given in a solid state, as particles of some form. The most common types of particles are crystals of the particular agent, since these are generally stable and easy to produce. The particle size of a substance has a significant effect on absorption by the oral route. Reduction of particle size gives a larger surface area to volume ratio, which decreases the time necessary for equilibration to a saturated free concentration. In general, reduced particle size is also associated with a more rapid and complete drug absorption (Foster, 1991).

5.3.2b. Absorption of hydrophobic compounds

The first major region of absorption of any orally administered drug is the stomach. The stomach is a large muscular bag which is designed to begin the process of digestion by secreting lytic enzymes, secreting acid and thorough mixing (Bowman and Rand, 1980). The mixing process increases absorption by augmenting dissolution. However, the low pH environment present generally limits drug absorption to soluble acidic compounds, since these compounds become un-ionised, as outlined above. The gastric contents then empty into small intestine. This region is physiologically highly adapted for absorption of nutritional compounds. The most significant adaptation is the very high surface area of this region, produced by finger-like projections called villi, which allows the absorption of compounds which would otherwise be unfavourable in this environment (Taylor and Kennewell, 1993). The small intestine secretes

many emulsifying agents, amongst many other substances, to allow the absorption of fats and fat soluble compounds, and is therefore the principal site of absorption of strongly hydrophobic drugs (Bowman and Rand, 1980). The most important of these emulsifiers are bile acids and salts secreted into intestine by the liver, via the gall bladder and bile duct. The bile acids are oxidation products of cholesterol and the salts are produced by conjugation with taurine or glycine. These compounds possess hydrophobic and hydrophilic groups and form micelles with lipid soluble compounds which solvate these compounds in an aqueous environment. In so doing, the overall fat solubility is augmented and these substances dissolve even in the presence of a largely aqueous environment (Bowman and Rand, 1980). The micelles are then brought through the epithelial lining of the intestinal wall by a combination of fusion with the cellular membrane and pinocytosis by these cells. The lymphatic and blood systems drain this part of the GIT. The lymphatic system carries mixed micells of fats and lipoproteins, termed chylomicra, away to be distributed to the body. However, with a few very rare exceptions, most drugs enter the blood stream which then flows directly to the liver (Bowman and Rand, 1980). This is the principle metabolic organ of the body, where hydrophobic compounds are usually made more polar, by a series of enzymes including the cytochrome P450 family, to allow their removal in an aqueous environment.

The presence of food in the GIT stimulates the physical and biochemical mechanisms involved in the absorption of nutrients. This includes the movements of the intestine, and the many substances needed for nutrient absorption, both of which are important for drug absorption. The presence of fats, in particular, in food stimulates release of bile and provides a large volume for the solubilisation of hydrophobic drugs (Bowman and Rand, 1980). All these factors normally significantly increase the absorption of fat soluble drugs.

5.3.2c. Lymphatic absorption

Absorbed compounds with a molecular weight in excess of 10 KDa have a higher affinity for the lymphatic system and will preferentially enter the circulation via this route (Muranishi, 1991). The blood system has a 500 fold greater flow than the lymphatic system, and it has been calculated that selective entry of a low molecular weight drug into the lymphatic system necessitates that the drug has a partition coefficient into chylomicron lipid of at least 50,000 (Charman and Stella, 1986; Tucker, 1993). Despite this fact, examples of selective lymphatic absorption of strongly hydrophobic compounds exist, and, it has been suggested that drug delivery vehicles, such as oleic acid, can increase the partition of certain pharmaceuticals into the lymphatic system (Muranishi, 1991).

5.3.3. Measurement of drug absorption

Despite the large surface area and specialist transport processes present for absorption of substances from the GIT, the amount of a particular dose absorbed may be less than 100 % of the administered amount. This incomplete absorption is particularly common with drugs that are not easily absorbed and/or which are relatively insoluble in the GIT, due to physicochemical or formulation properties. With compounds that are rapidly eliminated after absorption, an accurate estimate of the amount of absorption can be made by measuring the blood level, or the eliminated dose present in the urine. Alternatively, measurement of the fraction of the dose present in the faeces gives a measure of absorption. Measurement of faecal drug levels by conventional methods can often be difficult due the presence of a heterogenous mixture of interfering substances. A convenient way to measure drug absorption can be to include a small amount of radiolabelled compound in the dose. The amount eliminated can therefore be assessed by measuring the radioactivity of the faecal samples (Reynell and Spray, 1956).

With compounds that are absorbed and then rapidly eliminated into the faeces, measurement of the unabsorbed percentage of drug is more complex, since the amount of drug present in the faeces represents the unabsorbed fraction plus an amount eliminated from the body. This problem was overcome in this project using a dietary marker, chromium oxide. This substance is not absorbed by the GIT (Davignon *et al.*, 1968; Hildebrant and Marlett, 1990; Czubayko *et al.*, 1991; Sauer *et al.*, 1991). When administered with a drug, the chromium travels through the GIT and its presence in the faeces represents the transit time from mouth to anus. Any drug appearing in the faeces with the chromium represents the unabsorbed fraction. Any drug found in the faeces after the chromium has been eliminated represents the fraction of drug that has been absorbed and then eliminated into the faeces, for example, by the bilary excretion route. Chromium is also useful in these experiments because rats and mice are coprophagous, that is, they eat some of their own faecal pellets (Kraus, 1980). Any drug present in the faeces could therefore be reabsorbed. Recycling of faecal contents will show up as a reemergence of chromium.

5.3.4. Distribution of hydrophobic compounds

After absorption, any drug must be transported by the blood system to its site of action. For hydrophilic compounds this transport may be in the form of free drug in the plasma. However, many drugs are bound to other blood constituents, especially serum albumin, which has both low and high affinity sites for acidic, basic and hydrophobic compounds. When this

binding is of high affinity, the drug is essentially unavailable for activity, since only the free form can interact with the appropriate receptor or system, and this fraction represents an inactive drug depot. A significant consequence of protein binding is that the apparent plasma solubility of the drug is increased (Bowman and Rand, 1980).

In very rare cases, it has been suggested that lipoproteins especially β-lipoprotein may act as a carrier for hydrophobic drugs (Conalty and Jina, 1971; Ichihashi *et al.*, 1992). β-lipoprotein, also known as low density lipoprotein (LDL), is a large colloidal mixture of an apolipoprotein, triglycerides, free fatty acids, phosphatides and cholesterol. This mixture makes the LDL complex very hydrophobic while remaining in solution in the plasma. This property is vital since the principle function of LDL is the transport of cholesterol to various cells in the body (Cullis and Hope, 1985). Although all cells in the body have a requirement for cholesterol, since it is a vital structural component of the cell membrane, four major groups of cells have particular affinities for LDL. These are the adipose, liver, reticuloendothelial and certain tumour cells (Cullis and Hope,1985, Vitols, 1991).

The adipose cells act as fat storage depots for the body, storing lipids which act as fuel, structural components, and chemical precursors for the rest of the body. The lipid deposited in adipose cells constitutes approximately 15 - 20 % of the average body weight, and can absorb large amounts of fat soluble drugs (Bowman and Rand, 1980). The equilibration of blood levels of hydrophobic compounds with adipose tissue is slow, due to low blood flow of this region of the body, and drugs binding to these cells are turned over very slowly (Bowman and Rand, 1980). Liver cells have a high turnover of cholesterol, principally as a precursor for many steroid based hormones and bile compounds (Bowman and Rand, 1980). Reticuloendothelial cells have a high uptake of lipoproteins possibly to control cholesterol and lipoprotein levels and deposition in the body (Fielding and Fielding, 1985). The high uptake of LDL by tumour cells is thought to be principally due to the requirements of cellular kinetics and proliferation (Samadi-Baboli *et al.*, 1989). Although LDL is not a significant carrier for many drugs, even hydrophobic agents, techniques have been developed to incorporate chemotherapeutic agents into LDL and synthetic congers (Iwanik *et al.*, 1984; Samadi-Baboli *et al.*, 1989; Filipowska *et al.*, 1992). The selective uptake by tumour and reticuloendothelial cells has been shown to improve the therapeutic profile of these agents in cancer treatment and the chemotherapy of selected diseases (Iwanik *et al.*, 1984; Chaudhuri *et al.*, 1989; Samadi-Baboli *et al.*, 1989; Filipowska *et al.*, 1992).

5.4. NOVEL METHODS OF DRUG TARGETING AND DELIVERY

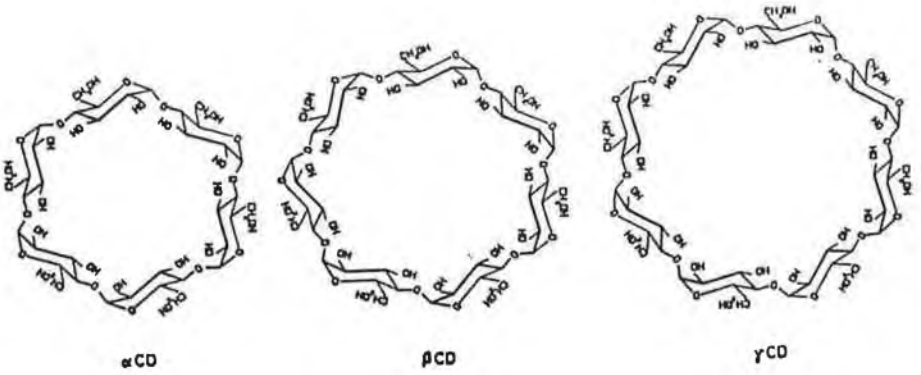
Drug-based therapies have been developed for many diseases and afflictions, and have undoubtedly had a dramatic effect of the general standard of health of man and animal kind. However, even with modern sophisticated pharmaceutical technology many diseases remain poorly treated. In many instances, very active agents exist for clinical or radical cures of these afflictions, but poor pharmacological profiles limit or prevent their general use. The long list of limitations includes:- formulation difficulties, including instability of the active compound in storage or administration; poor absorption, often due to the inherent biological barriers we possess; inappropriate distribution, to the wrong body systems, or, in too low a concentration to the correct target receptor; unwanted metabolism to less desirable compounds, prematurely stopping the clinical effect, or producing undesirable consequences; and, too rapid excretion and elimination. However, advances are being made which may allow improved or broader activity of existing drugs, or, the use of compounds that have been useless up to now, due to some of the reasons outlined above.

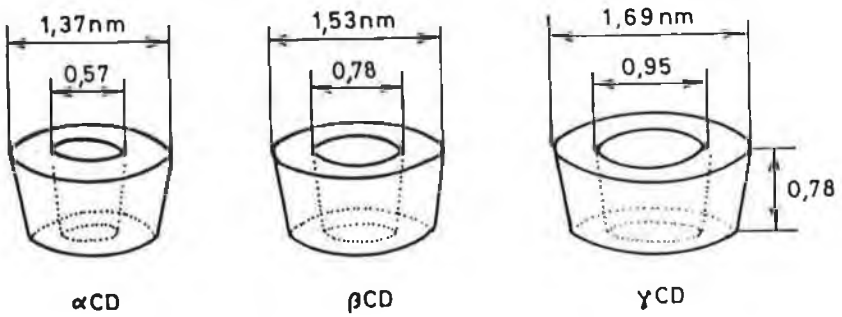
These new developments include:- new forms of administration, including transdermal delivery (Taylor and Kennewell, 1991); chemical coupling of drugs to other compounds to change the net physicochemical properties (Bodor and Brewster, 1983), leading to organ-specific delivery; incorporation into biopolymers for controlled and selective drug release (Saffran, 1992); coupling to receptor specific compounds, especially antibodies, for cell specific drug delivery (Engert and Thorpe, 1992); incorporation into lipid vehicles, including liposomes, to change drug pharmacokinetic and pharmacodynamic properties (Cullis and Hope, 1985); and incorporation into macromolecular complexes, such as cyclodextrins, to change the net physicochemical properties of a drug (Szejtli, 1988). The production and properties of drug-cyclodextrin complexes is discussed below since such complexes were made with clofazimine in this project in an attempt to improve the pharmacokinetics of this drug.

5.4.1. Cyclodextrins and their applications in pharmaceuticals

Cyclodextrins are cyclic oligomers of glucose produced by the action of the enzyme cyclodextrin transglycosylase, an amylase, on starch. Three types of cyclodextrin are used in pharmaceutical preparations, α-cyclodextrin, which has six glucose units, β-cyclodextrin (β-CD) has seven and γ-cyclodextrin, eight (Bender and Komiyama, 1978; Szejtli, 1988) . The linkage of each glucose unit in cyclodextrins produces a structure with a cone-like shape as shown in Figure 5.2. The narrow end of the cone contains the glucose primary hydroxyl groups while the

Figure 5.2. The chemical structures of the three principal naturally occuring cyclodextrins. α-cyclodextrin consists of a ring of 6 glucose units, β-cyclodextrin, seven units, and γ-cyclodextrin, eight units. In each case a cone shaped structure is produced. The inside of the cone is apolar and relatively hydrophobic due to the ether linkages and the carbon backbone. As a consequence of the confirmation of the glucose units, all secondary hydroxyl groups are found on one edge of the cone, and all primary hydroxyls on the other.





wide end contains the secondary hydroxyl functionalities. Skeletal carbons and ether oxygen groups make the interior of the molecule hydrophobic, while the outside remains relatively water soluble. The net result is a cone shaped molecule with a hydrophobic microenvironment in the core, which can accommodate hydrophobic molecules, while the whole molecule is water soluble (Szejtli, 1988).

Cyclodextrins are useful in pharmaceutical technology because small organic molecules, or organic groups of larger molecules can form an inclusion complex by reversibly inserting into the core of the cyclodextrin (Duchene, 1988). Once complexed, the compound takes on the bulk properties of the cyclodextrin, including, especially, the high aqueous solubility (Weiszfeiler *et al.*, 1988). In addition to increasing drug solubility, cyclodextrin complexation also has applications in :- increasing drug stability, camouflaging undesirable compound tastes and odours, increasing oral bioavailability, reducing direct drug-associated tissue damage, converting liquid drugs to crystalline form and allowing new methods of drug administration (Duchene, 1988; Nagai, 1987; Szejtli, 1988). Cyclodextrins are non-toxic by the oral route, and parenteral toxicity is only associated with high doses of poorly soluble derivatives (Szejtli, 1988; Gerloczy *et al.*, 1994).

Although these compounds are relatively soluble in aqueous media, the secondary hydroxyl groups can interact with each other, via hydrogen bonds, to stabilise the crystal lattice, reducing the potential solubility, especially of β-CD, in water. This problem is overcome by chemically modifying to produce hydroxyalkyl derivatives (Muller *et al.*, 1988; Brewster *et al.*, 1989). These reactions produce cyclodextrins, such as hydroxypropyl-β-cyclodextrin (HPCD). These compounds are very water soluble (>100 % w/v) while maintaining, and in some cases, augmenting, the complexation properties of cyclodextrins. The high solubility and lack of toxicity, makes HPCD very useful as a vehicle for the parenteral delivery of poorly soluble pharmaceuticals. Parenteral delivery can produce high plasma drug levels while circumventing problems of stability, absorption and/or rapid metabolism (Brewster *et al.*, 1989). The complexed agents still maintain their full potency, since, the complex dissociates to deliver the active agent.

5.5. MONITORING OF TOXICITY

An unfortunate consequence of therapy with all drugs are the associated toxic side effects. With some drugs, those with a high therapeutic index, the dose at which toxic complications outweigh the therapeutic effect is very high. Unfortunately with some compounds,

there is little selectivity, and a serious degree of toxicity is inherent with the dosage necessary for clinical effect. Toxicity has many different degrees of severity and can be very varied among individual patients due to the inherent biochemical variation we all possess (Timbrell, 1982). Before any new agent can be released into the patient population, it must first be tested in a variety of systems with increasing complexity and similarity to the human system. Data from these experiments will normally yield sufficient information on the type and complexity of toxicity likely to be experienced *in vivo*. A typical regime after demonstration of efficacy in a disease model would include cell culture and isolated tissue studies, followed by acute, sub-chronic and chronic testing of high doses in mice, rats and/or other suitable small mammals. Assuming that a compound passes these tests, higher animals will be involved, and ultimately toxicity will be assessed in human volunteers. Even with such rigorous assessment, the full toxicological picture will only emerge after many years of widespread use (Foster, 1991).

5.5.1. Toxicity testing

Two forms of toxicity are often evident, a dose-responsive and a non-dose-related reaction. The non-dose related response is usually idiosyncratic and associated with a low dose administration to selected individuals. Examples in this class include drug allergies and carcinogen-induced cancers. This form of response can be very difficult to anticipate from non-human experiments and only appears with a statistically sufficient population. The more usual forms of toxicity are dose related. In these cases, the toxic response can be directly related to the concentration of drug present at one time or over a cumulative time period (Timbrell, 1982). Usually a particular cellular system is the target, and this system is the first to show symptoms of a toxic effect. This form of toxicity is generally simpler to work with and model, and often empirical estimations can be made on a theoretical basis. Examples of this form of toxicity include organ specific toxicities such as hepatotoxicity or nephrotoxicity, or, cell specific toxicities such as haemolysis or neurotoxicity. Overt toxicity is generally easy to recognise and quantify, and has been used in the past for comparison of toxicity between compounds. The most infamous of these procedures has been LD_{50} estimations of toxicity (Briggs and Oehme, 1980). The LD_{50} is the dose of agent required to kill 50 % of test subjects. This value gives crude acute data on the inherent lethality of a particular agent, but gives little insight on the likely consequences of chronic low dose administration. From academic and ethical considerations this form of assessment has been largely superseded by a more mechanistic approach giving more relevant information on the consequences of conventional doses.

5.5.2. Toxicity assessment

Any system in the body can potentially be a target for the toxic effects of an agent. To measure and describe of any form of toxicity necessitates the demonstration of a statistically significant difference in a particular system after drug exposure. The choice of marker selected to quantify the toxic effect is critical. It should be sensitive and accurate to the level of damage, without interference, convenient to measure and should also be species relevant. The principal form of toxicity measured include haematological toxicity, hepatotoxicity and nephrotoxicity but other forms such as reproductive toxicity, teratogenicity and mutagenicity will also have to be investigated for any new pharmaceutical agent.

The blood system, liver and kidneys are most likely to demonstrate the toxic potential of an agent since many drugs are concentrated or react with systems in these environments. The damage produced by a toxic insult can take many specific forms, but certain markers can be chosen to quantify both broad and specific types of damage (Boyd, 1962; Clampitt and Hart, 1978). A common and sensitive indicator of damage is increased cellular permeability to macromolecules (Boyd, 1983). Certain enzymes are only found in background levels outside particular cells (Balazs *et al.*, 1961). Using selective assays for the measurement of these enzymes allows the demonstration and measurement of selective toxicity in a cell. Other common markers include changes (increases or decreases) in the numbers and morphology or form of cell types. This is a common adaptive response to toxic attack and cells may also stop normal growth patterns and/or die. Cells may produce or secrete abnormal macromolecules due to interference in key production or transport systems, or loss of cellular control mechanisms.

Damage to the kidney is usually reflected by changes in the type and relative concentration of certain compounds present. Under normal conditions, the glomerulus only allows low molecular weight compounds to filter through to the urine and the presence of large substances such as enzymes and especially haemoglobin can be an indicator of kidney damage. Therefore, urinary monitoring is a sensitive indicator of nephrotoxicity, and of the general state of the whole body.

More subtle changes can often be seen by monitoring toxic effects on higher level functions. Although an animal cannot directly say that it is feeling unwell, changes in established behavioural patterns including food and water intake, pasificity or agressivness and, especially, activity can indicate unwanted general effects. All of these markers can be changed by other factors, such as age, and it is therefore important to have appropriate controls.

5.5.3. Urinary NMR profiles as a toxicity indicator

A recent method of demonstrating and quantifying toxicity, especially nephro- and hepatotoxicity is the use of NMR to identify changes in a number of cell-system-specific urinary compounds and metabolites (Sanins *et al.*, 1990; Murgatroyd *et al.*, 1992). The principles and practice of NMR are outlined in section 3.11.1. NMR has traditionally been used in chemical analysis with deuterated solvents. Analysis of compounds in non-deuterated solvents is now possible with Fourier transform data manipulation, more powerful magnets and radiofrequency oscillators, and, special saturation methods (Nicholson and Wilson, 1991). The method used in this project reduced the contaminating water signal by reconstitution in deuterated water (D_2O) and presaturating the residual water signal. Saturation of the water signal is accomplished by irradiating the sample with a high intensity radio field at the exact frequency of absorbance of water at the same time as the varying radio frequency is applied. The water absorbance becomes saturated and can be filtered out of the complete signal. This gives a profile showing the NMR absorbance of all the components present at a concentration of greater than approximately 10 mM (Sanins *et al.*, 1992).

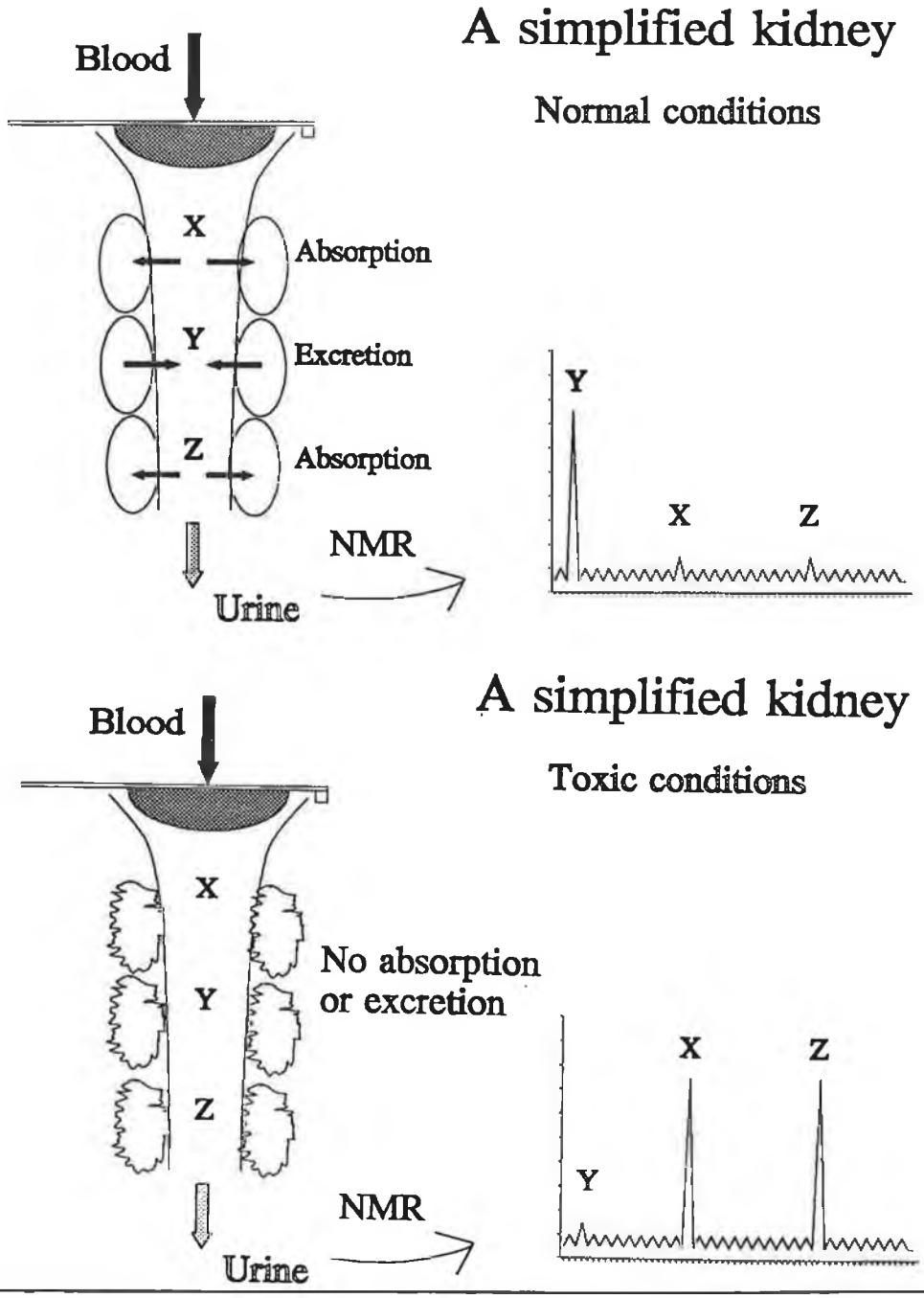
5.5.3a. The principle of urinary NMR analysis

The principle of this system is outlined in Figure 5.3. Specific cells in the kidney nephron are responsible for the absorption or secretion of selected compounds in the urine. For many of these compounds, the transport process involved are active and energy requiring. Any damage to these cells can cause the disruption of these process (Gartland *et al.*, 1989). For example hippurate is actively secreted into normal urine. Damage to kidney cells interrupts this secretion and the hippurate signal disappears from the urinary profile. Many different compounds are processed in this way by the different cell types present. The result of a toxic insult will be a "fingerprint" since increases and decreases in the levels of certain compounds are associated with specific cell types (Sanins *et al.*, 1992; Holmes *et al.*, 1992). The level of toxic damage can be quantified, both by the relative intensity of the change in the compound signal and by the identification of the damaged systems, since certain systems are more sensitive to toxic disruption (Gartland *et al.*, 1990).

5.5.4. Advantages of NMR analysis

NMR analysis is as sensitive as other modern methods of toxicity assessment but has several advantages over existing techniques. From the point of view of the subject the method

Figure 5.3. A diagrammatic representation of the principle of the NMR analysis of urine. (A) Blood is filtered through the glomerulus which acts as a molecular sieve, only allowing smaller molecular weight compounds through. The cells of the nephron absorb certain compounds while others are actively secreted into the glomerular filtrate, which then becomes urine. Analysis by NMR shows peaks due to compounds present in relatively high concentration, while absorbed compounds hardly figure in the spectrum. (B) Any damage to these cells affects these absorption and secretion processes, changing the relative concentration of the compounds, and producing a different and characteristic spectral profile.



is non-invasive and needs no clinical procedures, such as dye injection, to obtain a result. NMR analysis is very rapid and needs a minimum of sample preparation. Other methods of sample treatment, such as adjusting the pH to 3.5 and adding urea, minimise the sample preparation necessary (Murgatroyd *et al.*, 1992). A very large variety of organic molecules can be measured at the same time, from a small sample, without a bias towards, for example, chromogenic compounds or a particular chemical class of compound. This also means that unexpected results will be picked up without a battery of unnecessary tests. Other methods, such as HPLC, can be readily coupled for more specific and sensitive analyses. Other liquid biological samples, such as plasma, cerebrospinal fluid and bile can also be analysed (Wevers *et al.*, 1994; Wilson *et al.*, 1989). This method is very suited to simultaneous investigations of metabolism and toxicity, since many drug metabolites are found in the urine in concentrations well above the sensitivity threshold for analysis (Nicholson and Wilson, 1989; Kriat *et al.*, 1991; Lommen and Groot, 1993).

5.6. MOUSE EXPERIMENTS

The established model for measuring the pharmacokinetics of phenazines was oral gavage of a drug solution into the stomach of a mouse, generally for a period of 21 days. Initially, groups of mice were gavaged by this method for 21 days with the different phenazines tested. The tissue levels of each agent are shown in Figures 5.4.(i) - (v). B663 levels were measurable in all tissues, with the highest levels in the fat. High levels were also evident in the kidney, liver, lung, and spleen. The lowest level of B663 were found in muscle tissue. With the B749 group high drug levels were also seen in the kidney, liver, lung and spleen tissue, but notably, the fat concentration was very low. The animals in the B3954 group showed very low levels of this agent, with the only significant levels being found in the kidney, liver and spleen. Comparison with the B663 and B749 drug levels shows that proportionally very little drug was present in the tissue. The highest tissue levels of phenazine were associated with the B4090 group. The levels of phenazine in this group were several times those found in the B663 kidney, liver and spleen. However, the level of B4090 found in the fat was proportionately very low. High levels of phenazine were also found with B4100 in kidney, liver, lung and spleen, with low levels of drug in the fat tissue. Although these drug levels were greater than those seen in B663, B749 and B3954 tissue samples, the amount present in all tissues was less than that seen B4090. Interestingly, levels of phenazine were found in the cerebral tissue of all animals except those of the B3954 group. Table 5.1. shows the values measured.

Autopsy of these animals, showed that the B663-treated group was significantly discoloured. The skin, particularly around the ears and paws of the animals, was orange coloured. All fatty tissue and mesentery were very orange coloured and the intestine was a deep orange-red colour. The liver and spleen were also lighter in colour than normal with an orange tinge. The orange colouration of body fat was also evident in an animal which died after only 4 days of treatment. The fat of the B749 dosed animals had a similar colour to normal. The fatty mesenteric tissue around the liver, spleen and intestine had a pink-red discolouration and the intestine was red coloured. The colour of the B3954-treated animals was the same as a normal mouse. The intestine of the B4090 mice was wine coloured, and the fat had a slight orange tinge. The fat of the B4100 mice was also slightly orange coloured with a strong pink colour to the intestine.

Figure 5.4.(i). The tissue levels in mice following administration of 20 mg/kg/day of B663 by gavage for 21 days. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each tissue is the mean of the values measured in three animals.

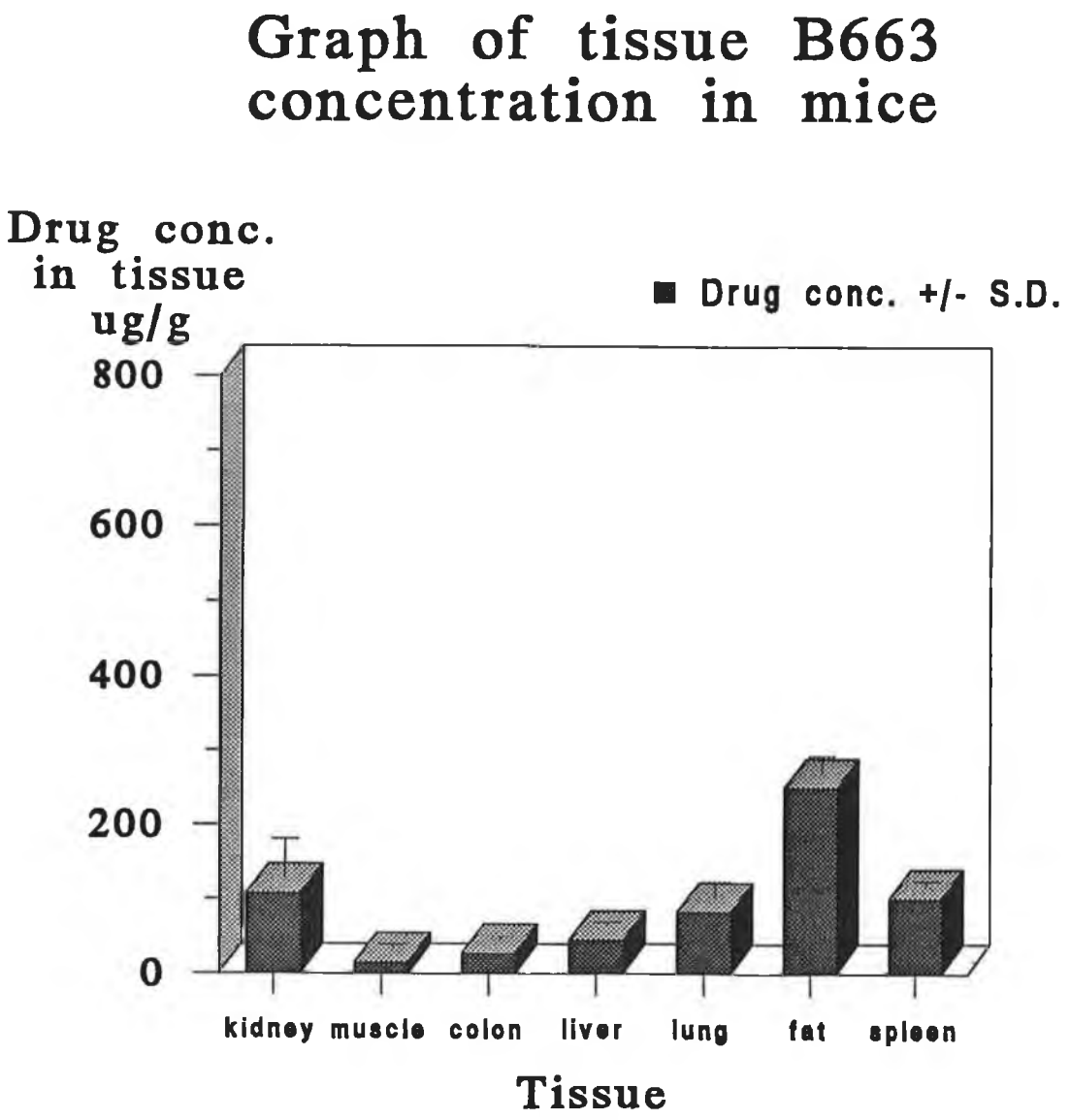


Figure 5.4.(ii). The tissue levels in mice following administration of 20 mg/kg/day of B749 by gavage for 21 days. The drug concentrations were measured using the HPLC quantification method. The concentration of B749 for each tissue is the mean of the values measured in three animals.

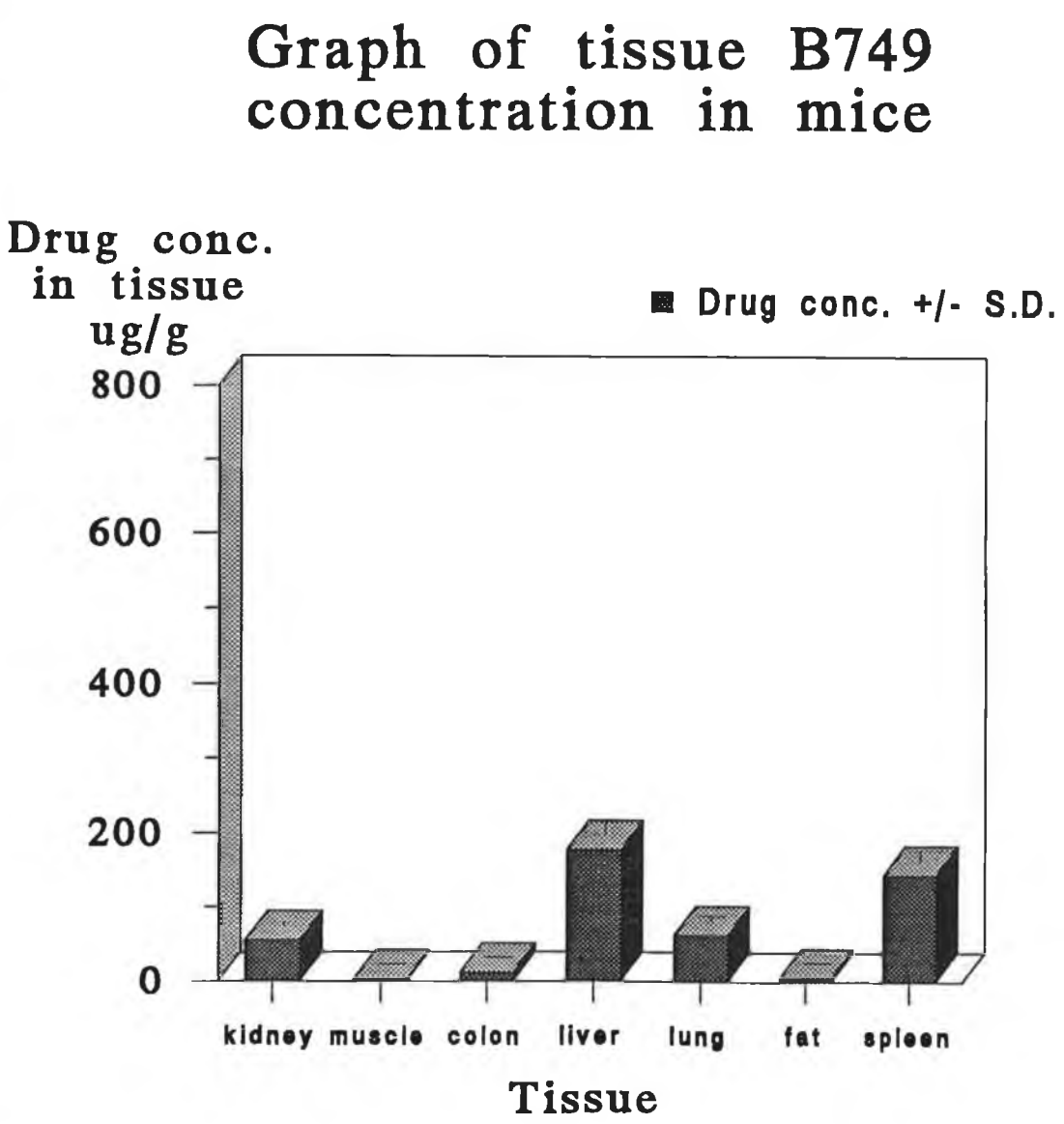


Figure 5.4.(iii). The tissue levels in mice following administration of 20 mg/kg/day of B3954 by gavage for 21 days. The drug concentrations were measured using the HPLC quantification method. The concentration of B3954 for each tissue is the mean of the values measured in three animals.

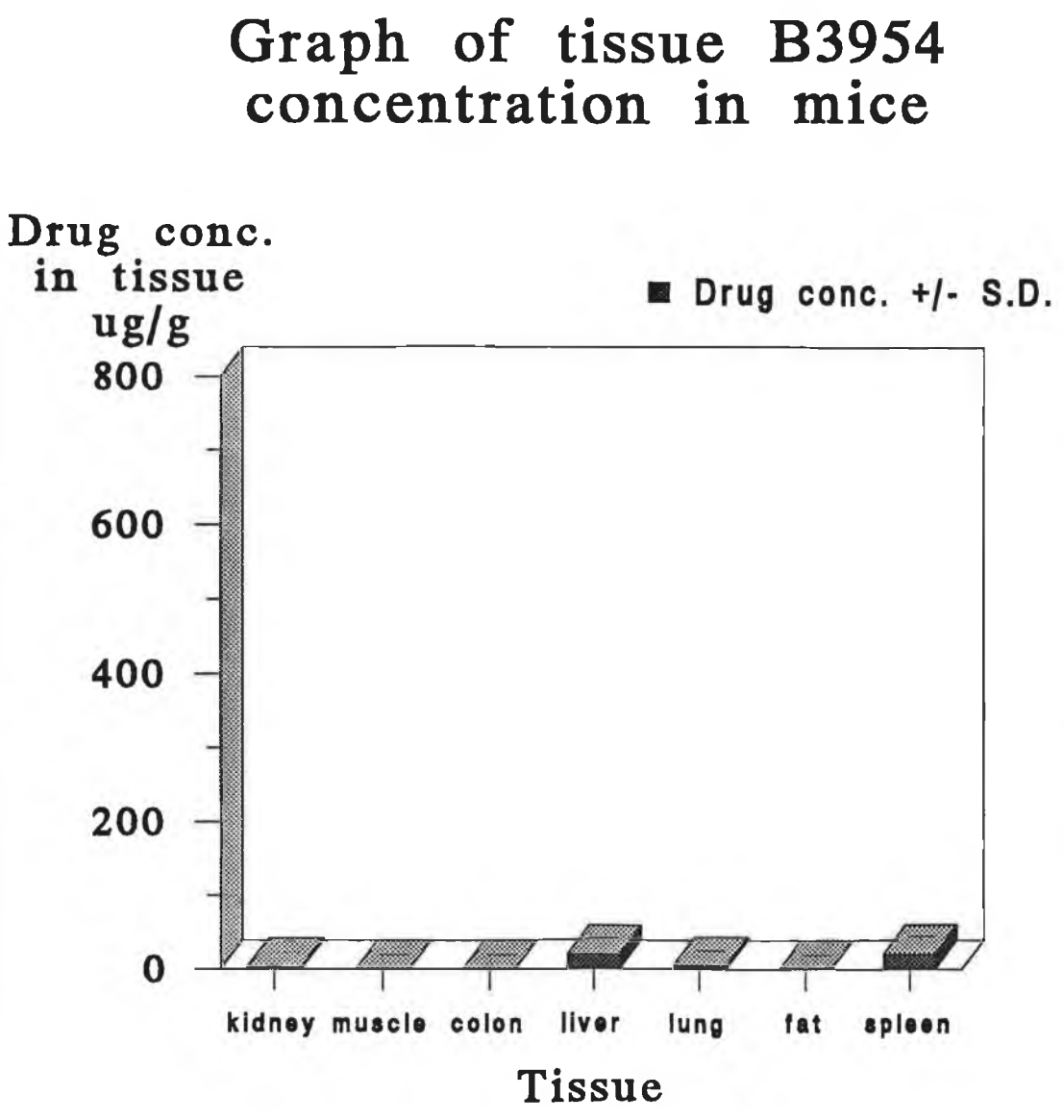


Figure 5.4.(iv). The tissue levels in mice following administration of 20 mg/kg/day of B4090 by gavage for 21 days. The drug concentrations were measured using the HPLC quantification method. The concentration of B4090 for each tissue is the mean of the values measured in three animals.

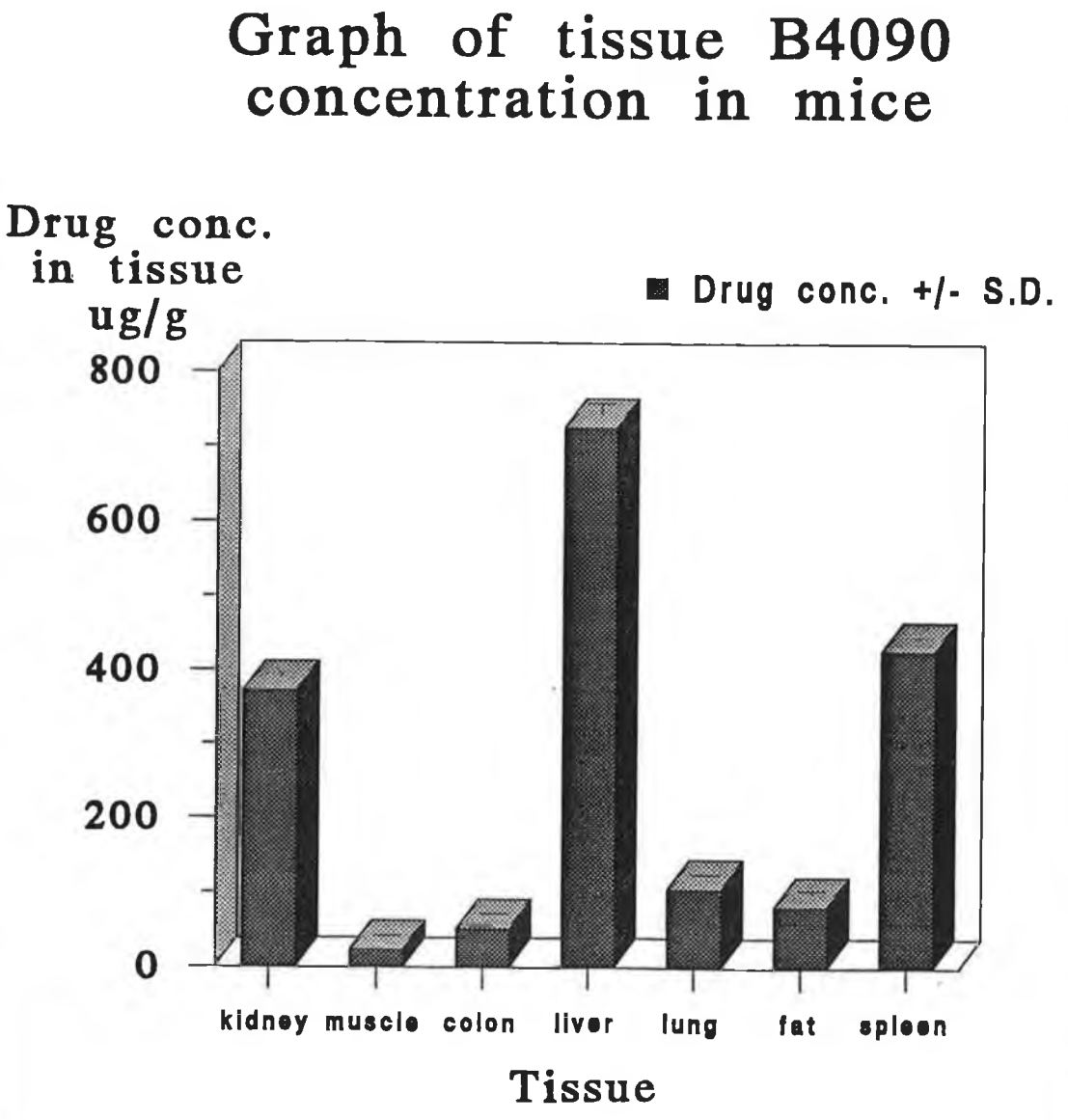


Figure 5.4.(v). The tissue levels in mice following administration of 20 mg/kg/day of B4100 by gavage for 21 days. The drug concentrations were measured using the HPLC quantification method. The concentration of B4100 for each tissue is the mean of the values measured in three animals.

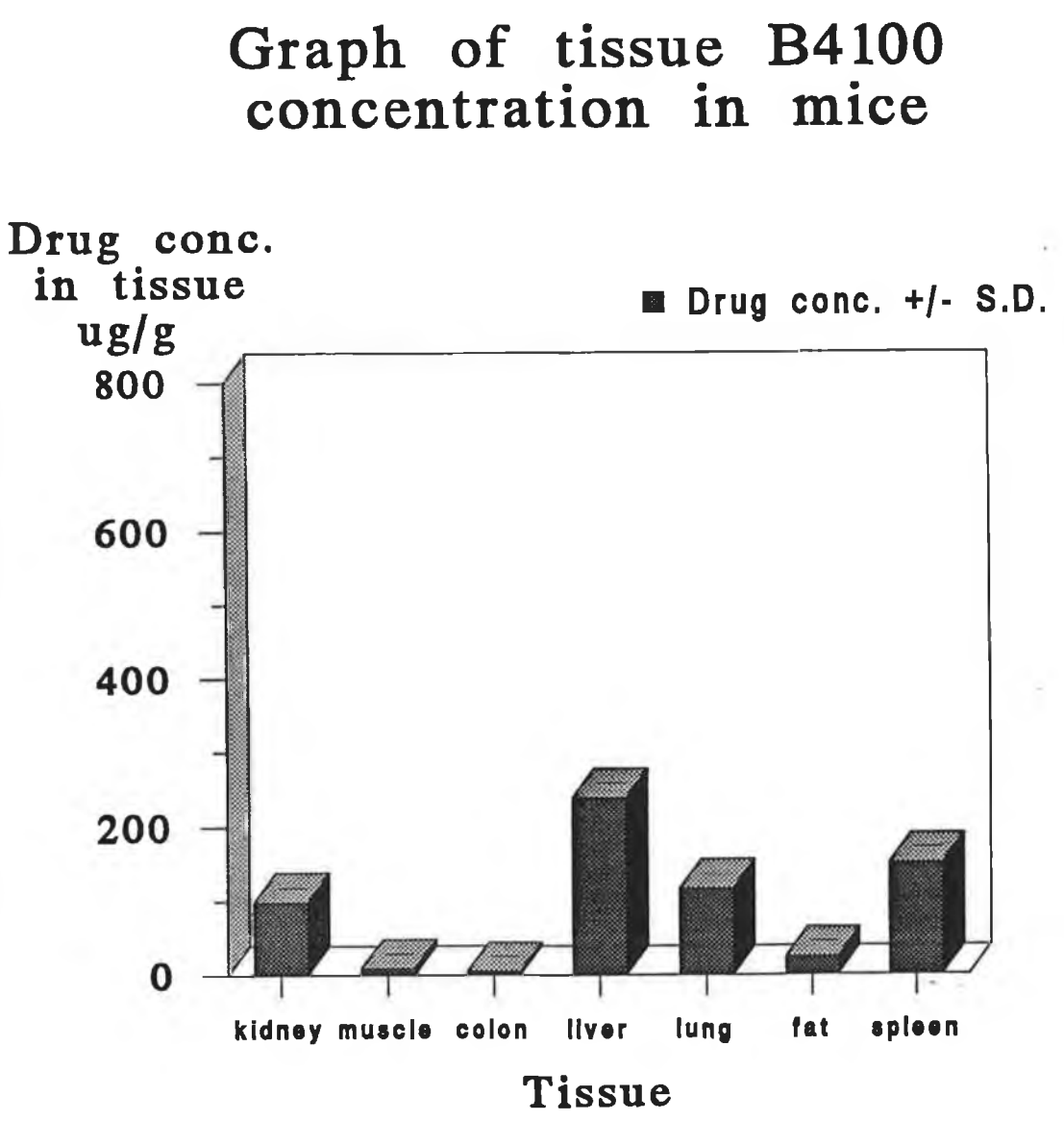


Table 5.1. The concentration of phenazines in the cerebral tissue of mice. The animals had received a dose of 20 mg/kg of B663, B749, B3954, B4090 or B4100 for 21 days by oral gavage. In each case the number of animals assayed was 3.

Drug	Mean cerebral concentration (µg/g)	Standard deviation (µg/g)
B663	4.47	1.22
B749	1.16	1.64
B3954	- *	- *
B4090	0.72	0.02
B4100	1.89	0.30

* Below limit of detection

Samples from mice receiving B663 as part of an investigation of therapeutic efficacy against *Mycobacterium avium* by Dr. Ji in the Faculte de Medecine, Pitie-Salpetriere, Paris, were also investigated (Figure 5.5). Despite receiving an oral dose of 20 mg/kg of B663 six times a week for 12 weeks, a comparison of the tissue levels shows that they are less than those seen in the B663 group from the phenazine study (Figure 5.4.(i).). Variability in B663 tissue levels between animals is also evident in these results.

To investigate if these agents could be administered in another way, B663 was incorporated into mouse food and given to the animals at the same dose for the same period of time, 21 days. As shown in Figure 5.6, administration of the drug in food gave superior tissue drug levels.

5.7. TREATMENT OF MALARIA-INFECTED MICE

To investigate if any of these agents had any anti-malarial activity, groups of mice were dosed with phenazines for two weeks and then inoculated with *Plasmodium bergheii.* The control and the three phenazine-treated groups of mice all became ill on the sixth day and had to be sacrificed. This indicated that an approximate dosage of 20 mg/kg of B663, B4090 or B4100, had no effect anti-malarial effect in this model. The sulfasalazine group remained clear of infection until the drug was removed, when they too became susceptible and had to be killed.

5.8. RAT EXPERIMENTS

To investigate if the higher drug levels of the tetramethylpiperidine-substituted phenazines was due to superior absorption of these agents, it was decided undertake a more sophisticated experimental investigation. Administration of phenazine agents to rats in specially built cages also allowed a more complete investigation of their pharmacokinetics and potential toxicity.

5.8.1. Tissue distribution of B663, B4090 and B4100

Figures 5.7.(i) - (iii) show the tissue distribution of these agents. As expected, B663 gave high tissue levels with the highest concentration associated with the fatty tissue. Autopsy of these animals showed that all tissues had an orange colouration, especially the fat which was intensely orange. This colour was evident on the ears and paws of the animals, and a red

Figure 5.5. The levels of B663 in mice following administration by oral gavage of 20mg/kg/day of B663 six days a week for 12 weeks. The animals were receiving B663 as part of a drug testing programme by Dr. Ji, in the Faculte de Medecine, Pitie-Salpetriere, Paris. The levels of clofazimine are less than those seen in figure 5.4.(i). due to a different gavage formulation. The results are the mean of two analyses. The bar represents the standard deviation of each duplicate.

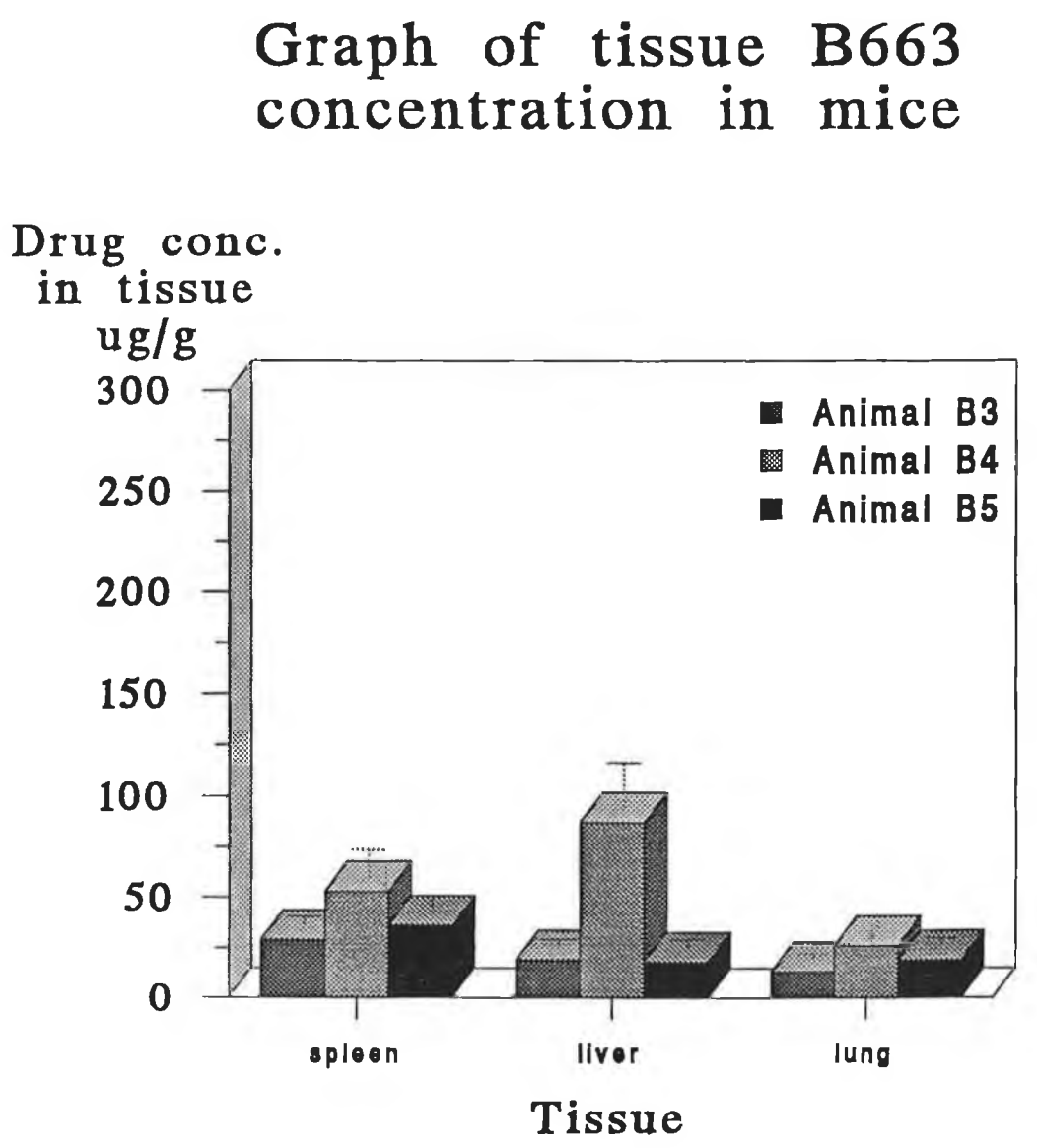
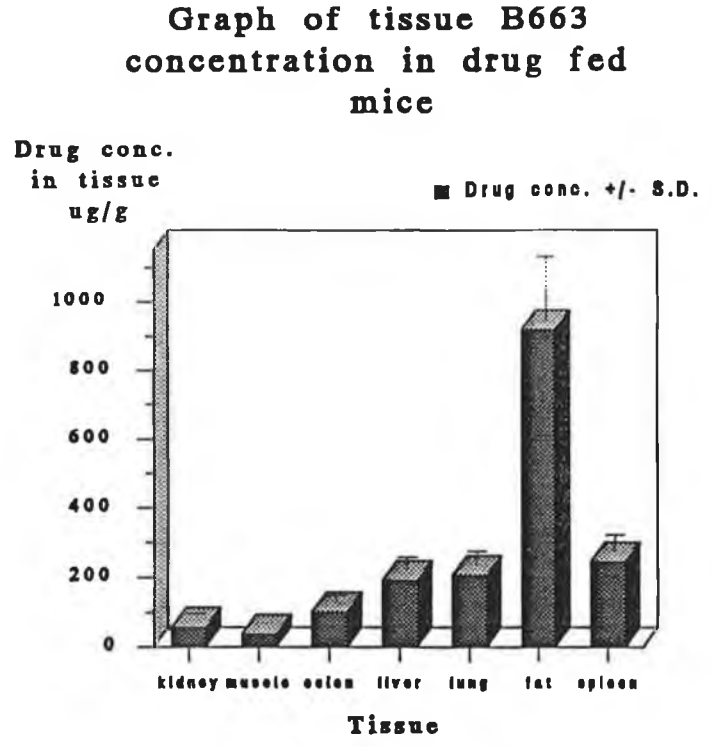


Figure 5.6. A comparison of the tissue levels of B663 in mice following, (A) a dose of 20 mg/day of B663 in food for 21 days, and (B) administration of 20 mg/kg/day of B663 by gavage also for 21 days. The drug concentrations were measured using the HPLC quantification method. The levels of B663 in each tissue are higher in the group receiving the drug by food. Each tissue concentration is the mean of the values measured in three animals.



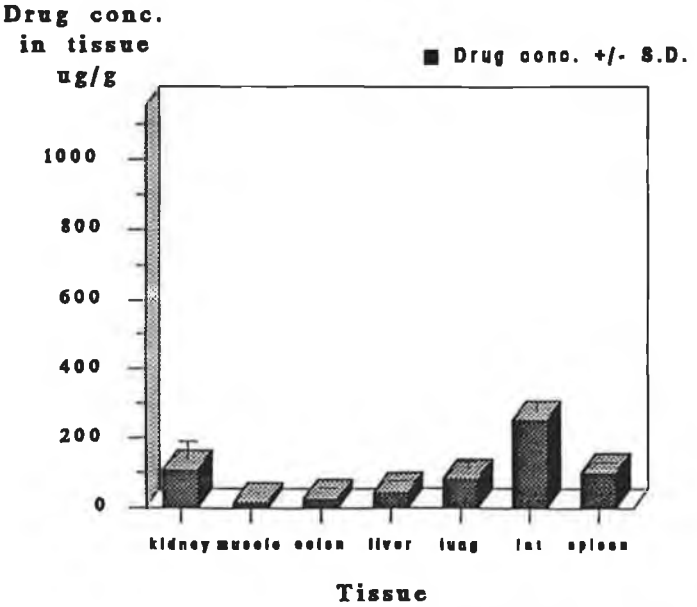


Figure 5.7.(i). The tissue levels in rats following administration of B663 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each tissue is the mean of the values measured in three animals.

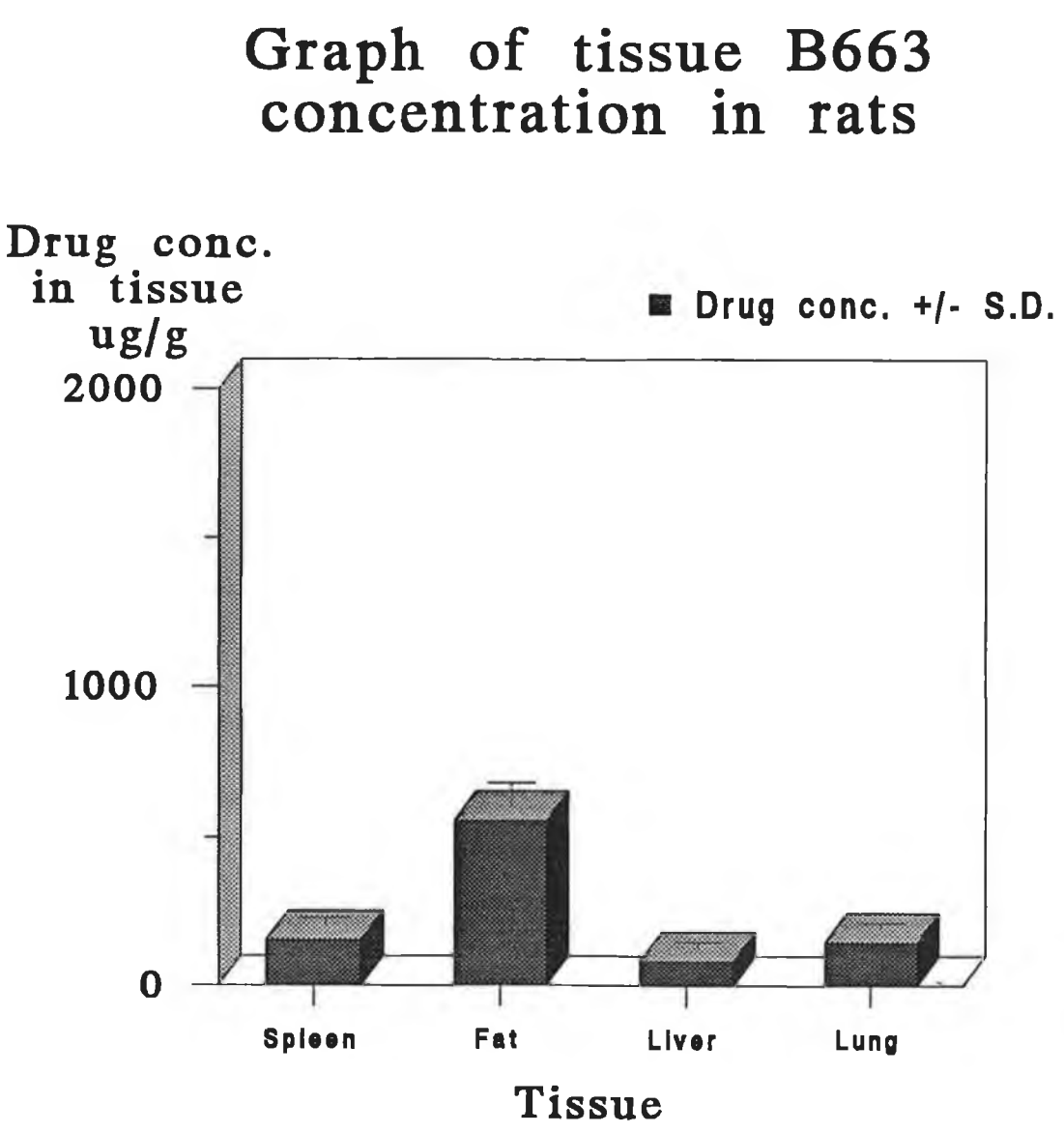


Figure 5.7.(ii). The tissue levels in rats following administration of B4090 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B4090 for each tissue is the mean of the values measured in three animals.

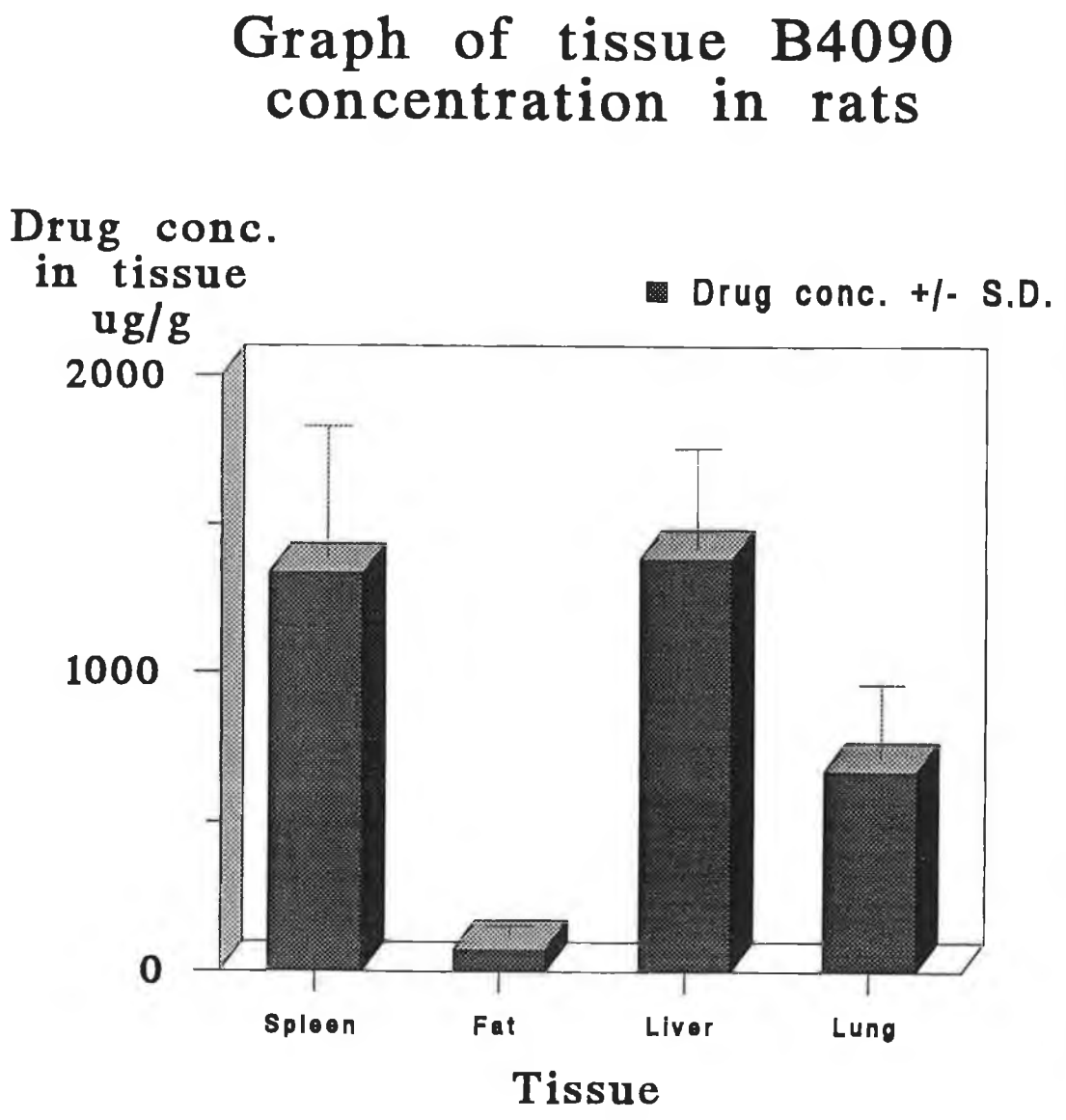
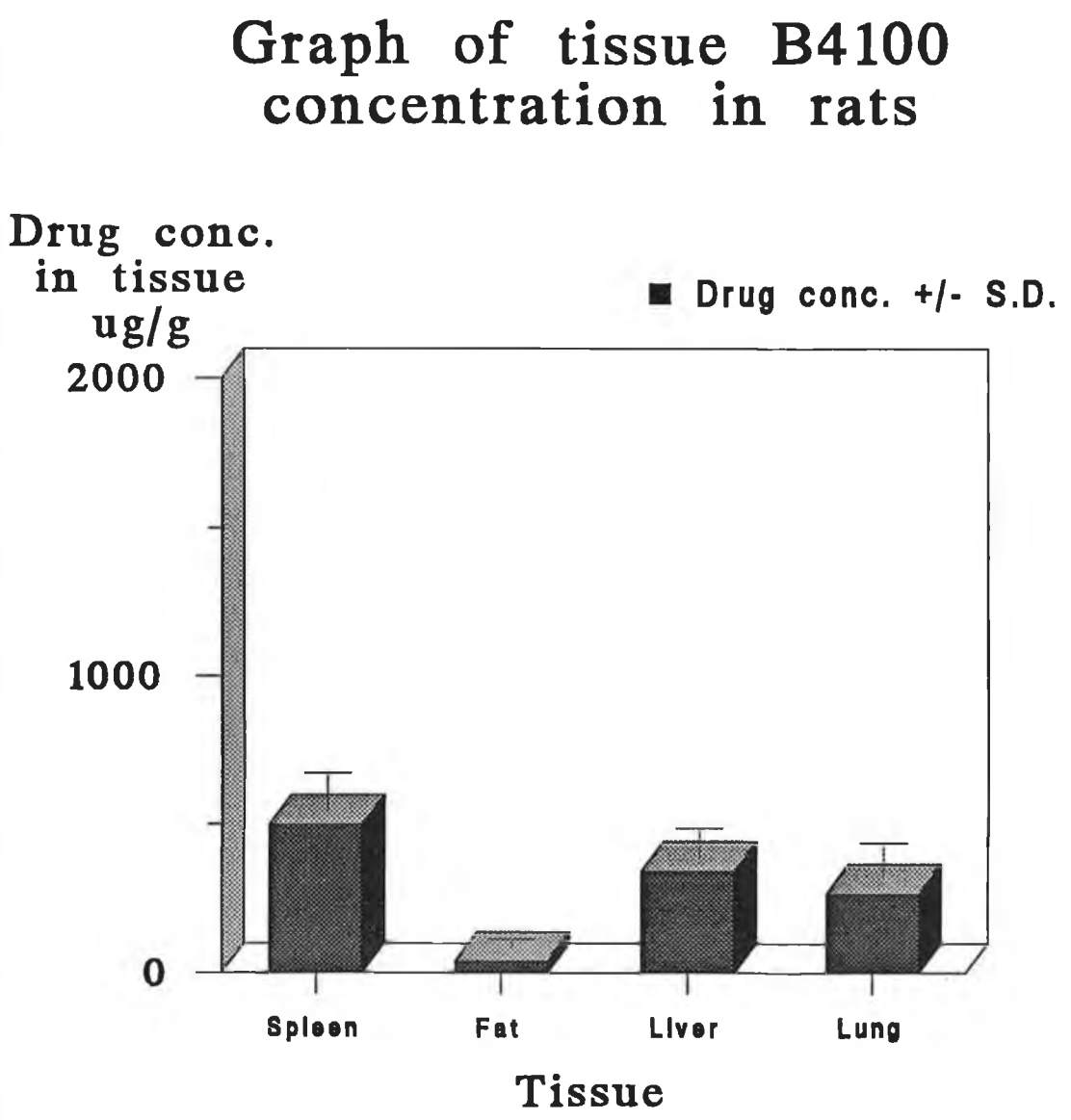


Figure 5.7.(iii). The tissue levels in rats following administration of B4100 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B4100 for each tissue is the mean of the values measured in three animals.



colouration was also seen in patches of their fur. Very high drug levels were seen in the liver, lung and spleen tissues of the B4090 group. Despite this, very little drug was found in the fatty tissue. The intestinal tissue of these animals was strongly red-purple coloured and the fat had a very slight pink tint. High spleen, liver and lung drug levels were also seen in the B4100 animals, but as with the mice, the B4100 tissue drug levels were less than those of the B4090 group. These animals had a red coloured intestine and the fatty tissue was slightly orange coloured. As with the mice, levels of phenazine were also measurable in cerebral tissue (Table 5.2.). Measurement of the serum levels of these agents showed that B663 gave the highest blood concentration (Table 5.3.). Serum levels of B4090 were also measurable. However, the levels of B4100 were very low and near the limit of detection for the HPLC method. Serum samples were also subject to ultrafiltration through a 3 KDa molecular weight cut-off membrane. Drug could only be measured in the high molecular weight retentate of these samples, indicating near complete plasma protein-binding of the drugs.

5.8.2. Absorption of B663, B4090 and B4100

Measurement of the amount of drug present in the faeces following a single oral dose of agent gave a measure of the amount of drug eliminated. To allow for the possibility that a certain fraction of the faecal phenazine might be produced by elimination of some of the absorbed dose, a non-absorbable dietary marker, chromium oxide, was included in the initial drug dose. Any drug present at the same time as the chromium, therefore, represents non-absorbed drug.

Figures 5.8.(i) - (iv) show the egestion profiles of phenazine and chromium in the four groups. Analysis of the chromium results shows that the typical transit time for these animals was 24 hours. The profiles are also similar for the control and phenazine-treated groups. Recycling of the egested chromium is evident from the undulation of the faecal chromium levels after the main dose has been eliminated. The cumulative amount of drug produced when near 100 % of the chromium had appeared in the faeces was taken as the non-absorbed drug fraction. This was taken from the initial dose and expressed as a percentage to give the absorption values quoted in Table 5.4. These results show that most of the B663 administered by this route was absorbed. More B4100 remained unabsorbed, but interestingly, nearly half of the administered dose of B4090 was not absorbed. This is particularly surprising since this compound gave by far the highest overall tissue drug levels.

Table 5.2. The concentration of phenazines in the cerebral tissue of rats. The animals had been fed with food containing 0.035 % (w/w) B663, B4090 or B4100 for 20 out of 26 days. In each case the number of animals assayed was 3.

Drug	Mean cerebral concentration (μg/g)	Standard deviation (μg/g)
B663	0.38	0.13
B4090	0.39	0.05
B4100	0.21	0.03

Table 5.3. The concentration of phenazines in the serum of rats. The animals had been fed with food containing 0.035 % (w/w) B663, B4090 or B4100 for 20 out of 26 days. In each case the number of animals assayed was 3.

Drug	Mean serum concentration (µg/ml)	Standard deviation (µg/ml)
B663	0.78	0.27
B4090	0.21	0.01
B4100	0.003	0.001

Figure 5.8.(i). The profile of the dietary marker, chromium, egestion in faeces following administration of a single dose of 0.6 % (w/w) chromium in food to a group of control rats receiving no phenazine. The chromium content of the faeces was measured by the spectrophotometric method. Most of the chromium appears after 24 hours, indicating that this is the dietary transit time in these animals. Chromium continues to be egested due to the coprophagous nature of these rodents.

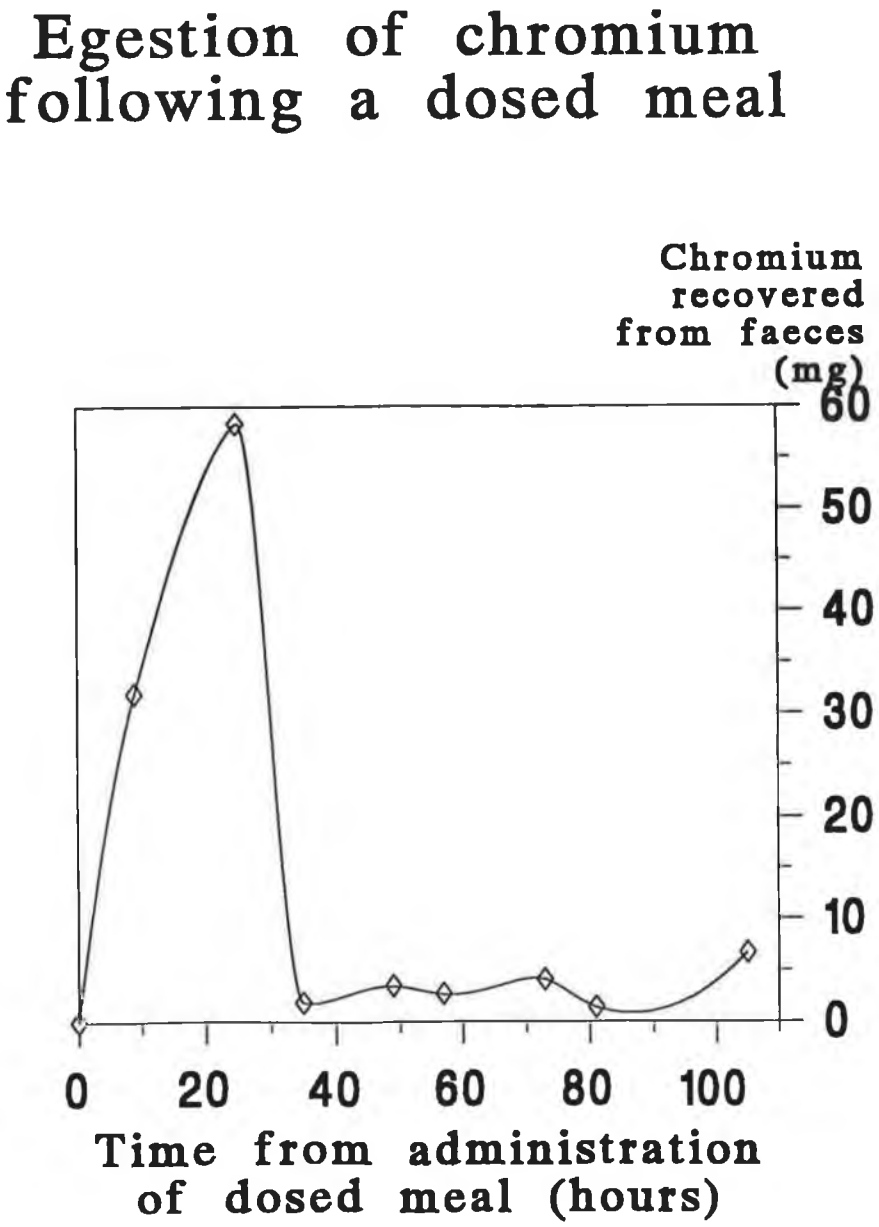


Figure 5.8.(ii). The profile of chromium egestion in faeces superimposed on the egestion of B663 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B663 in food to a group of rats. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method. The B663 profile can be seen to follow that of chromium.

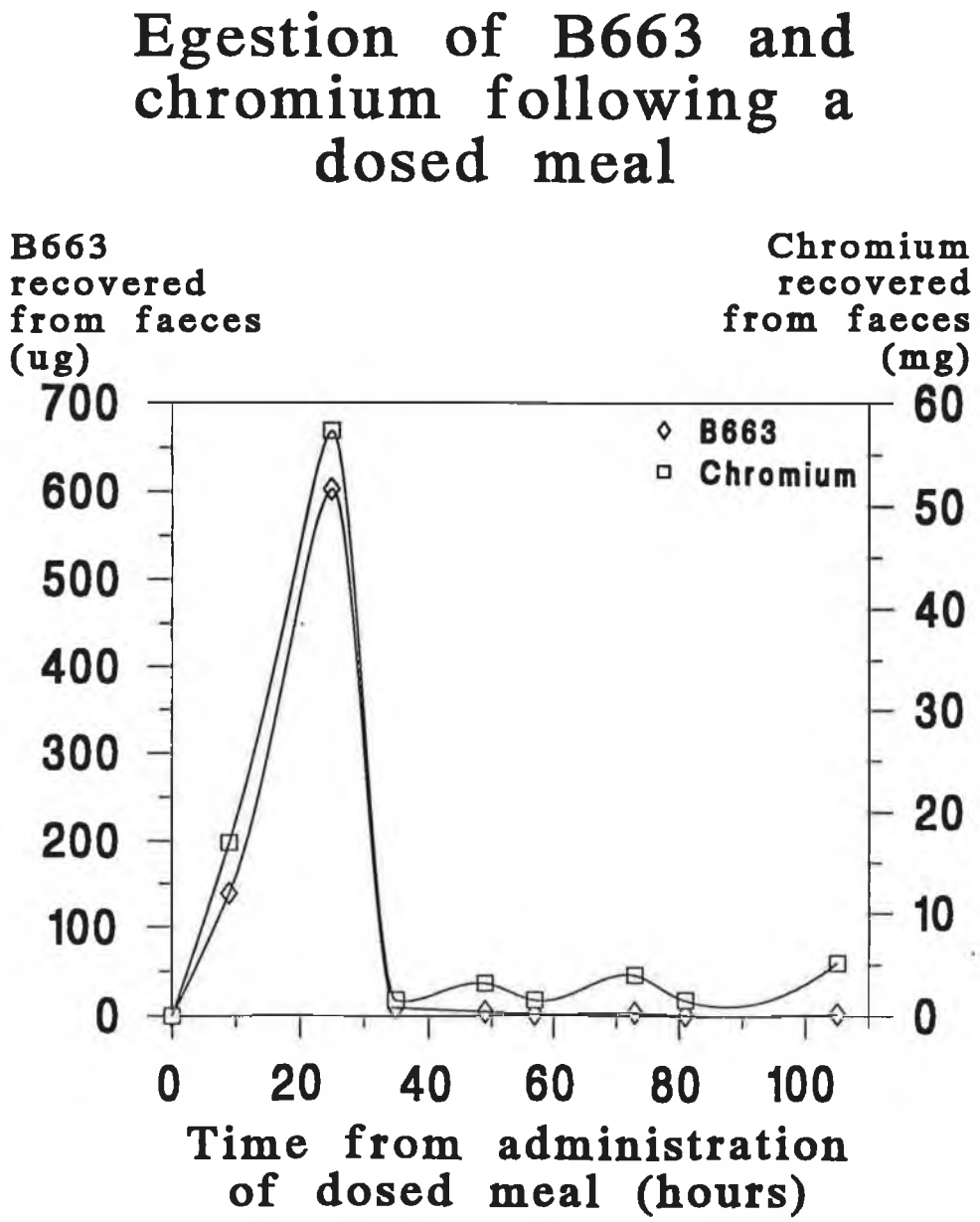


Figure 5.8.(iii). The profile of chromium egestion in faeces superimposed on the egestion of B4090 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B4090 in food to a group of rats. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method. A second hump is evident at 49 hours due to coprophagia.

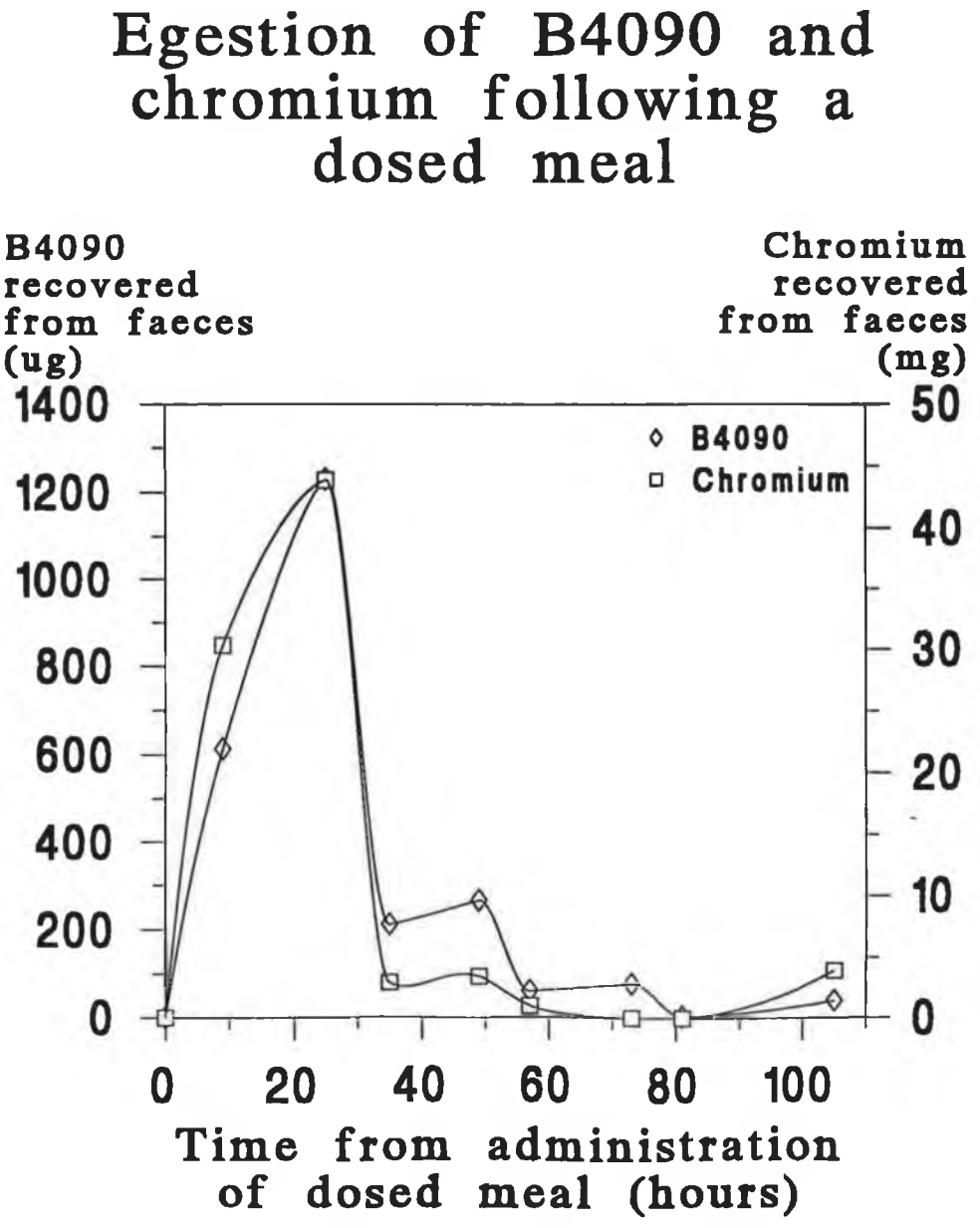


Figure 5.8.(iv). The profile of chromium egestion in faeces superimposed on the egestion of B4100 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B4100 in food to a group of rats. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method.

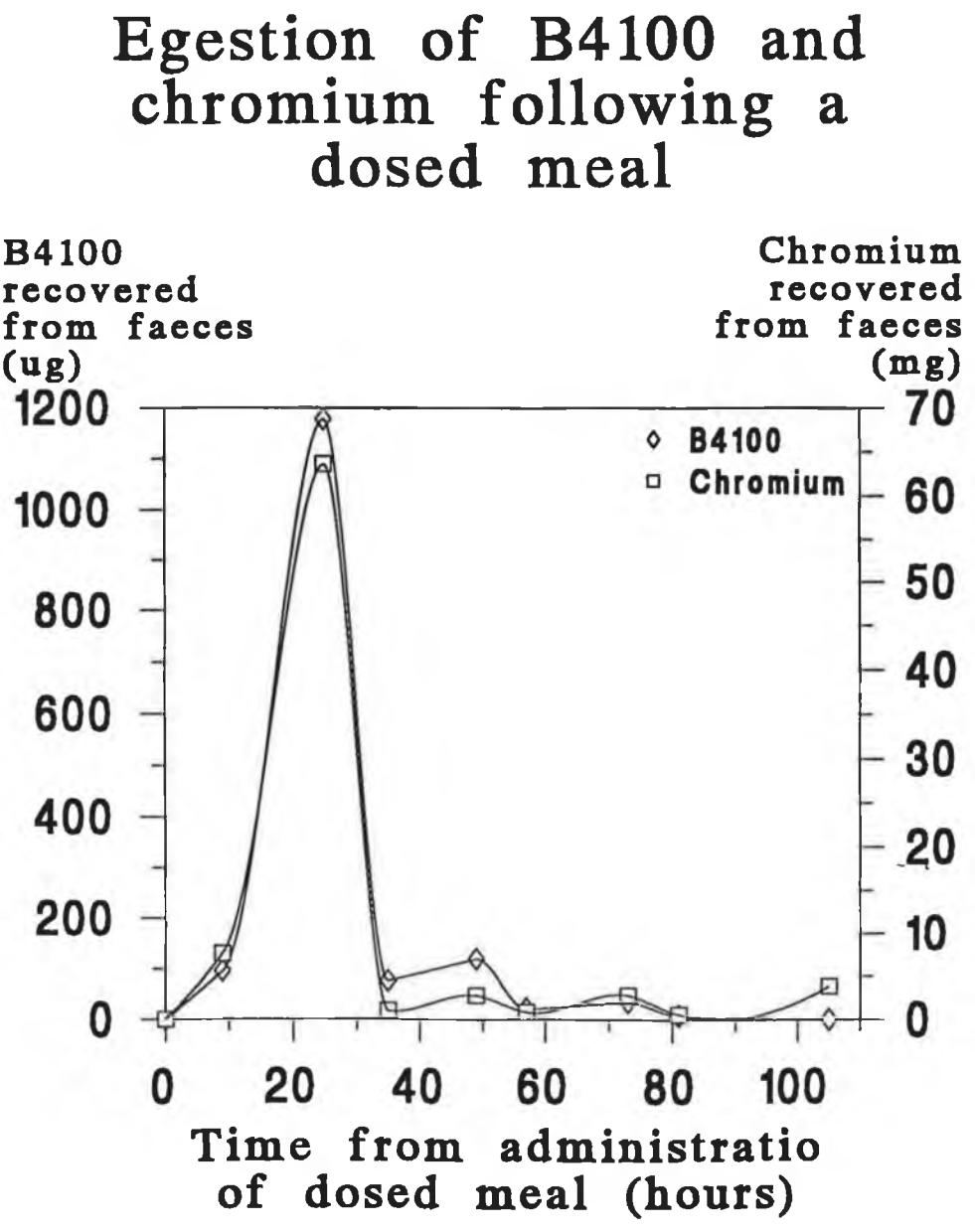


Table 5.4. The egested and absorbed percentage of B663, B4090 or B4100 in rats. The animal drug groups were given a single dose of food containing 0.035 % (w/w) phenazine and 0.6 % (w/w) chromium oxide. Chromium was used as a control to monitor transit of the drugged food. The amount of phenazine and chromium egested in the pooled faeces was measured and the amount of phenazine recovered expressed as a percentage of the original dose. The remaining dose was the absorbed percentage.

Drug	% Egested	% Absorbed
B663	14	86
B4090	49	51
B4100	25	75

5.8.3. B4100 and B4090 toxicity measurement

Potential toxicity of these compounds was assessed by measuring several different indicator parameters and comparing these values with the B663 and control groups. The parameters measured were behavioural and feeding characteristics, blood enzymes and cell numbers, animal weights over the experiment and post mortem tissue weights, and urinary markers of toxicity. The urine was analysed by NMR (and these results are reported in section 5.9.1.) and using combination test strips sensitive to pH, glucose, ascorbic acid, ketones, nitrite, protein, bilirubin, urobilinogen and blood. None of the values for these parameters were different between the groups or changed over the course of the experiment. No changes were noted in pecking order, activity, cleaning and condition or aggressiveness in the groups over time. No differences in water intake was noted between the groups although general water intake did vary due to variations in environmental conditions. Feeding characteristics varied between the groups as shown in Figure 5.9.(i). The average daily consumption of treated or drugged food was seen to decrease slightly over the course of the experiment. This difference increased from the B663 to B4090 to B4100 group. Comparison with the change in the amount of non-treated food in Figure 5.9.(ii) shows that there was a corresponding increase in the amount of this food eaten. This also had a corresponding effect on the weight gain of these animals over the course of the experiment (Table 5.5.). Post mortem tissue weights were very similar among all the groups of animals.

The change in aspartate aminotransferase (AST), alanine amino transferase (ALT) and sorbitol dehydrogenase (SDH) blood enzymes was measured over the course of the experiment. AST levels increased in all groups over the course of the experiment, but ALT and SDH were similar between groups before and after the experiment. Blood samples were also analysed by Dr. Hugh Larkin in the Veterinary school of University College Dublin for the following parameters before and after the experiment:- packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total white blood cell count (WBC), neutrophils, eosinophils, lymphocytes, monocytes and protein content. Table 5.6.(i). - (iv). shows all the results obtained. The only apparent difference seen was with the neutrophil count of the B4090 and B4100 groups.

Figure 5.9.(i). The average daily intake of drugged food for the control, B663, B4090 and B4100 treatment groups of rats. In all groups the intake of treated food decreased over time. However, this effect was more noticeable in the B663, and especially, the B4090 and B4100 groups.

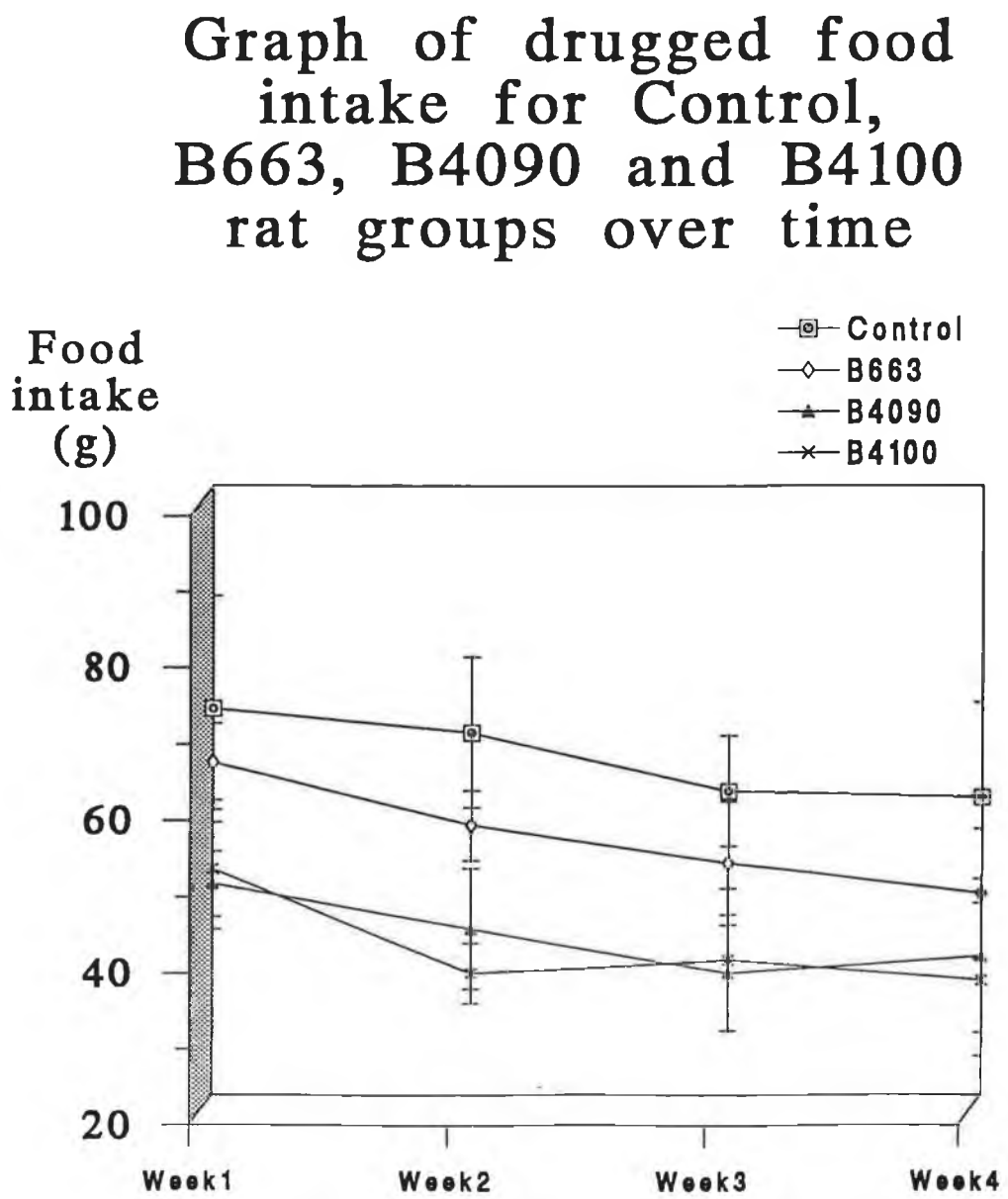


Figure 5.9.(ii). The average daily intake of normal animal food for the control, B663, B4090 and B4100 treatment groups of rats. In the control group the consumption hardly varied. However, in the phenazine groups the consumption increased over time particularly with B4090 and B4100 animals whose intake of drugged food was lowest, as shown in figure 5.9.(i).

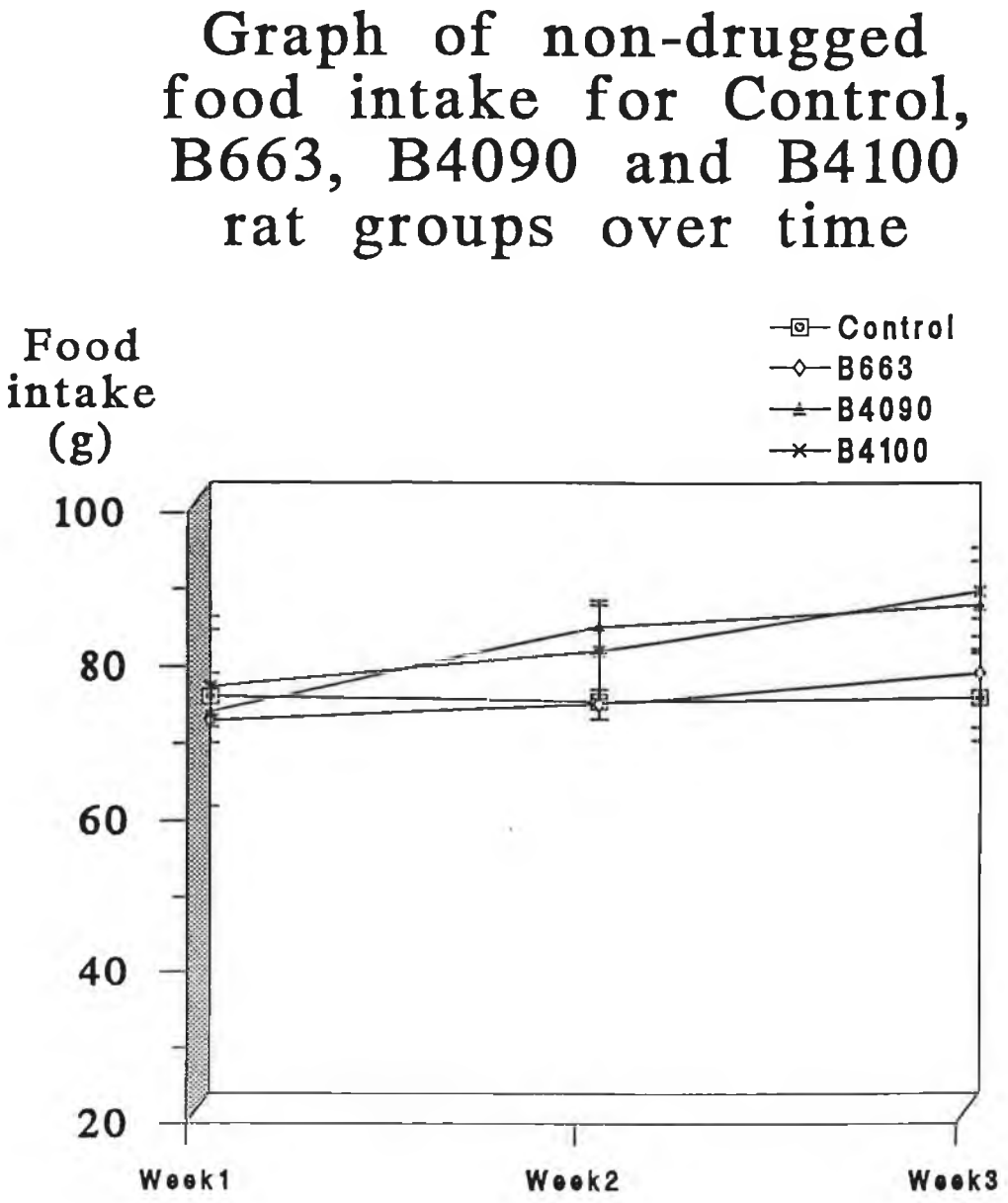


Table 5.5. The percentage change in weight of each animal in the different phenazine treatment groups after 26 days of the treatment schedule.

Animal	Initial weight (g)	Change in weight % $\frac{\text{(final wt. - initial wt.)}}{\text{initial wt}}$ 100
Control 1	257.1	9.5
Control 2	288	5.4
Control 3	264.9	4.9
B663 1	257.5	7.5
B663 2	252	4.9
B663 3	250	2.7
B4090 1	257.5	0.9
B4090 2	248.3	-5.0
B4090 3	250.8	6.6
B4100 1	257.2	2.6
B4100 2	230.1	-0.6
B4100 3	229.6	0.9

Table 5.6.(i). The values of the various haematological parameters before and after the feeding experiment for the control rat group. In each case the number of animals assayed was 3.

Drug	Control				
Parameter	**Before**	**S.D.**	**After**	**S.D.**	**Units**
AST	36.5	2.7	353.9	66.0	U/L
ALT	101.4	24.2	62.2	5.2	U/L
SDH	4.76	1.4	5.5	3.1	mU
PCV	0.4	0.0	0.5	0.0	L/L
Hb	134.0	3.0	150.0	8.7	g/L
RBC	8.4	0.4	8.7	0.71	10^{12}/L
MCHC	320.0	10.0	336.0	15.3	g/l
MCV	50.5	0.5	51.3	0.6	fL
WBC	7.4	1.9	5.9	1.5	10^{9}/L
Neut	1.5	0.2	0.9	0.3	10^{9}/L
Eos	0.05	0.05	0.16	0.11	10^{9}/L
Lymph	5.7	1.6	4.8	1.4	10^{9}/L
Mono	0.1	0.1	0.03	0.06	10^{9}/L
Prot	68.0	1.0	65.0	5.0	g/L

Table 5.6.(ii). The values of the various haematological parameters before and after the feeding experiment for the B663 rat group. In each case the number of animals assayed was 3.

Drug	B663				
Parameter	**Before**	**S.D.**	**After**	**S.D.**	**Units**
AST	68.4	33.6	401.6	11.6	U/L
ALT	129.1	22.2	72.0	16.6	U/L
SDH	5.4	3.1	7.9	3.5	mU
PCV	0.4	0.0	0.4	0.0	L/L
Hb	142.3	12.3	144.0	8.2	g/L
RBC	7.7	0.5	7.6	0.3	10^{12}/L
MCHC	356.6	5.7	360.0	0.0	g/l
MCV	50.6	1.2	52.7	2.1	fL
WBC	8.0	2.4	7.6	1.8	10^{9}/L
Neut	1.8	0.4	1.0	0.3	10^{9}/L
Eos	0.03	0.05	0.07	0.05	10^{9}/L
Lymph	6.2	1.8	6.4	1.4	10^{9}/L
Mono	0.03	0.05	0.1	0.1	10^{9}/L
Prot	64.3	4.5	65.0	2.0	g/L

Table 5.6.(iii). The values of the various haematological parameters before and after the feeding experiment for the B4090 rat group. In each case the number of animals assayed was 3. An increase in the neutrophil count of these animals was noted *.

Drug	B4090				
Parameter	**Before**	**S.D.**	**After**	**S.D.**	**Units**
AST	35.4	2.4	340.1	81.1	U/L
ALT	84.0	12.5	81.0	10.0	U/L
SDH	5.0	0.8	13.5	4.0	mU
PCV	0.4	0.0	0.4	0.0	L/L
Hb	147.7	3.2	134.0	15.7	g/L
RBC	8.2	0.2	7.2	0.9	10^{12}/L
MCHC	340.0	20	356.7	5.8	g/l
MCV	52.3	1.5	53.3	0.6	fL
WBC	6.1	3.0	9.3	2.2	10^{9}/L
Neut	1.2	0.2	4.5*	0.5	10^{9}/L
Eos	0.1	0.1	0.1	0.1	10^{9}/L
Lymph	4.7	2.9	4.81	2.6	10^{9}/L
Mono	0.03	0.05	0.0	0.0	10^{9}/L
Prot	71.7	5.9	66.0	3.6	g/L

Table 5.6.(iv). The values of the various haematological parameters before and after the feeding experiment for the B4100 rat group. In each case the number of animals assayed was 3. An increase in the neutrophil count of these animals was noted *.

Drug	B4100				
Parameter	**Before**	**S.D.**	**After**	**S.D.**	**Units**
AST	74.0	67.3	206.8	37.6	U/L
ALT	73.4	22.2	54.1	11.2	U/L
SDH	5.4	2.1	11.0	1.6	mU
PCV	0.4	0.0	0.4	0.0	L/L
Hb	144.7	12.7	132.3	20.3	g/L
RBC	8.3	0.8	7.0	1.0	10^{12}/L
MCHC	333.3	20.8	340.0	20.0	g/l
MCV	51.6	4.0	56.0	4.4	fL
WBC	6.3	0.8	11.3	0.6	10^9/L
Neut	1.3	0.2	4.8*	0.7	10^9/L
Eos	0.2	0.2	0.0	0.1	10^9/L
Lymph	4.8	0.5	6.1	0.5	10^9/L
Mono	0.06	0.05	0.30	0.20	10^9/L
Prot	71.3	1.5	68.3	1.5	g/L

5.8.4. Tissue distribution of B4103 and B4154

Using the same apparatus and methodologies described for the tetramethylpiperidine derivatives, the absorption and tissue distribution of B4103 and B4154 were investigated. Figure 5.10.(i) - (iii) shows the tissue levels of B663, B4103 and B4154. The drug tissue level profile of B663 is very similar in shape and magnitude to the previous experiment, as might be expected. B4103 produced higher levels of drug in the spleen, liver and lung, but lower level of drug in the fat tissue. The tissue levels of B4154 were low relative to the other two compounds, with the highest concentration seen in the fat tissue. The cerebral and serum levels of these three compounds are shown in Tables 5.7 and 5.8 respectively. The level of B663 present in the cerebrum was similar to that found in the TMP distribution study, but the serum level was much lower in this experiment. Both B4103 and B4154 gave higher levels in the brain than B663. Serum levels of these agents were less than the level seen with B663. Autopsy of the B663 group was similar to that described earlier. The fat of B4103 was slightly tinted orange and the intestine had a light red colouration. The orange colouration of the B4154 group was more a little more intense than that in the B4103 group, with a similar level of red colouration evident in the intestine.

5.8.5. Absorption of B4103 and B4154

Figures 5.11.(i) - (iii) show the absorption profiles of these compounds. In this experiment the transit time was shorter with most of the egested chromium and phenazine agents appearing in the faeces after 10 hours. Table 5.9 shows the calculated drug absorption figures derived from this data. The percentage absorption of B663 was very similar to that seen in the previous experiment. The absorption level of B4154 was lower than the B663 value but B4103 gave the highest absorption of any of the compounds tested in this project.

5.8.6. B4103 and B4154 toxicity measurement

No changes in behaviour or urinary parameters were noted over the course of the experiment. These animals had normal eating patterns and weight gains unlike those of the TMP study animals. Blood markers of toxicity were not investigated for this group.

Figure 5.10.(i). The tissue levels in rats following administration of B663 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. These results are part of the investigation of tissue levels of B4103 and B4154. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each tissue is the mean of the values measured in three animals.

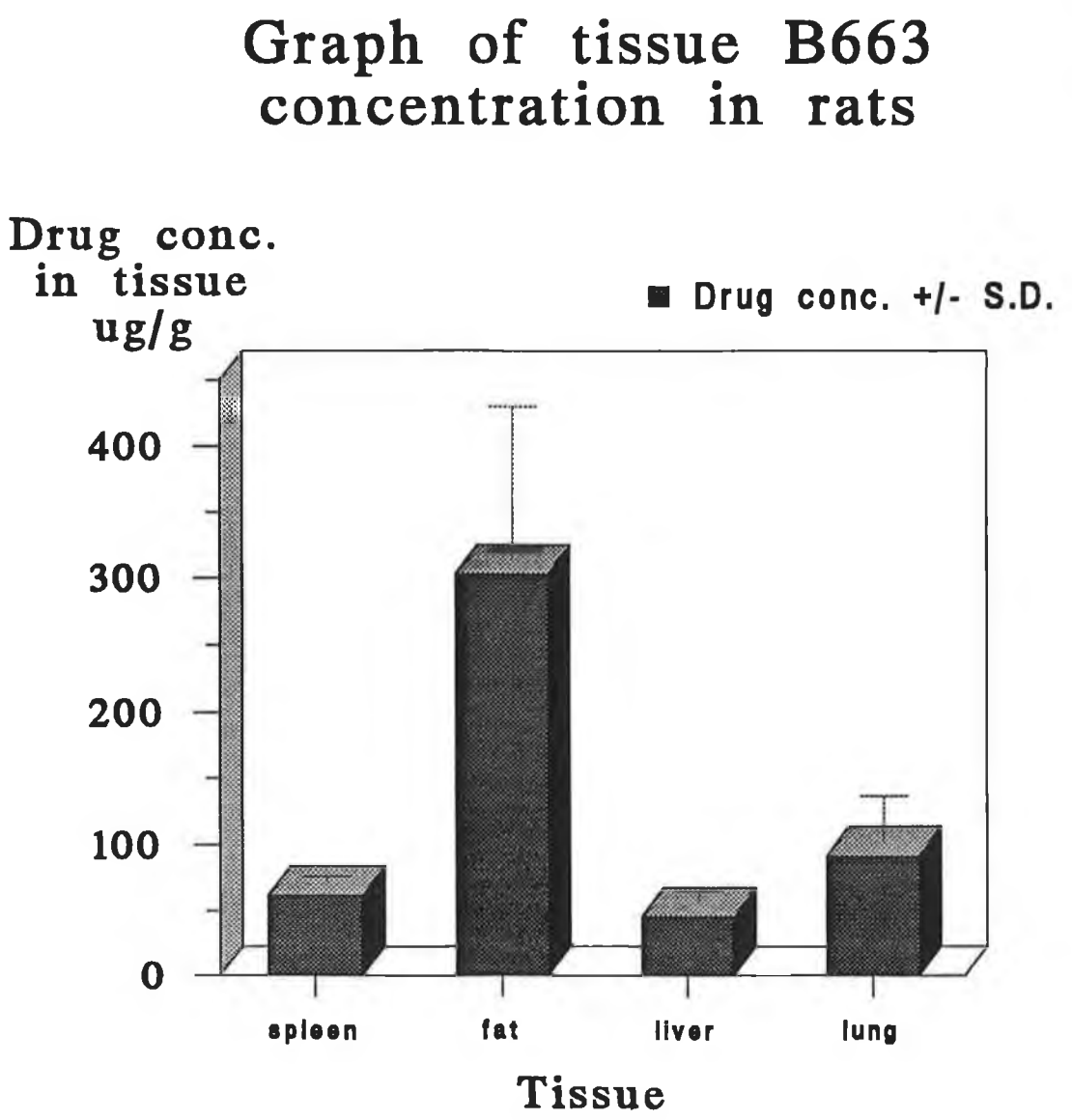


Figure 5.10.(ii). The tissue levels in rats following administration of B4103 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B4103 for each tissue is the mean of the values measured in three animals.

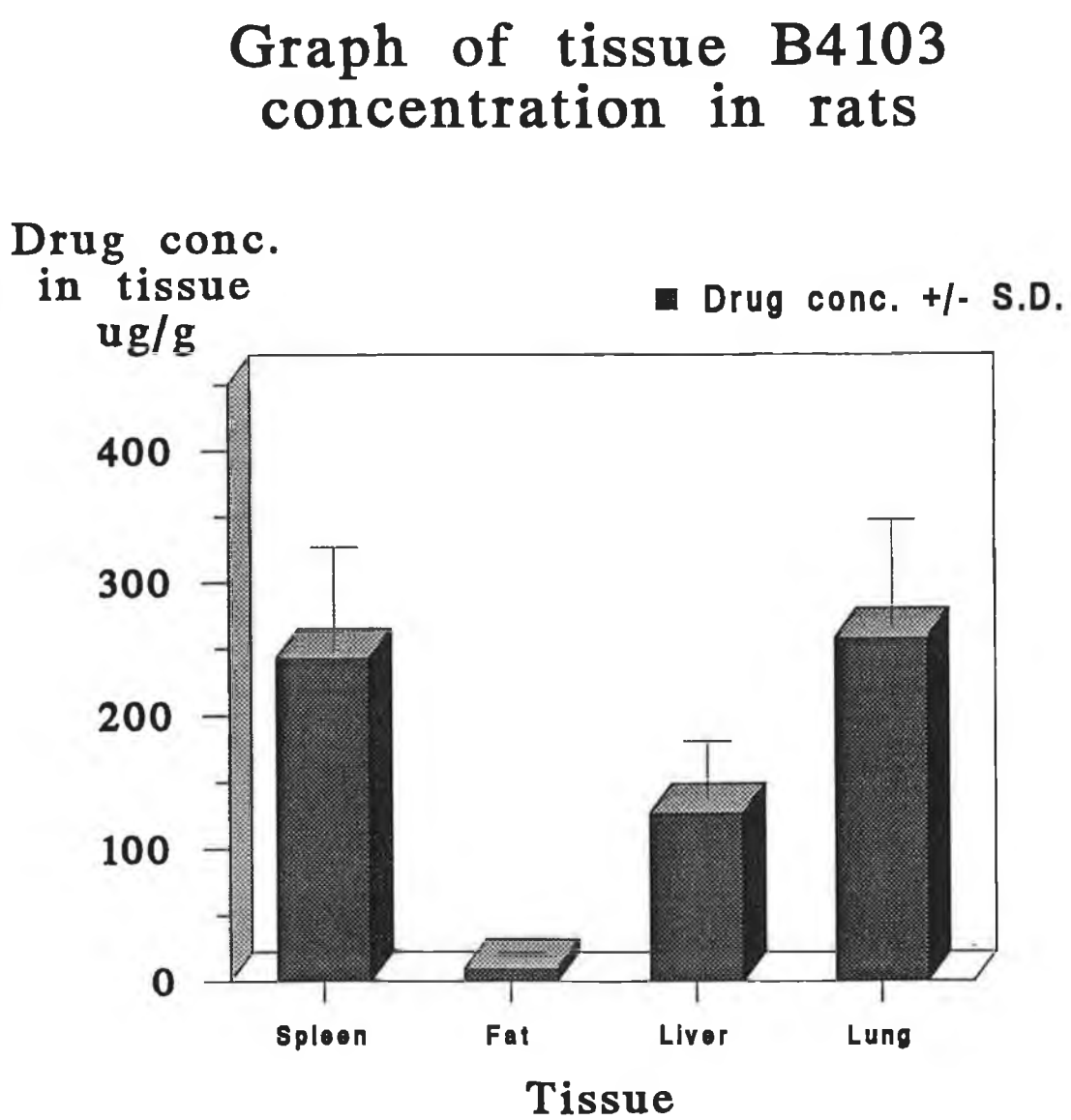


Figure 5.10.(iii). The tissue levels in rats following administration of B4154 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B4154 for each tissue is the mean of the values measured in three animals.

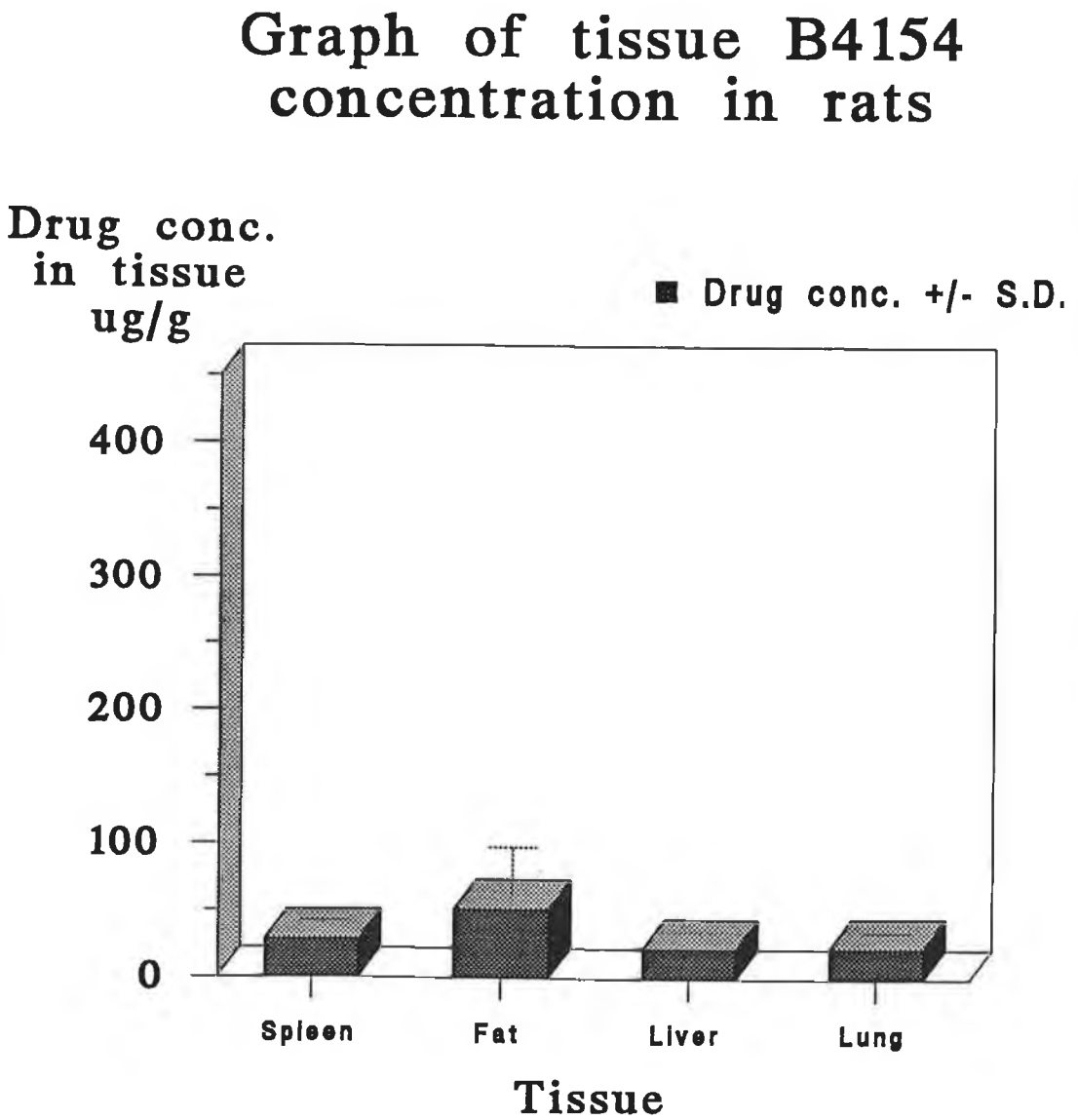


Table 5.7. The concentration of phenazines in the cerebral tissue of rats. The animals had been fed with food containing 0.035 % (w/w) B663, B4103 or B4154 for 20 out of 26 days. In each case the number of animals assayed was 3. Drug levels were measured using the HPLC procedure described in the methods chapter.

Drug	Mean cerebral concentration (μg/g)	Standard deviation (μg/g)
B663	0.47	0.13
B4103	5.54	0.23
B4154	7.70	0.23

Table 5.8. The concentration of phenazines in the serum of rats. The animals had been fed with food containing 0.035 % (w/w) B663, B4103 or B4154 for 20 out of 26 days. In each case the number of animals assayed was 3. Drug levels were measured using the HPLC procedure described in the methods chapter.

Drug	Mean serum concentration (µg/ml)	Standard deviation (µg/ml)
B663	0.18	0.08
B4103	0.09	0.02
B4154	0.04	0.01

Figure 5.11.(i). The profile of chromium egestion in faeces superimposed on the egestion of B663 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B663 in food to a group of rats as part of the investigation of B4103 and B4154. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method. The B663 profile can be seen to follow that of chromium.

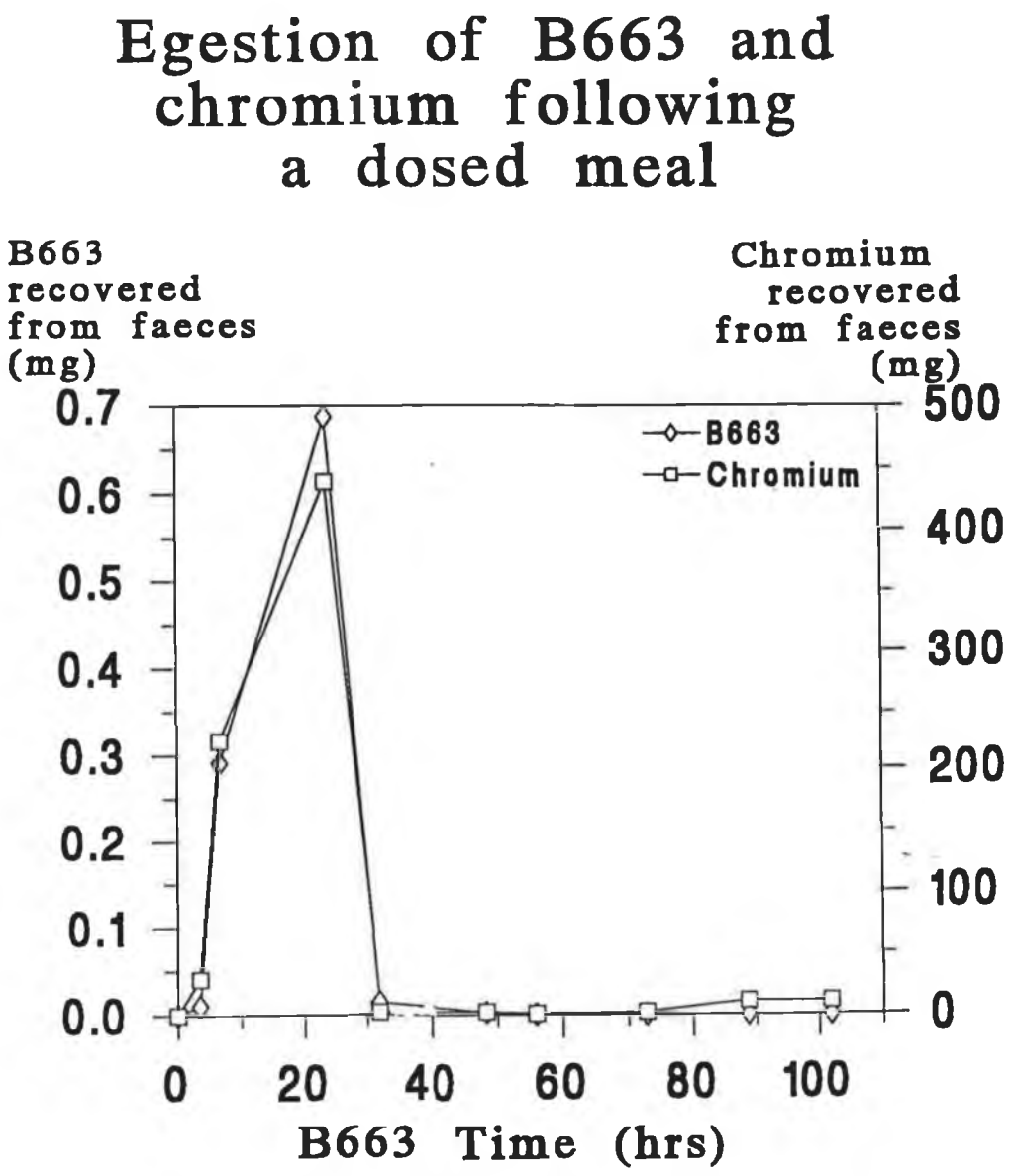


Figure 5.11.(ii). The profile of chromium egestion in faeces superimposed on the egestion of B4103 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B4103 in food to a group of rats. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method. Unlike the B663 group (figure 5.1.(i)), a large portion of the dietary contents were egested after 10 hours.

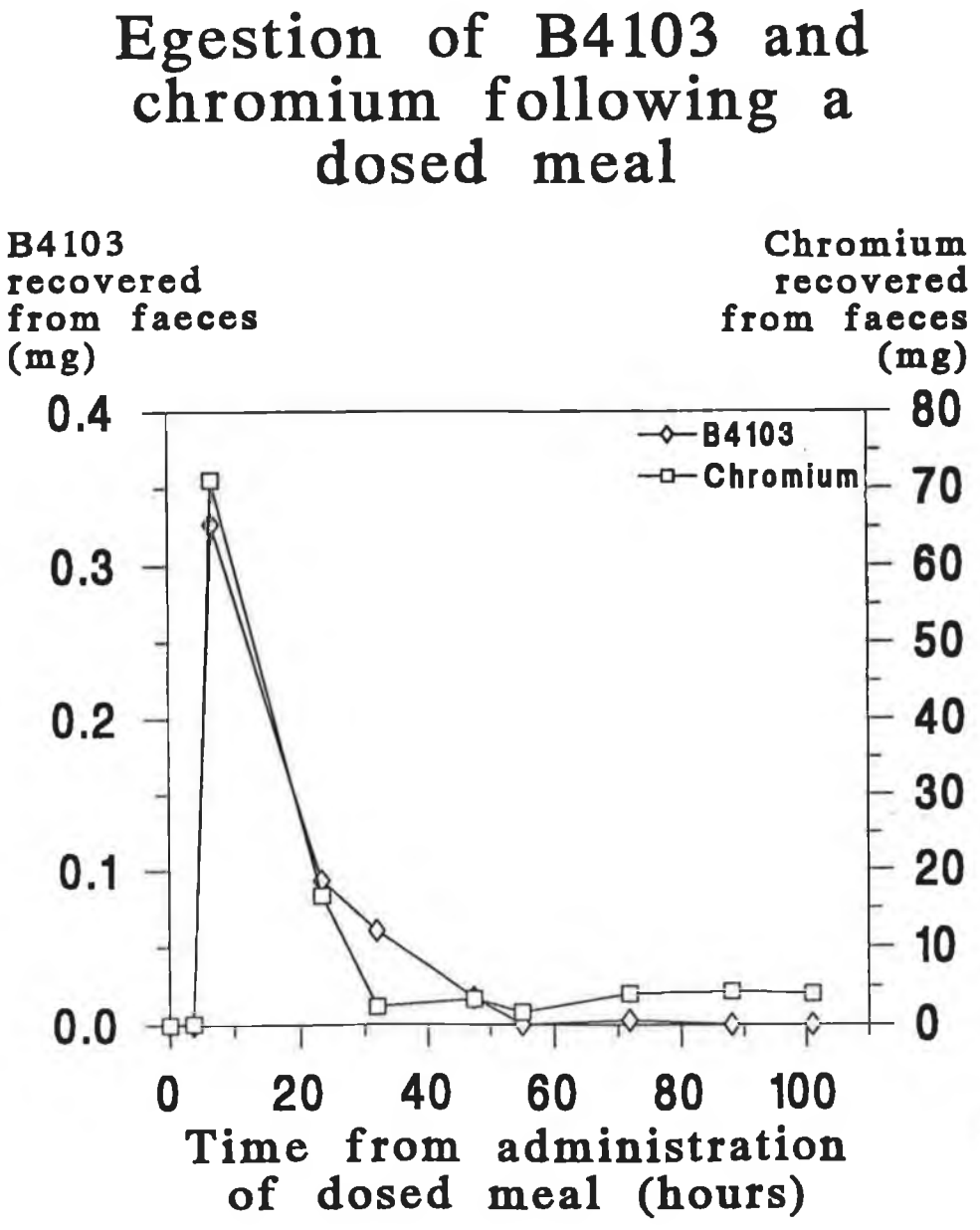


Figure 5.11.(iii). The profile of chromium egestion in faeces superimposed on the egestion of B4154 following administration of a single dose of 0.6 % (w/w) chromium and 0.035 % (w/w) B4154 in food to a group of rats. The chromium content of the faeces was measured by the spectrophotometric method and the amount of phenazine present quantified by the HPLC method. Unlike the B663 group (figure 5.11.(i)), and similar to the B4103 group (figure 5.11.(ii)), a large portion of the dietary contents were egested after 10 hours.

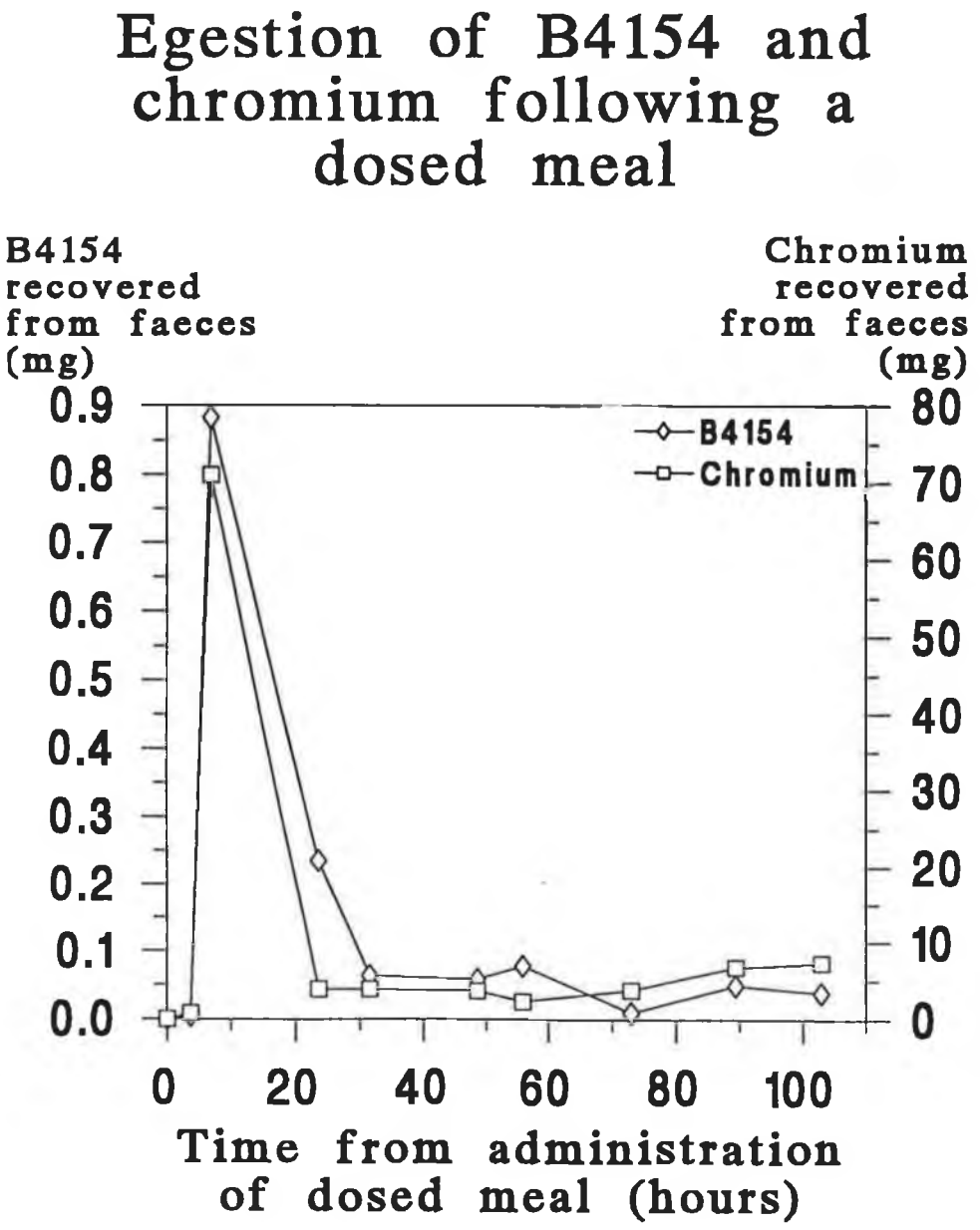


Table 5.9. The egested and absorbed percentage of B663, B4103 or B4154 in rats. The animal drug groups were given a single dose of food containing 0.035 % (w/w) phenazine and 0.6 % (w/w) chromium oxide. Chromium was used as a control to monitor transit of the drugged food. The amount of phenazine and chromium egested in the pooled faeces was measured and the amount of phenazine recovered expressed as a percentage of the original dose. The remaining dose was the absorbed percentage.

Drug	% Egested	% Absorbed
B663	17	83
B4103	8	92
B4154	24	76

5.9. INVESTIGATIONS OF TOXICITY USING NMR

5.9.1. NMR of rat urines

Urine sample from all of the rat groups were investigated by NMR. Figure 5.12.(i) shows a typical urinary profile from a control rat. The important component peaks present are indicated in the figure. Figure 5.12.(ii) shows a comparison of the urinary spectrum from a control animal with that of a rat exposed to a single small dose of carbon tetrachloride, a known toxicant. Changes which are evident include the absence of peaks due to hippurate, urea and trimethylamine N-oxide (TMAO), and increases in the peaks produced by glucose, lactate, and ethanol. Figure 5.12.(iii) shows a comparison of the control with the spectra from all the rat groups investigated. As this figure shows, the urinary profiles were very similar for all the groups tested.

5.9.2. NMR of rabbit urines

To further investigate the potential of this method, urine samples from rabbits were analysed. Figure 5.13 shows a comparison of the spectrum from a control healthy rabbit with that from an animal that died from what was later shown to be nephropathy. The control spectrum shows some species differences from that of the rat equivalent in the levels of creatinine, TMAO, taurine, citrate and succinate. The spectrum from the nephropathic rabbit shows a decrease in urea, phenylalanine and tyrosine, with increases in lactate, citrate and glycine. *Post mortem* pathological analysis by Dr. Peter Nowlan of the Bioresources unit, Trinity College Dublin, had the following results. There were periportal aggregates of lymphocytes in the liver, with bands of interstitial fibrosis in the kidney. At a functional level, there were adhesions of the glomerular tufts of the Bowman's capsules and proteinaceous casts in the tubule. The chronic interstitial nephritis described, caused an elevated level of urea in the blood which made the animal ill. The most likely cause of this disease was a chronic infection with the encephalazooan parasite *E. cuniculi* although high levels of circulating antibody may have contributed to the morbidity.

5.10. DISTRIBUTION OF CLOFAZIMINE-CYCLODEXTRIN COMPLEXES

Figure 5.14.(i). - (vi). shows a comparison of the B663 levels in the clofazimine, clofazimine-β-cyclodextrin and clofazimine-hydroxypropyl-β-cyclodextrin groups. As expected,

Figure 5.12.(i). The urinary NMR spectrum from a control group of rats. The urine sample was freeze dried and reconstituted in an equal volume of deuterated water (D_2O), with the pH adjusted to 7.0 using HCl/NaOH. The spectrum was analysed using the presat water suppression programme on a 400 MHz spectrometer. The significant peaks evident are labelled. Trimethylamine N-oxide (TMAO) gives the peak of greatest intensity. a complex doublet peak due to partially deuterated water (HOD) is also evident. Urea and hippurate are particularly important, from a toxicological perspective, indicating that the secretory systems of the kidney are functioning.

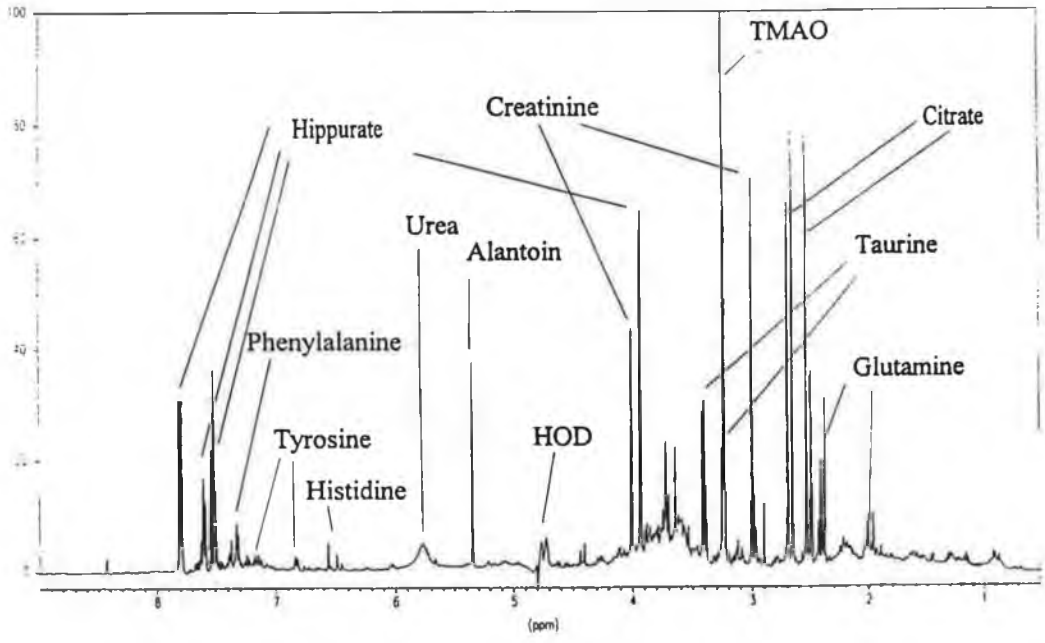


Figure 5.12.(ii). A comparison of the urinary NMR spectrum from a control group of rats (spectrum (a)) with that of a rat given a dose of carbon tetrachloride, a toxicant(spectrum (b)). The urine samples were freeze dried and reconstituted in an equal volume of deuterated water (D_2O), with the pH adjusted to 7.0 using HCl/NaOH. The spectra were analysed using the presat water suppression programme on a 400 MHz spectrometer. The loss of secretory capacity is illustrated by the absence of compounds including hippurate and urea. Damage to the absorptive functions and aerobic metabolic pathways is indicated by the presence of glucose, ethanol and lactate.

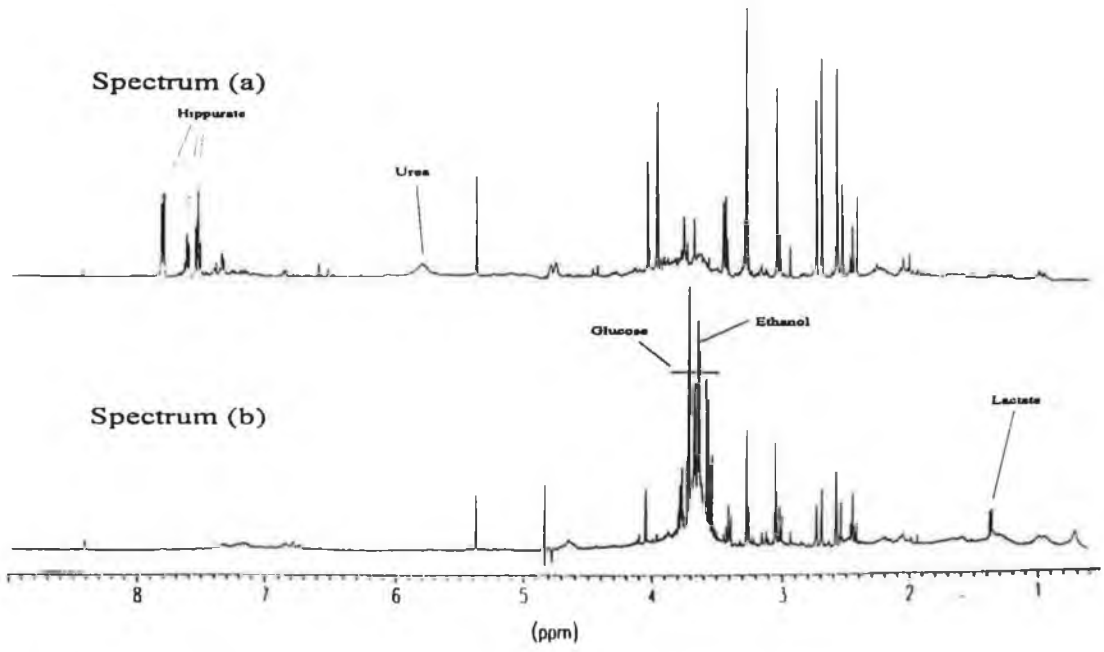


Figure 5.12.(iii). A comparison of the urinary NMR spectrum from all the groups of rats investigated. The urine samples were freeze dried and reconstituted in an equal volume of deuterated water (D_2O), with the pH adjusted to 7.0 using HCl/NaOH. The spectra were analysed using the presat water suppression programme on a 400 MHz spectrometer. The similarity of the spectra indicates that there is no evidence of a significant toxic effect with these compounds after 26 days of treatment.

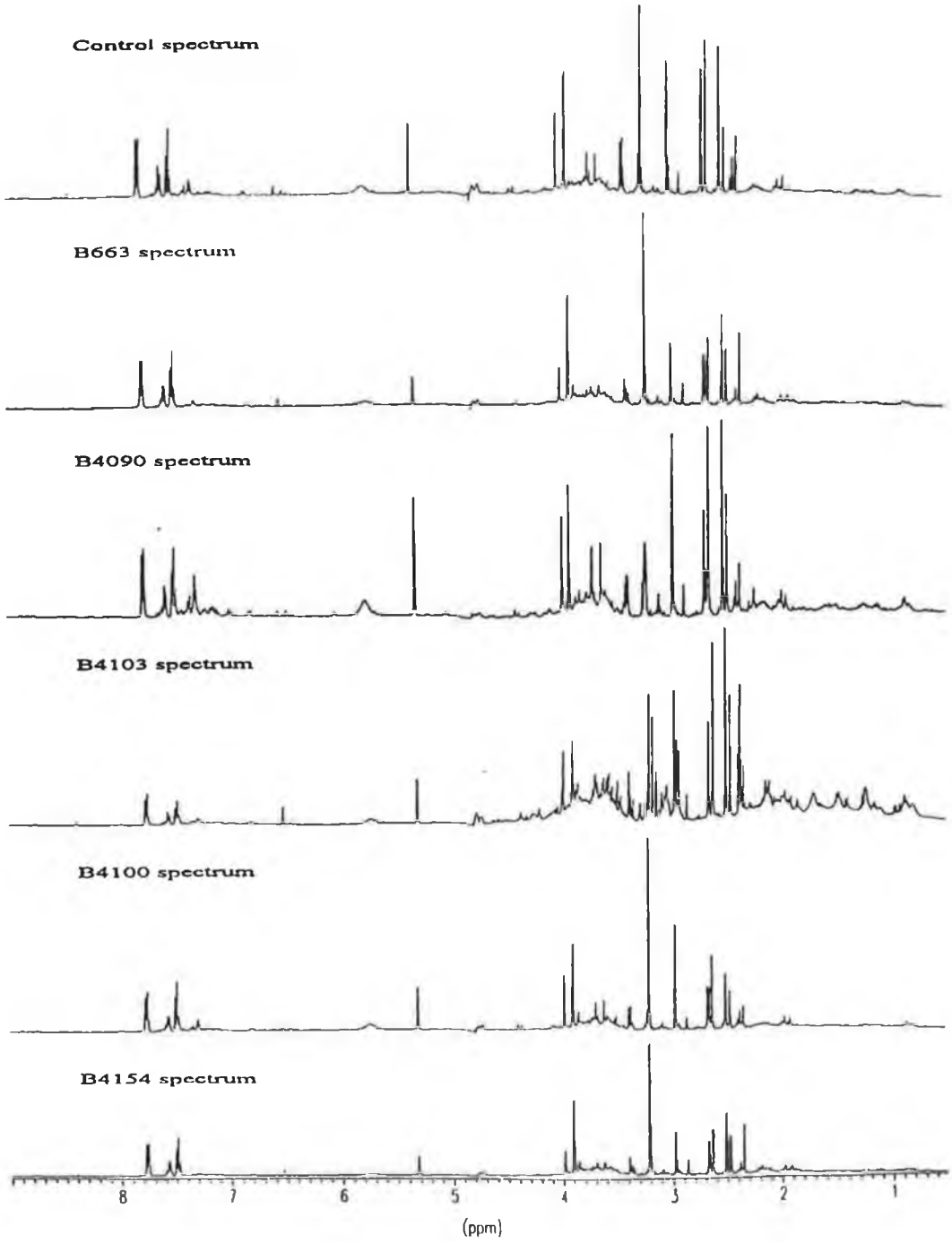


Figure 5.13. A comparison of the urinary NMR spectra from a control rabbit and a rabbit which later died from nephropathy. The urine samples were freeze dried and reconstituted in an equal volume of deuterated water (D_2O), with the pH adjusted to 7.0 using HCl/NaOH. The spectra were analysed using the presat water suppression programme on a 400 MHz spectrometer. Some species specific differences are evident when compared to the rat spectrum in figure 5.12.(i). The kidney disease in the nephropathic rabbit can be seen to affect the secretion of certain amino acids, increasing the levels of glycine present, while decreasing the amount of certain aromatic amino acids seen. Disruption of the aerobic metabolic pathway in kidney cells of this animal is also evident with the increases seen in certain key metabolic intermediates.

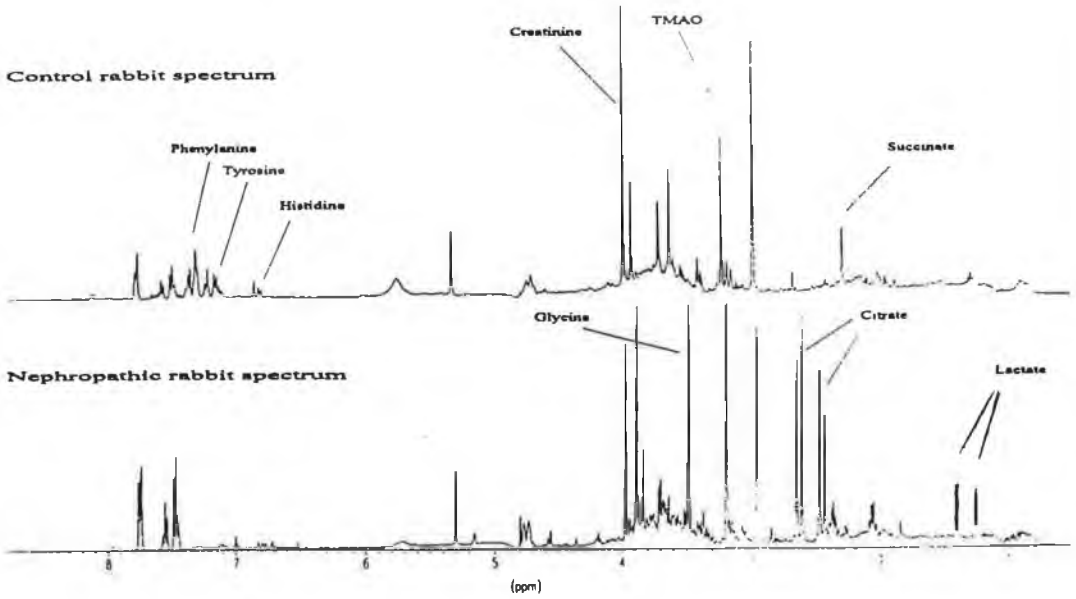


Figure 5.14.(i). The fat levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd) in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.

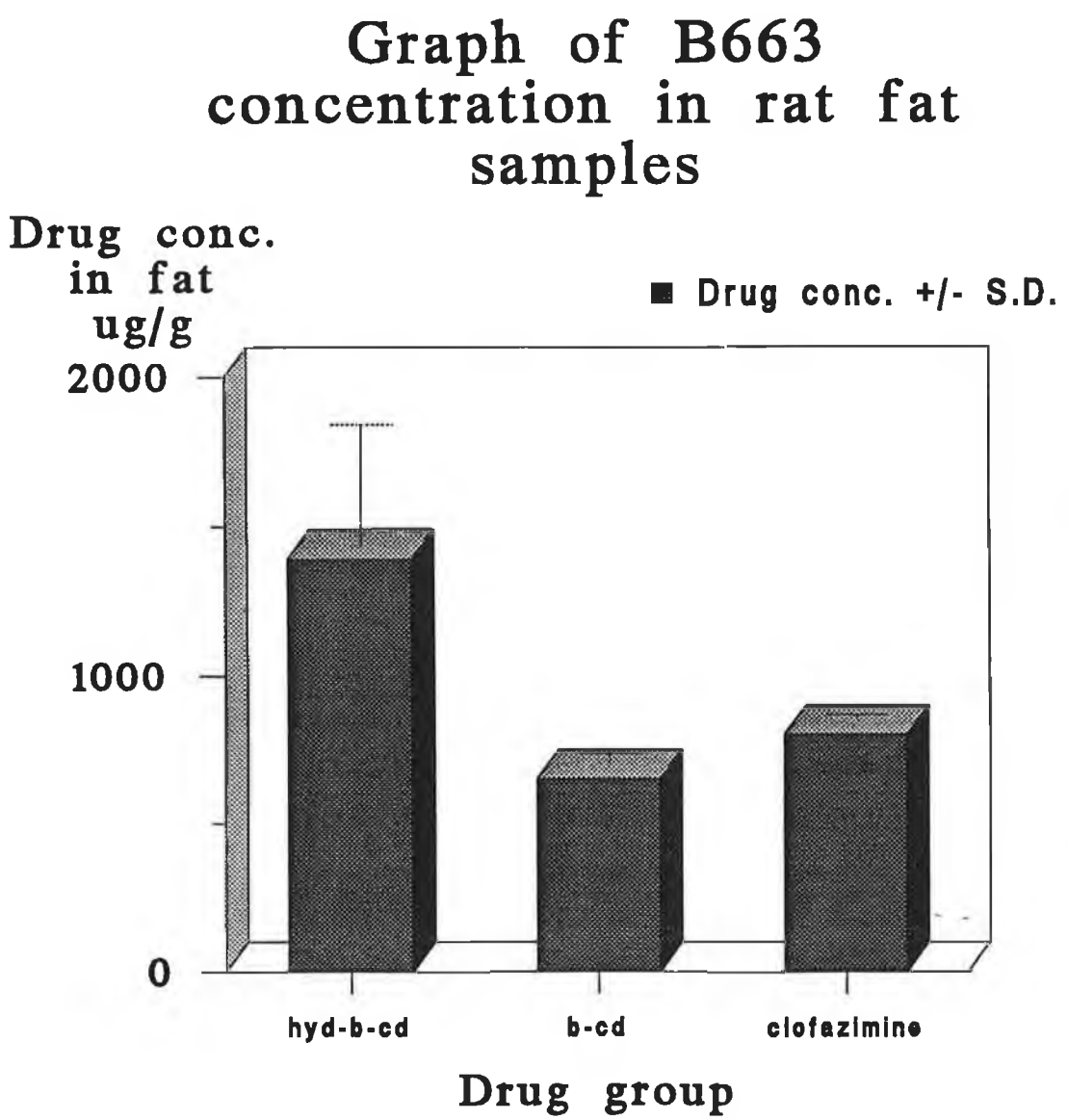


Figure 5.14.(ii). The spleen levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd) in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.

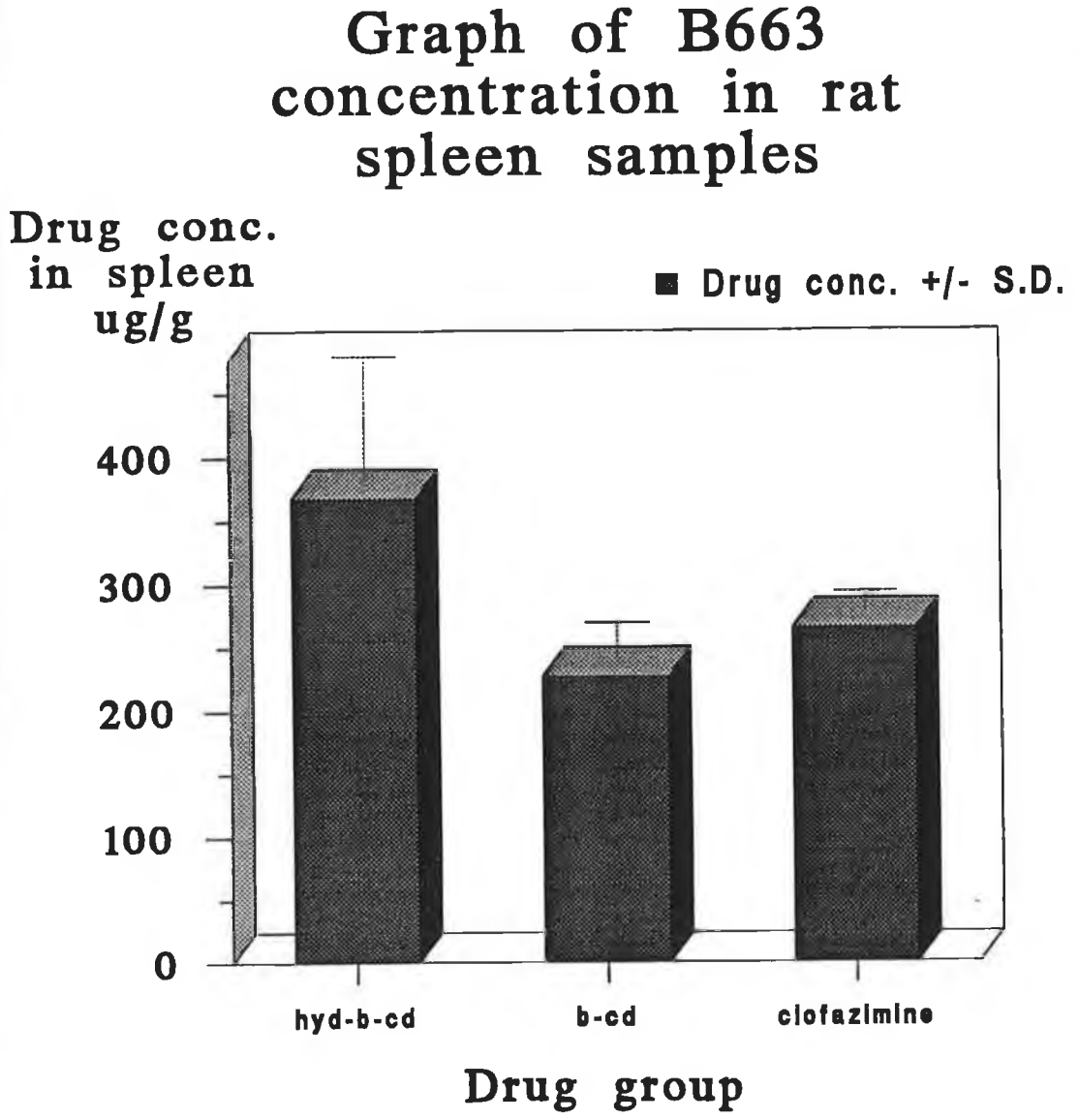


Figure 5.14.(iii). The liver levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd)in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.

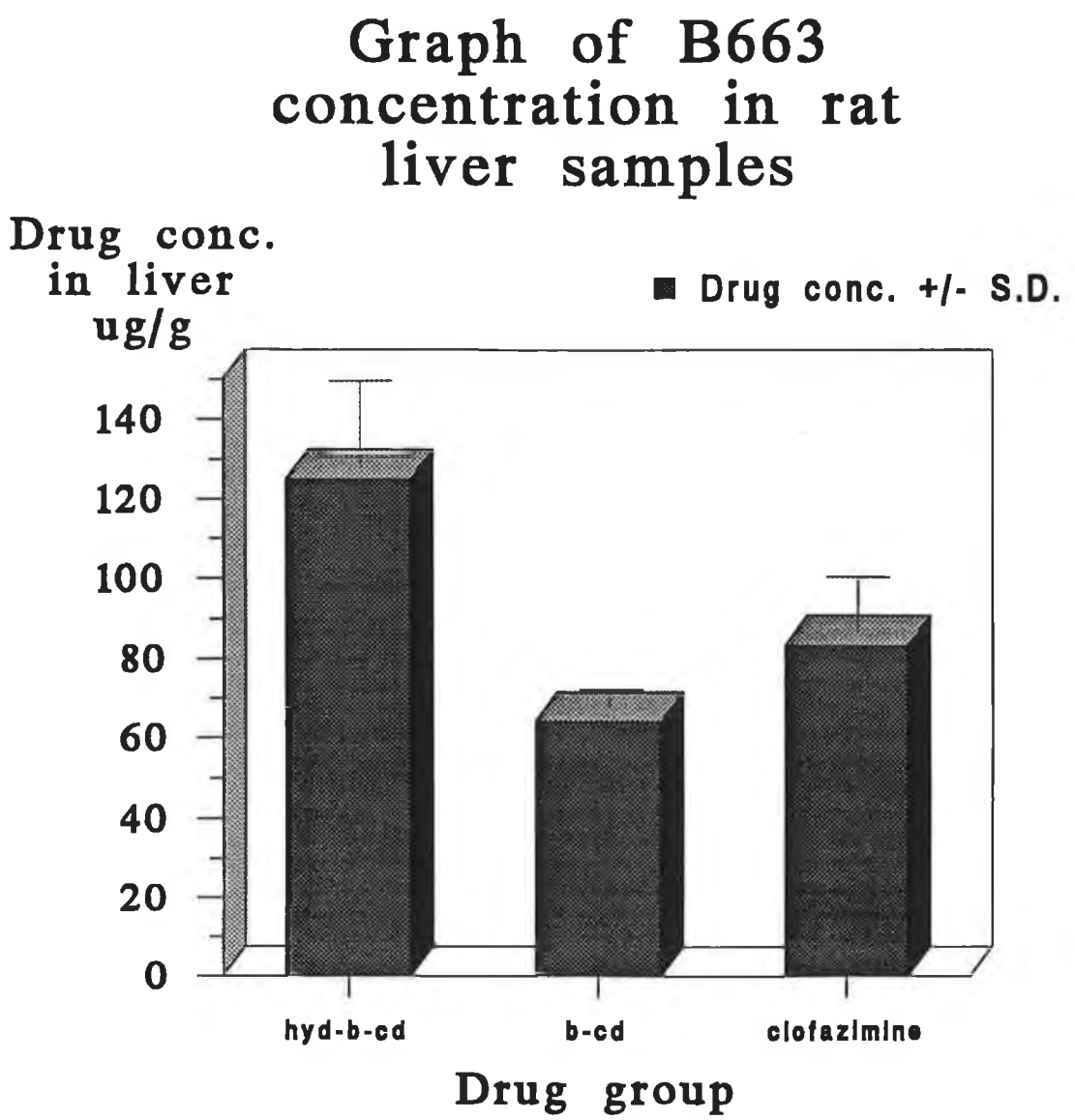


Figure 5.14.(iv). The lung levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd) in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.

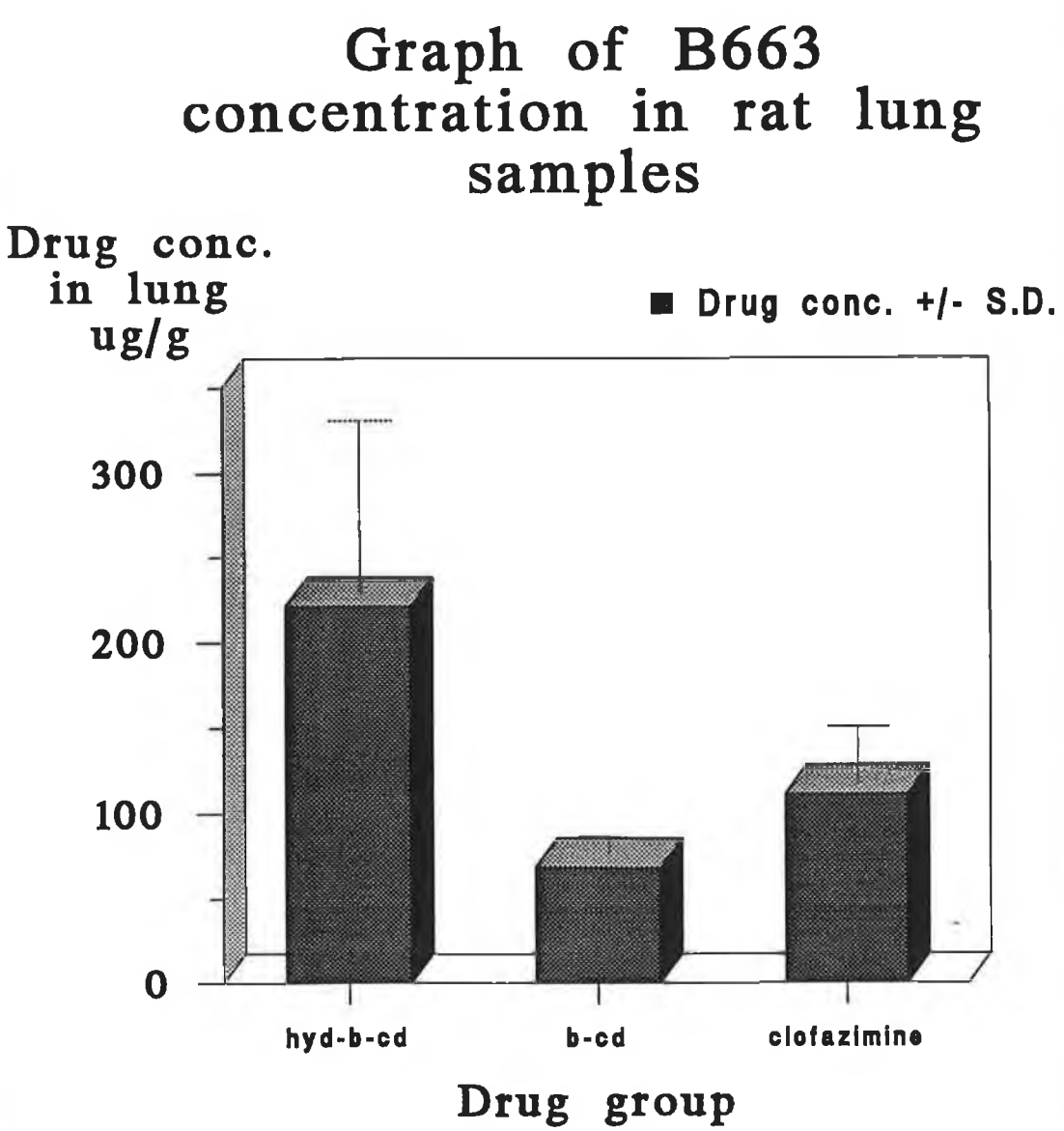


Figure 5.14.(v). The kidney levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd) in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.

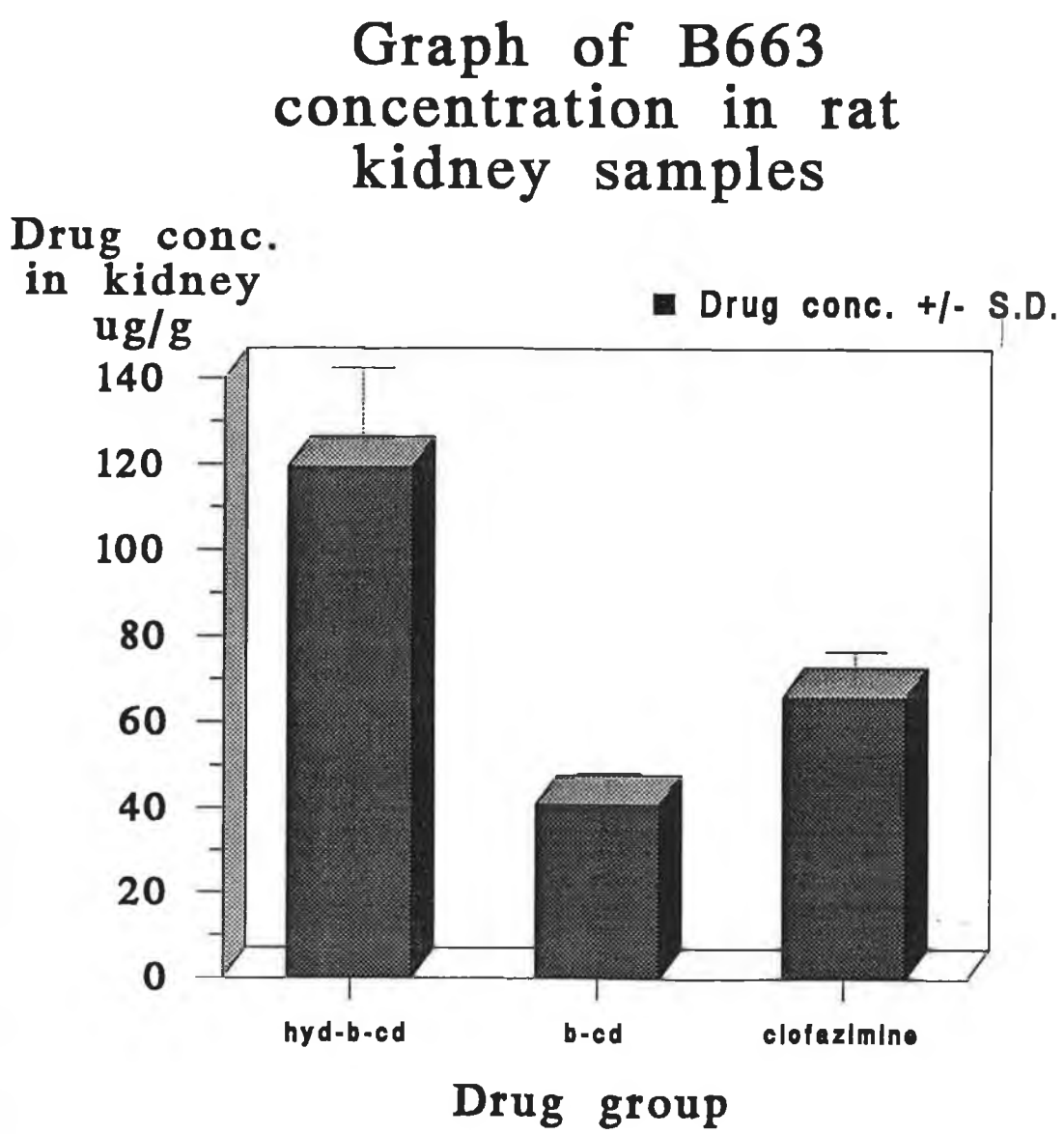
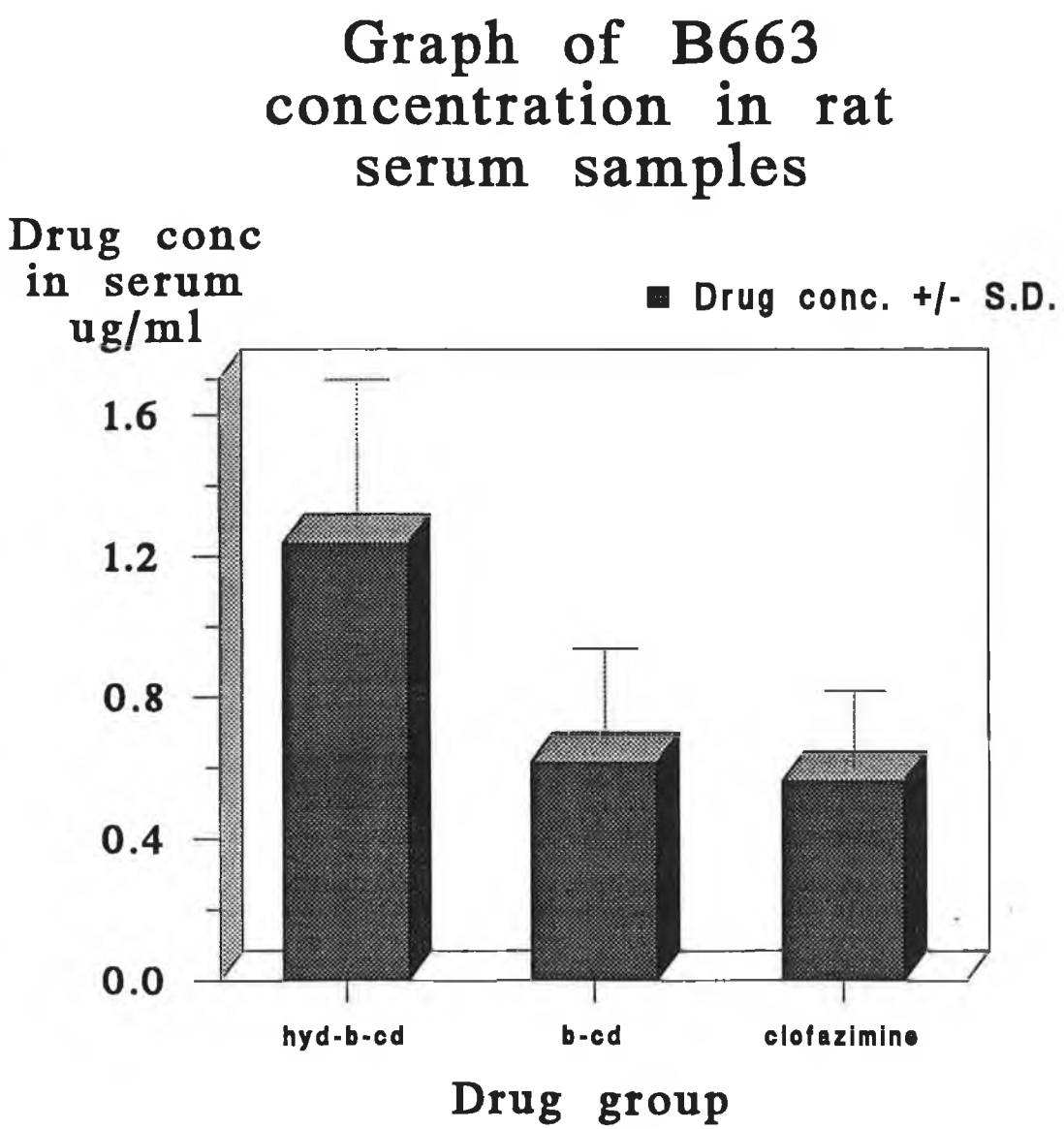


Figure 5.14.(vi). The serum levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd)in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The concentration of B663 for each sample is the mean of the values measured in the three animals in the group.



the highest concentration of B663 was found in the tissue fat of the animals. The highest tissue levels of B663 were consistently from the hydroxypropyl-β-cyclodextrin group (Figure 5.14.(vii)). The clofazimine group generally gave the next highest drug levels, compared to the lowest levels in the β-cyclodextrin group, although there was very little difference between the values measured in the serum and spleen of these two groups.

5.10.1. GIT levels of clofazimine

Washed tissue samples from sections of the GIT with mesentery removed were also analysed for clofazimine levels from an animal in the clofazimine control group. As shown in Figure 5.15., the highest drug concentration was found in the ileum, with the lowest concentration in the fundus of the stomach.

5.11. ABSORPTION OF CLOFAZIMINE IN CYCLODEXTRIN COMPLEXES

Table 5.10 shows the egestion and absorption results from this experiment. The clofazimine group gave the lowest percentage absorption of the groups. This value is lower than had been seen with other clofazimine absorption groups. The clofazimine used in the cyclodextrin complexes and in the control was from the same batch, but this batch was different from that used in all the other experiments. The percentage absorption was highest in this study with the hydroxypropyl complex with the β-cyclodextrin giving a similar % absorption value to clofazimine on its own. Toxicity of the complexes was not investigated since there is an extensive literature on the toxicity-free characteristics of orally administered cyclodextrins.

5.12. INTRAVENOUS ADMINISTRATION OF A CLOFAZIMINE-CYCLODEXTRIN COMPLEX

Experiments with the cyclodextrin complexes showed that the β-cyclodextrin complex with clofazimine was not especially water soluble, but the hydroxypropyl complex was very water soluble. In sterile physiological saline, a proportionally large amount of cyclodextrin was dissolved as indicated by an intense red colouration. Not all the added complex would dissolve in water, so in the first experiment an excess of the complex was mixed with saline and the undissolved fraction removed. A 1 ml sample of the injected filtrate contained 267.5 ug of clofazimine so the total dose received on this occasion was 1.337 mg (approximately 5 % of the amount of clofazimine present in the suspension). Analysis of the blood samples over 48 hrs

Figure 5.14.(vii). The tissue levels of B663 in rats following administration of B663, B663-β-cyclodextrin (b-cd), or, B663-hydroxypropyl-β-cyclodextrin (hyd-b-cd) in the diet at a concentration of 0.035 % (w/w) B663 for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The results show that the hydroxypropyl cyclodextrin complex consistently gave the highest tissue levels of B663. The concentration of B663 for each sample is the average of the values measured in the three animals in the group.

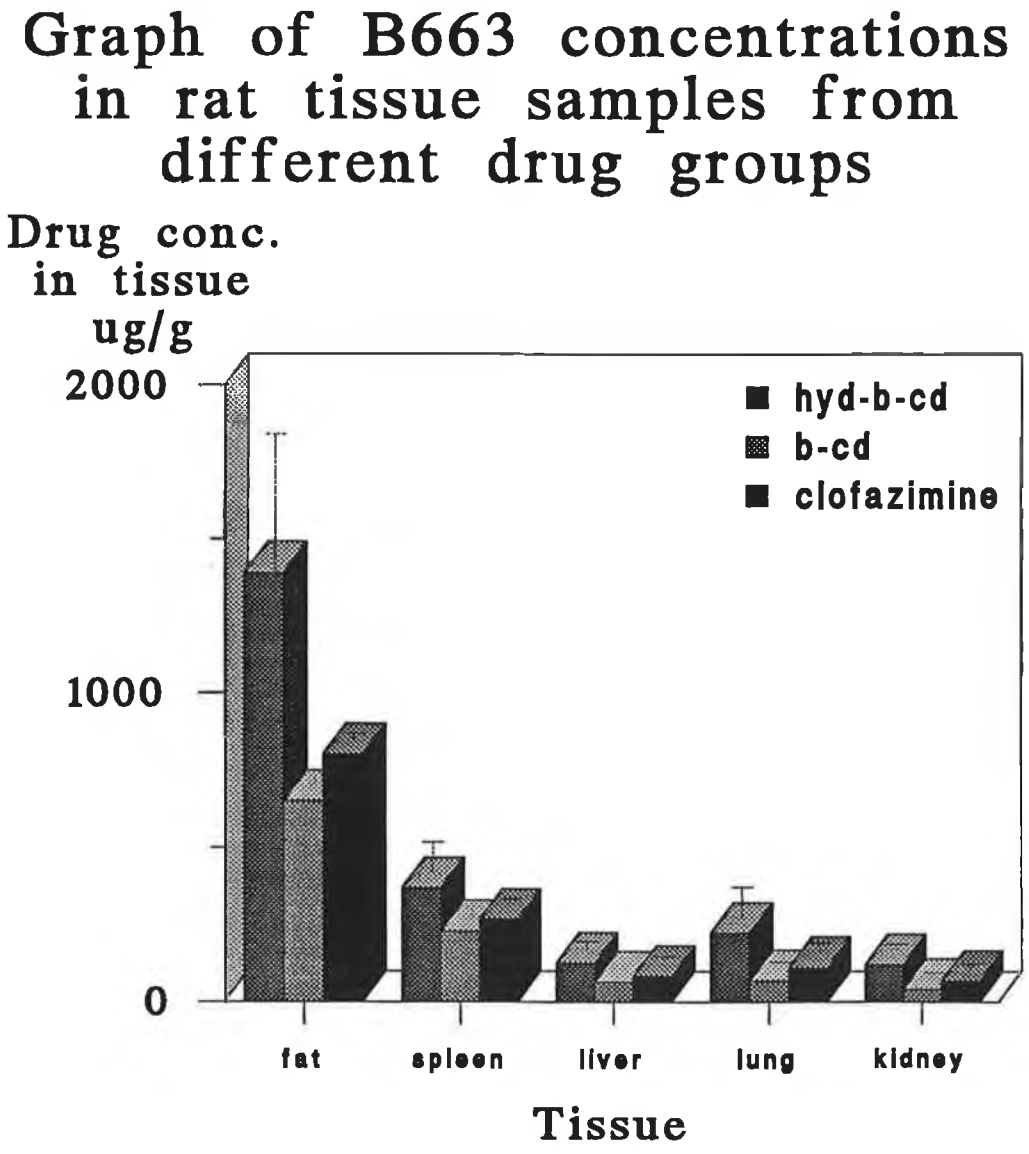


Figure 5.15. The levels of B663 in different regions of the GIT from a rat which had been receiving B663 in the diet at a concentration of 0.035 % (w/w) for 20 days of a 26 day study. The drug concentrations were measured using the HPLC quantification method. The highest concentration is seen in the ileal tissue suggesting that this is the principal region of B663 absorption.

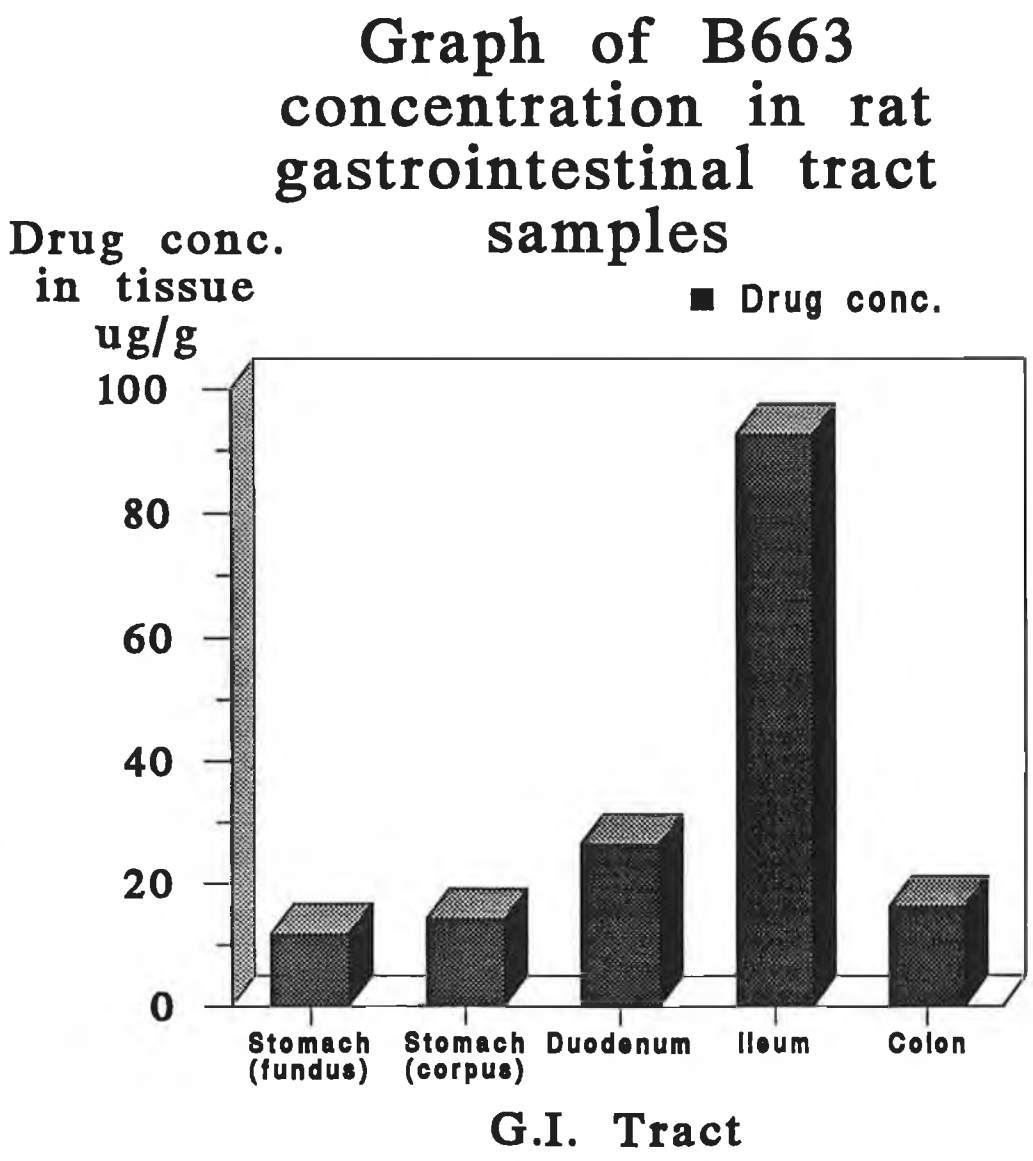


Table 5.10. The egested and absorbed percentage of B663 from B663, B663-β-cyclodextrin (β-CD-B663) or B663-hydroxypropyl-β-cyclodextrin (Hyd-β-CD-B663) dosed food in rats. The animal drug groups were given a single dose of food containing 0.035 % (w/w) phenazine and 0.6 % (w/w) chromium oxide. Chromium was used as a control to monitor transit of the drugged food. The amount of phenazine and chromium egested in the pooled faeces was measured and the amount of phenazine recovered expressed as a percentage of the original dose. The remaining dose was the absorbed percentage.

Drug group	% Egested	% Absorbed
B663	31	69
β-CD-B663	28	72
Hyd-β-CD-B663	19	81

showed that the plasma concentration of clofazimine never went above 50 ng/ml. No untoward effects were noted in the animal. To investigate what had happened to the injected dose of clofazimine, a large dose of the complex was later administered. Table 5.11. shows the resulting levels of clofazimine in the blood and tissue immediately after administration. The results show that some of the drug was found in the blood but most of the administered dose had been deposited around the body particularly in the spleen, kidney and lung.

Table 5.11. The concentration of phenazines in the serum and various tissues of a rabbit following a I.V. dose a saturated solution of 15 ml of B663-hydroxypropyl-β-cyclodextrin. Drug levels were measured using the HPLC procedure described in the methods chapter.

Rabbit tissue	B663 concentration
Fat	3.7 (µg/g)
Spleen	32.7 (µg/g)
Kidney	26.6 (µg/g)
Lung	50.0 (µg/g)
Liver	19.0 (µg/g)
Serum	0.38 (µg/ml)

DISCUSSION

The experiments outlined in this chapter are all of a preliminary nature, investigating the basic properties of the agents investigated. In each case the number of animals used was very small and as a result the data from each experiment cannot be compared by the conventional statistical models. With certain results, for example, the increased tissue drug levels associated with B4090 administration, a very clear difference is evident. However, with observations such as the comparison of tissue levels in the cyclodextrin study, any differences present are clouded by the biological variation present. Therefore, the results are largely qualitative in nature, indicating trends, rather than quantifying the effect. Multiplication of the experimental design would provide a statistical weight to all of the findings of this project.

5.13. PHENAZINE DISTRIBUTION STUDIES IN MICE

As outlined in the introduction of this chapter, the pharmacokinetic and pharmacodynamic properties of a drug are of equal importance for the net therapeutic outcome. Other authors have investigated the phamacodynamic properties of phenazines in *vitro* and *in vivo* models of microbial infection (Grumbach, 1960; Barry, 1969; Barry *et al.*, 1970; Franzblau and O'Sullivan, 1988; Franzblau *et al.*, 1989; O'Sullivan *et al.*, 1988, 1990 and 1992; Van Landingham, *et al.*, 1993; Zeis *et al.*, 1987 and 1990; Savage *et al.*, 1989). However, the most active agent in a test tube may be totally ineffective if it cannot get to its site of action. The major side effects of phenazines, skin discolouration and crystal deposition (Hastings, 1976; Moore, 1983; Jopling, 1976; Desikan *et al.*, 1975; Desikan and Balakrishnan 1976; Mason *et al.*, 1977 ;Levy and Randall, 1970), are principally the result of kinetic rather than dynamic properties of phenazines. The work in this chapter was undertaken to investigate the disposition of phenazines, in the hope of finding a phenazine with the optimal kinetic properties. Mice have been extensively used for this type of study in the past because they appear to distribute phenazines throughout their bodies in a similar way to humans, and they give qualitative data quickly and simply (Barry *et al.*, 1960; Conalty and Jackson, 1962; O'Sullivan *et al.*, 1990). The standard method of dosing these agents has been oral gavage, since this delivers a defined dose in a controlled fashion.

Mice treated in this way with clofazimine demonstrated the typical distribution profile (Barry *et al.*, 1960; Conalty and Jackson, 1962; Stern, 1969; O'Sullivan *et al.*, 1990). High levels were especially evident in the fatty tissue and also in organs with a significant reticuloendothelial component. This profile is also seen in man (Desikan and Balakrishnan,

1976; Mansfield, 1974). The visual observation of high fat levels in a mouse after only four days of treatment may have important kinetic implications. This result implies that when given over a period of time, clofazimine is rapidly sequestered into the body fat. In experiments by Barry *et al.*, (1960) where B663 was given to mice and the tissue levels measured over different periods of time, drug levels were seen to initially rise in tissues with a high reticuloendothelial content. Over several hours the drug levels in these tissues declined but steadily increased in fat tissue. As outlined in the introductory chapter, it is this high fat concentration of clofazimine which is largely responsible for the stigmatising orange colouration seen in most cases of clofazimine therapy. In leprosy, the causative organism, *Mycobacterium leprae* is principally found in the cells of the reticuloendothelial system. Therefore, a new therapeutic phenazine should ideally produce a high concentration in the reticuloendothelial system, while exhibiting low levels in the fatty tissue.

With B749 the tissue profile was different. High levels were seen in the kidney, lung, spleen and especially the liver, with lower levels evident in the fat tissue. This low fat concentration has also been noted in previous experiments (Barry and Conalty, 1965). B749 has a tertiary amine substituent which reduces its fat solubility. Reducing this fat solubility allows higher concentrations to build up in other tissues due to the high affinity for reticuloendothelial cells.

B3954 produced very poor tissue levels in mice. This analogue has a substituted piperidine on the rimino-nitrogen, which reduces fat solubility. Unfortunately, due to experimental design, this type of experiment give no suggestion as to why the tissue levels seen were so low. The most likely possibilities are that the rimino-substituent in this analogue reduces its absorption from the GIT, and/or, causes increased excretion from the body.

B4090 gave very high drug levels in the kidney, spleen and, especially, the liver tissue. The fat levels of this agent were low, particularly, when compared to the levels in other tissues. A similar profile was seen with B4100, although the overall tissue concentrations produced was less. Again, the limitations of this experiment prevented an explanation of the underlying cause of this difference.

With the exception of B3954, levels of phenazine were also found in the brain tissue of these mice. Only Barry *et al.*, (1960) have previously demonstrated the presence of phenazines in brain tissue, although the hydrophobic nature of phenazines should allow transport through the blood-brain barrier. Mansfield, (1974) and Desikan and Balakrishnan, (1976) failed to find

levels of clofazimine in the human brain. However, the level measured in these experiments is below their limit of detection. The presence of clofazimine in the brain may explain the giddiness side effect of therapy which has been described (Browne and Hogerzeil, 1962; Waters, 1969).

5.14. IMPLICATIONS OF PHENAZINE LEVELS IN MICE

These experiments permit the selection of a potentially more active phenazine for further testing. The tissue profile of B3954 indicates that it would have little therapeutic use since it produces very low drug levels. B749 would be potentially useful since it gives similar tissue levels to clofazimine, without the discolouration associated with high fat concentrations or crystallising inside cells (Barry and Conalty, 1965). However, the most interesting compounds are the tetramethyl (TMP) compounds B4100 and especially B4090. The low fat levels of B4090 combined with its less intense colour (see section 3.13.4) indicates that this compound would produce little direct discolouration. However, the secondary darkening often produced by clofazimine therapy (Browne, 1965; Pettit, 1967; Levy and Randall, 1970) might still be evident. These compounds have been shown to be more active *in vitro* and do not crystallise inside cells (O'Sullivan *et al.*, 1992; Van Landingham *et al.*, 1993). This indicates that these compounds could be more active against leprosy with less side effects.

5.15. LIMITATIONS OF MOUSE EXPERIMENTS ADMINISTERING PHENAZINES BY GAVAGE

The underlying reason behind the differences in tissue levels of each agent were unclear, so it was decided to investigate absorption and distribution in more detail. The oral gavage of mice has certain inherent limitations. The drug can only be given as an aqueous solution/suspension. The technique can produce variations from normal drug absorption due to the method of dosage, and deaths due to trauma and direct damage are common. Phenazines are poorly soluble in the gavage solution and absorption is therefore limited by the crystal size of the administered dose. The importance of this insolubility was indicated by the differences in the tissue levels between the mice in this experiment and those of Dr. Jis programme. This effect has been noted by other authors (Noufflard and Berteaux, 1961). Despite receiving a larger dose, the tissue levels were lower due to the lack of detergent in the gavage solution. Attempts to further increase the solubility of the gavage solution, by adding ethanol at a final concentration of 10 % (v/v), successfully dissolved more drug but caused dehydration in the animals, preventing use. Inclusion of a drug in animal food is a simpler, less stressful method

of administration for stable drugs. In the case of clofazimine administration, this system is closer to the human method of dosing, since clofazimine is taken after feeding to improve absorption. The comparison of animals receiving a similar dose of B663 in food versus those receiving B663 by gavage indicates improved drug absorption and this was also noted by Barry *et al.*, (1959 and 1960). The increased absorption of clofazimine taken with food, over clofazimine has been clearly shown for human subjects (Schaad-Lanyi *et al.*, 1987). In these cases, a high fat content in the meal appears to promote absorption. This finding suggests that absorption into the lymphatic system is significant in B633 absorption in the human case. However, Barry *et al.*, (1960) have shown that the fat content of food has little effect on phenazine absorption in mice.

5.16. EXPERIMENTS WITH MALARIA

In a paper by Sheagren, (1968) it was suggested that clofazimine had a significant anti-malarial effect in mouse models of malaria. Since a similar model was available in the school it was decided to investigate if this effect could be seen with B663, B4090 or B4100. The animals were given two weeks of drug treatment to allow the drug levels to build up in their bodies. Unfortunately, no difference was evident in the time before onset of serious morbidity, between the negative control and phenazine treatment groups. In the initial paper, it was found that a dose of 1.8 mg per mouse was needed to suppress disease progression in 50 % of mice. This represents a dose of 6.3 g for an average man which would be expected to cause significant side effects if given for any prolonged period of time. The mice in this programme received approximately 0.4 mg of drug (human equivalent 1.4 g) per day, since it was hoped that a lower dose given before infection might prove as active with one or more of the phenazines. The lack of activity in this experiment indicates that the anti-malarial effect seen is only evident at very high doses which could not be administered in human therapy. The difference in findings may be due to the fact that phenazines drugs are not usually found in erythrocytes where the malarial parasite lives. As the administered dose increases, perhaps the concentration of clofazimine reaches a therapeutic level inside the infected erythrocytes.

5.17. RAT EXPERIMENTS

It was decided to use rats as an experimental model for a number of reasons. Physiologically and anatomically they are very similar to mice, but their larger size allows larger samples, especially of blood, to be taken without having a detrimental effect on the animal. Rats are also a standard species in initial metabolic and toxicological screens, since a vast amount of

physiological data on them has been produced over many years (Briggs and Oehme, 1980). A special metabolism cage was constructed to give more information on feeding and absorption over a prolonged period of time. Conventional metabolism cages are designed for using single animals for short periods of time, usually a maximum of a few days (Kraus, 1980; Knight *et al.*, 1984). The apparatus outlined in section 2.6.1. permitted convenient measurements of food and drug intake, allowing estimation of absorption and feeding patterns over the longer period of time needed for sub-chronic investigations. Other investigators have used tissue levels to compare the absorption of phenazines (Byrne *et al.*, 1989). However, as the rat absorption results have shown tissue levels do not correlate with absorption values.

5.18. DISTRIBUTION OF PHENAZINES IN RATS

Comparison of the individual phenazine levels in rats and mice indicated a similar tissue distribution profile in both species. The actual drug concentration was generally higher in the rats probably due to superior absorbance associated with drug incorporation into food. B4090 gave the highest tissue level of any of the agents tested. The profile of tissue levels was similar for the three TMP derivatives tested, B4090, B4100 and B4103, with high levels in the spleen liver and lung and very low fat drug concentrations. This provides further evidence of the likely therapeutic improvement which would be seen with B4090 treatment instead of clofazimine. Within this group the presence of a double chlorination of the anilino and phenyl groups or a trifluoro methyl substitution seemed to have little effect on the drug level in the tissues measured. The chlorination of the seven position of the phenazine nucleus seems to increase tissue levels. Unfortunately, these high tissue levels are associated with low serum drug levels. The tissue levels of B4154 were inferior to the TMP derivatives, and the more lipophilic nature of this compound is indicated by proportionally high fats drug levels. However, this compound also gave the highest cerebral drug concentration.

5.19. SERUM LEVELS OF PHENAZINES

The phenazines were initially developed as a treatment for tuberculosis. As outlined in section 1.2. of the introduction, many phenazines, especially clofazimine, were very active *in vitro* and in some *in vivo* models of T.B. In higher animals and man clofazimine proved to be ineffective against common forms of the disease. Clofazimine is active against a number of infections caused by mycobacteria, and its lack of efficacy in the treatment of T.B. implies that it is not reaching sufficient concentration in the environment of T.B. It has been suggested that, unlike the bacteria inhibited by clofazimine, T.B. can exist outside the intracellular environment

(O'Sullivan *personal communication*). An ancillary aspiration of this project was to find a suitable method of increasing the serum levels and serum activity of these compounds to produce an effect against T.B. Barry *et al.*, (1959) have also shown an approximate correlation between both the plasma and overall body concentration and the mean survival time of T.B. infected mice. None of the compounds tested reached serum concentrations as high as clofazimine. The low blood levels in B4090 and especially B4100 are possibly the result of a greater affinity for tissue components. However, Barry *et al.*,(1959, 1960 and 1965) noted that less hydrophobic phenazines produce lower plasma concentrations, even in portal plasma from the intestine. These authors also noted that clofazimine produced very high levels in lymph, especially when given in a lipid vehicle, while a less hydrophobic, more polar, compound, B720, produced very low lymph levels. This indicates that although high tissue affinity may explain some of the variance in kinetic distribution between phenazines, the whole picture is still unclear.

Conalty and Jina, (1971) demonstrated that β-lipoprotein, now known as low density lipoprotein (LDL), was the principal carrier of clofazimine in the blood. Transport in rat blood was investigated using ultrafiltration membranes with a 3 KDa molecular weight cut-off. Any free phenazine would pass through this membrane since they have a molecular weight below the cut-off value. With B663 and the TMP derivatives B4090 and B4100, drug could only be measured in the high molecular weight retentate of the membrane. This indicates that phenazines do not exist in a free form in the blood. This finding is also supported by the observation that addition of serum reduces the activity of phenazine compounds under *in vitro* conditions (Barry *et al.*, 1948), indicating that a serum component sequesters free phenazine. Clofazimine is transported by LDL and this would therefore appear to be the most likely carrier of phenazines in general. However, other lipoproteins are also present in the blood. The affinity of the various lipoproteins varies for specific cell types, and variations in the type carrying a specific phenazine could explain some of the differences seen in drug levels associated with particular phenazine agents.

5.20. ABSORPTION OF PHENAZINES

It was an unexpected finding that high tissue levels were not the result of better absorption. The highest tissue levels were seen with B4090 where only approximately half of an administered dose was absorbed. With the other compounds which produced lower tissue concentrations of drug, absorption was higher, up to 92 % in the case of B4103. Following the faecal concentration of phenazine for 105 hours with a non-absorbed control of chromium, indicated that there was very little faecal elimination of these compounds after the absorption

of a single dose. Although the egestion was shorter in the B4103-B4154 experiment, both sets of transit times are within reported values (Thompson and Hollis, 1958; Varga, 1976; Hildebrandt and Marlett, 1990). The difference in this one experiment is possibly due to some form of environmental upset. Possible explanations for the differences in overall tissue levels seen include an increased excretion as the concentration of phenazine increases in the body or changes in the absorption pattern over the course of the experiment. The strong colouration of the intestine evident with these drugs might also suggest that this tissue could act as an absorption depot for absorbed drug especially in the case of agents with low affinity for fatty tissue. Lower tissue drug levels may be the result of storage in the intestine, although this possibility was not investigated. In addition, a lower affinity for fatty tissue would also increase the pool of phenazine available to all the other tissues relative to that associated with clofazimine.

5.21. SITE OF CLOFAZIMINE ABSORPTION

Measurement of the drug levels in different regions of the GIT of a clofazimine-treated rat showed the highest concentration in the jejunum of the small intestine. This section of the GIT is the principal region of drug absorption, particularly of hydrophobic compounds (Bivin *et al.*, 1979), and the high concentration of clofazimine found here suggests that phenazines are mainly taken up in this area. Desikan and Balakrishnan, (1976) have noted very high clofazimine levels in small intestinal tissue of human patients which suggests a similar possibility in man.. Mansfield *et al.*, (1974) and Yawalker and Vischer, (1979) have shown high levels of clofazimine in human bile. Bile is released into the duodenum, proximal to the jejunum, and is a major contributor to the absorption of lipophilic substances. The high biliary concentration suggests that clofazimine may engage in entero-hepatic circulation, being absorbed by the jejunum and any fraction excreted in the bile, reabsorbed as it passes through the jejunum. This possibility could not be easily verified in rats, since they do not possess a gall bladder.

5.22. TOXICITY STUDIES

The general lack of toxic side effects associated with clofazimine therapy is well documented (Vischer, 1969; Stenger *et al.*, 1970; Hastings, 1976; Bulakh, 1983; Peters *et al.*, 1983). Any potentially new replacement would have to be as non-toxic, as well as more active, to compete. It is, therefore, of little use to develop a new phenazine for treatment without having some evidence of its innocuousness. In the experiments described, a dose well in excess of that normally administered to a patient was used. The typical dose of clofazimine is 100 mg

for an adult of average weight, 70 kg. The dose of 20 mg/kg given to these animals therefore extrapolates to a human dose of 1400 mg. Such an excess would be expected to magnify the symptoms of any inherent toxicity especially if given over longer period of time. Toxicity would be unexpected in phenazines since clofazimine is so non-toxic. However, the first phenazine tested in humans, B283, had relatively serious toxic side effects (Lane, 1951; Allday and Barnes, 1952). Besides the exceptions noted in the subsequent sections, all parameters remained unchanged and within the reported values (Kozma *et al.*, 1972; Ringler and Dabich, 1979; Loeb and Quimbey, 1989).

5.22.1 Blood indicators of toxicity

As outlined in section 5.5.2. of this chapter, the kidneys, liver and blood system are the most common organs to display toxicity. In the rat, circulating levels of SDH and ALT are sensitive and specific indicators of hepatotoxicity (Balazs *et al.*, 1961; Ringler and Dabich, 1979; Boyd, 1983). If any of the agents tested were hepatotoxic, a large increase in the measured activity of these enzymes in the blood would be expected (Korsrud *et al.*, 1972). This was not seen, indicating no hepatotoxicity. High levels of AST are indicative of soft tissue, including liver, damage, in this species (Boyd, 1983; Meeks, 1989; Hoffman *et al.*, 1989). The levels of this enzyme were found to have increased in all the animals at the end of the experiment. Since there was no difference in the magnitude of the increase seen between the food and B663 control, an age or food related factor is the most likely cause for this elevation (Ringler and Dabich, 1979).

The only change noted between the start and end of experiment in the blood cell analysis was in the number of neutrophils in the B4090 and B4100 groups. Agents which are selectively toxic to the immune system generally reduce the number of these specific cells in the blood. Neutrophils are usually increased in inflammatory and infectious states and some form of infection in the two groups is a possible explanation of the results seen. Alternatively, these TMP derivatives may in some way selectively stimulate the production of neutrophils. This would be advantageous in combatting many microbial diseases.

5.22.2 Behavioural indicators of toxicity

The lack of an effect on the behavioural patterns of the animals in the group suggests that the animals do not feel ill when taking any of these agents. Measurement of the cerebral concentrations of these drugs indicates that they can cross the blood brain barrier. However, the

absence of behavioural changes indicates that there is little or no interaction with the psychomotor centres in the brain.

5.22.3. Urinary indicators of toxicity

Variations in the constitution of urine are a sensitive indicator of kidney function and the general state of health of the body. In this project the urine was monitored by two techniques. The test strips provide data of a more qualitative nature on possible toxicity. The combination of the strips used allows a basic screen for the detection of diabetes, metabolic abnormalities, liver diseases, biliary and hepatic obstruction, haemolytic diseases and bacterial infections of the urinary tract (Ragan, 1989). Analysis by this method is imprecise, but is very effective as a rapid indicator for guiding more specific and precise tests. The absence of any change in any of the groups reinforces the other findings of no overt toxicity of these compounds.

5.22.4. Urinary analysis by NMR

NMR analysis of urine is a relatively new technique in the arsenal available to study toxicity. Again no evidence of toxicity was found in any of the groups tested, but the positive control of a carbon tetrachloride-treated animal demonstrates the type of results which could have been evident. The system as described here give mainly qualitative results as to the presence or absence a toxic effect. For example the absence of hippurate in the urine of the positive control urine is indicative of damage to the specific hippurate transport system found in the cells of the proximal tubule of the kidney (Bowman and Rand, 1980). More sophisticated analysis allows qualitative and quantitative analysis by characterising the resultant urinary fingerprint, as well as measuring the net increase or decrease in specific peaks (Gartland *et al.*, 1990). The result seen with the rabbit urine demonstrate that this technique is equally useful in the testing and diagnosis of pathological conditions especially as an adduct to other methods (Nicholson and Wilson, 1991; Anthony *et al.*, 1992; Lundina *et al.*, 1993; Bock, 1994).

5.22.5. Other toxicity tests

Before going on to human trials a larger scale of investigation involving other specific toxicity tests would be necessary. The animal results outlined in this chapter only provide a general indication of the likely level of toxicity which might be expected. The numbers of animals used are too small to give statistical weight or allow a broad generalisation of the

innocuousness of these compounds. Other forms of toxicity which would have to be investigated include mutagenicity, carcinogenicity, teratogenicity and cardiac toxicity.

5.23. CYCLODEXTRIN STUDIES

5.23.1. Effects of cyclodextrins on clofazimine absorption

Cyclodextrin complexes of clofazimine were prepared to study possible methods of modifying the absorption and distribution of clofazimine. Many authors have shown that cyclodextrin-drug complexes can have a significant effect on the pharmacokinetics of certain drugs, especially poorly soluble hydrophobic agents (Levai *et al.*, 1988; Jansen and Hilbers, 1988). In this study, only the hydroxypropyl-β-cyclodextrin-clofazimine complex appeared to increase absorption as compared to an equivalent dose of clofazimine. Unfortunately, these results are equivocal since although this cyclodextrin improved absorption relative to clofazimine from the same batch, the absorption value is very similar to that seen with B663 in previous experiments. The most likely factor to cause the reduced absorption seen with the clofazimine group in this experiment was differences in the size or form of the clofazimine crystals from this batch. If this was the case, then increased absorption seen with hydroxypropyl-cyclodextrin complexation represents a meaningful augmentation of clofazimine absorption.

5.23.2. Effect of hydroxypropyl-β-cyclodextrin on clofazimine distribution

The hydroxypropy-β-cyclodextrin complex also gave the highest tissue and serum concentrations of clofazimine. The animals in this group had a slightly greater food intake per unit weight than the other animals in the programme. This resulted in an increased drug intake of approximately 10 % compared with the other groups. While this may have had the effect of increasing the tissue concentration of clofazimine, this 10 % difference does not account for the magnitude of the difference in drug concentrations seen with the hydroxypropyl-cyclodextrin complex. The increased food intake in this group may reflect the masking of some undesirable taste associated with the clofazimine administration, or may be the result of a perceived improvement in the food taste for the animals. Masking of bad odours from compounds is a common application of cyclodextrin complexation (Szejtli, 1988). These results are consistent with the decreased food intake associated with B4090 and B4100 administration in food being caused by a drug associated malodour.

5.23.3. Effect of β-cyclodextrin on clofazimine distribution

The absorption and tissue levels produced by the β-cyclodextrin-clofazimine complex indicate that this form of dosage has no effect on the pharmacokinetics of clofazimine. With the hydroxypropyl complex the incorporation of clofazimine was easy to verify because one could see the resultant colouration of an aqueous solution. With the β-cyclodextrin complex this was not evident. The powdered complex appeared to be less soluble than β-cyclodextrin on its own, suggesting that clofazimine had stuck to the outside of the molecule, rather than forming an inclusion complex. Alternatively, the dissociation constant of the complex may be so low as to prevent the extensive release of clofazimine in the small intestine where it can be absorbed. When broken down in the large intestine and colon, the released clofazimine would not be readily absorbed since no specific transport mechanisms for hydrophobic agents exist in this area of the GIT.

5.23.4. Mechanism of hydroxypopyl-cyclodextrin increase in clofazimine absorption and distribution

Cyclodextrins are not absorbed from the GIT. The hydroxypropyl-cyclodextrin probably increases drug absorption by increasing the aqueous solubility of clofazimine. The complex with clofazimine would release single molecules of clofazimine when other parts of the intestinal environment have a higher affinity for either the drug or cyclodextrin. These single molecules would more readily form the micells with fats and bile compounds necessary for clofazimine absorption. The rapid solubilisation of clofazimine from the complex instead of slower crystal dissolution is probably responsible for the higher serum concentrations of clofazimine seen. Analysis of the solution prepared for rabbit injection suggests that only approximately 5 % of the hydroxypropylcyclodextrin-clofazimine powder was actually a water soluble complex. Purified complex from the mixture could therefore be expected to further increase both absorption and serum levels of clofazimine. Approximately 70 - 80 % of an administered clofazimine is absorbed in humans. Although hydroxypropyl-cyclodextrin could increase the absorbance of clofazimine, it is unlikely that the added cost of production would offset the small likely increase in efficacy. Complexation with poorly absorbed phenazines such as B4090 would be expected to have a significant effect, reducing the dosage necessary to produce the standard effect.

5.23.5. Distribution of an I.V. Dose of hydroxypropyl-cyclodextrin-clofazimine complex

Since the hydroxypropyl complex was so water soluble, we attempted to use it to produce high blood levels of clofazimine. This would be expected to be beneficial in the treatment of acute mycobacterial infections since conventional therapy takes several weeks to build up sufficient therapeutic clofazimine levels. The spectrum of clofazimine might also be extended to other diseases such as tuberculosis as outlined in section 5.19. Measurement of the serum levels resulting from the *intra venous* injection of the complex were therefore disappointing. Although a large dose of clofazimine was injected into the bloodstream, very little remained there. To investigate what was happening to the injected dose the experiment was repeated several weeks later with a larger dose of the complex. The pattern of drug deposition is consistent with precipitation of clofazimine from the complex particularly in the capillary beds of the body which have extensive blood flow. No untoward effect was noticed in the animal after dosing, but such precipitation could cause clot formation in a human body with potentially fatal consequences.

The rapid precipitation of clofazimine from the complex suggests that the dissociation of clofazimine from this cyclodextrin is too rapid in the blood. Many other cyclodextrins exist (Brewster *et al.*, 1989) and if a slower dissociating, aqueously soluble, complex could be produced it should be possible to produce high plasma levels of clofazimine which could reach the site of infection more rapidly and in a higher concentration. The therapeutic potential of such a formulation would be much better than currently exists.

5.24. OTHER FORMULATIONS OF CLOFAZIMINE

Other methods of increasing the dissolution of clofazimine have been investigated. Krishnan and Abrahams, (1991) have shown an increased dissolution of clofazimine in acidic, neutral and alkaline media using a maleic anhydride copolymer. In later experiments this formulation was given by gavage to pigs and successfully increased the blood levels of clofazimine (Krishnan and Abrahams, 1994). Kailasam *et al.*, (1994) , produced an implantable form of clofazimine in a polylactic co-glycolic acid polymer. Although, this polymer produced measurable levels of clofazimine over time, the level produced was significantly lower than seen with oral administration. Liposomal preparation of clofazimine have been made by Mehta *et al.*, (1993) and Sritharan, (1993). Sritharan showed that different liposomes could target clofazimine to different tissues. In the paper by Mehta *et al.*, (1993) incorporation, generally, did not lead to increased activity against a variety of mycobacteria. However, the potent activity of

clofazimine against mycobacteria, combined with the possibility of parenteral administration of these complexes, suggests that this formulation could extend the therapeutic usefulness of clofazimine. Therefore, several drug formulation technologies exist which could have a very significant effect on the therapeutic spectrum and efficacy of clofazimine. A combination of these methods with some of the phenazine analogues studied in this project may ultimately prove to be supremely effective.

5.25. SUMMARY

The basic pharmacokinetics of several different rimino-phenazine drugs were investigated. Tetramethylpiperidine derivative gave the highest overall tissue levels, whilst giving the lowest fat concentrations, despite a lower percentage of absorption compared to clofazimine. Administration of these agents also gave no indications of toxicity in rats. When combined with data from *in vivo* and *in vitro* investigations of antimicrobial activity, these results suggest that these derivatives might be more active with less side effects. Therefore, these compounds warrant further evaluation, initially in greater numbers in more sophisticated and varied animal models, and, perhaps, ultimately in man.

The cyclodextrin studies suggest that complexes with water-soluble cyclodextrin derivatives might increase the oral absorption of clofazimine, and, by extrapolation, other phenazines. It may also be possible to produce cyclodextrin complexes with phenazines which allow parenteral administration for acute anti-bacterial treatment. These complexes also warrant further development and investigation.

CHAPTER 6

OVERALL CONCLUSIONS

CONCLUSION

The work presented in this thesis has focused on three areas of rimino-phenazine research.

Chapter 3 described development and verification of a new extraction and quantification method for rimino-phenazines. A limited investigation of the purity of these agents was undertaken using a variety of chemical techniques and a procedure was developed for purifying these drugs. The combination of the new extraction and HPLC methods is a significant improvement on existing procedures allowing the accurate and precise measurement of all of the rimino-phenazine compounds tested. Therefore, this system would be suitable for a large scale evaluation of the pharmacology of any of these agents, including clofazimine.

Chapter 4 outlined the synthesis of phenazine-protein conjugates, the production of anti-phenazine antisera, using two of these conjugates, and the subsequent use and characterisation of the resultant antibody. Phenazine-protein conjugates were produced and characterised using the amino-acid substituted phenazines B3955 and B3976. These agents were linked to the proteins bovine serum albumin, thyroglobulin and keyhole limpet haemocyanin using glutaraldehyde and EDC/NHS as conjugating agents. Antisera was produced in two rabbits. This antisera could detect water-soluble phenazines in a variety of ELISA-based formats but could not detect the more hydrophobic phenazines. The antisera was also purified and characterised using a phenazine affinity column. The lack of sensitivity of the antibody to the hydrophobic phenazines limits its use and application, since all of the pharmacologically relevant rimino-phenazines are hydrophobic. The methods described are also applicable to the production of antibodies to other hydrophobic agents.

In chapter 5, the basic pharmacokinetics of some selected rimino-phenazines was investigated using the methods described in chapter 3. Tetramethylpiperidine-substituted phenazines gave the highest tissue levels and also gave proportionately lower drug levels in body fat. Investigations of several indicators of toxicity, including the newly developed method of urinary NMR analysis, did not indicate any evidence of toxicity associated with administration of these compounds in animals. A complex of clofazimine with hydroxypropyl-β-cyclodextrin produced a more water soluble formulation which increased the absorption of clofazimine. When given *intra venously*, this complex rapidly precipitated its clofazimine content. Although this clofazimine complex was not successful as an I.V. formulation of clofazimine, other cyclodextrin formulations might allow clofazimine administration by this route. Combined with

the increased anti-bacterial activity of these compounds *in vitro*, the selective effects on drug tissue levels outlined suggest that the tetramethylpiperidine-substituted compounds might be more active in the treatment of mycobacterial infections with less side effects. In the case of leprosy, many of the current drug treatments have associated therapeutic limitations. These include, serious associated toxic effects over the time course of the therapy, development of resistance by *M. leprae* to the drug, and, inherent difficulties in monitoring their usage in a third world situation. Therefore, further testing, ultimately leading to human trials, appears warranted, especially in the case of leprosy treatment.

CHAPTER 7

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PRESENTATIONS AND PUBLICATIONS

Seminars given

Beckman HPLC Systems User's group, D.C.U., 12th March 1993.

Biochemical Society Predoctoral meeting, U.C.C., Cork, 22nd September 1994.

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Papers

O,Connor, R., O'Sullivan, J.F. and O'Kennedy, R. (1995)
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A preliminary investigation of selected anti-mycobacterial phenazines.

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Posters presented

Federation of European Biochemical Societies, Trinity College Dublin, 13th August 1992.

XIV International Leprosy Congress, Orlando, Florida, 2nd September 1993 (2 posters).

The Biochemical Society, U.C.C, Cork, 23rd September 1994.

The Irish Society of Toxicology, Beaumont Hospital, 14th October 1994.