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Studies into factors affecting the antifilarial activity of albendazole

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STUDIES INTO FACTORS AFFECTING THE ANTIFILARIAL ACTIVITY OF ALBENDAZOLE

Submitted by Nornisah Mohamed, B.Sc. (Hon),

for the degree of Ph.D.

of the University of Bath

1993

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SUMMARY

Albendazole is a broad spectrum anthelmintic and is widely used in the treatment of helminth infections. Investigations have been undertaken to explore the possible use of this drug in the treatment of filariasis, although due to its poor aqueous solubility, albendazole is poorly absorbed in the gastrointestinal tract following oral administration.

In this project the possible role of using a triglyceride oil formulation to overcome the low bioavailability of albendazole associated with oral administration was investigated. These studies were undertaken in three related areas:

In analytical studies, sensitive, selective and reproducible HPLC methods were developed for the simultaneous determination of albendazole and its two major metabolites in bio-fluids and oil in order to provide suitable assay methods in the biological and formulation studies.

In formulation studies, the stability and solubility of albendazole were studied in the potential formulation vehicles. Degradation of albendazole *in vitro* via a simple thermal mechanism followed the same pathways as its metabolic pathways, namely oxidation and hydrolysis. Albendazole was formulated in a mixture of Miglyol:Tween 80 (60:40%, w/w) and the bioavailablity of this formulation was evaluated in animals.

Biological studies primarily involve *in vivo* studies in rats and some work was carried out in monkeys and humans. The levels of albendazole and its metabolites were highly variable in all species studied. There was no significant difference in bioavailability of albendazole when administered as "oily" formulation compared to PEG 400 formulation in rat and monkey. However enhanced absorption was observed in animals when compared to that of a healthy volunteer who received albendazole in tablet form in a comparable dose. The C_{max} and AUC were 6-7 and 10-14 times higher in animals.

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Finally my gratitude to my husband, my parents and family for their patience, understanding and encouragements.

ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

ABZ..... albendazole

ABZSO... albendazole sulphoxide

$ABZSO_2$.. albendazole sulphone

ACN..... acetonitrile

AUC..... area under concentration-time curve

^oC..... degrees Celsius

C_{max}.... maximum concentration

Concn... concentration

DEC..... diethylcarbamazine

DMSO.... dimethyl sulphoxide

DSC..... differential scanning calorimetry

EtOH.... ethanol

Fig..... figures

g..... grammes or gravity

HCl..... hydrochloric acid

HPLC.... high performance liquid chromatography

hr..... hours

kg..... kilogrammes

MBZ..... mebendazole

MeOH.... methanol

mg..... milligrammes

ml..... millilitres

min..... minutes

Mig:Twn. Miglyol:Tween 80 (60:40%, w/w)

MS..... mass spectrometry

µg..... microgrammes

µl..... microlitres

ng..... nanogrammes

NMR..... nuclear magnetic resonance

PEG..... polyethylene glycol

r..... correlation coefficient

R_f..... refractive index

R_T..... retention time

RSD..... relative standard deviation

SD..... standard deviation

SDS..... sodium dodecyl sulphate

SPE..... solid phase extraction

$t_{1/2}$.... half-life

T_{max}.... time to reach maximum concentration

v/v..... volume to volume

w/w..... weight to weight

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 FILARIASIS

"Filariasis" is comprised of several tropical diseases caused by species of nematode worms. The most important filarial diseases are associated with *Wuchereria bancrofti, Brugia malayi, Brugia timuri, Onchocerca volvulus* and *Loa loa,* and are transmitted by bloodsucking insects. The adult worm develops at specific tissue sites and the female discharges millions of microfilariae which appear either in circulating blood or migrating freely in the subcutaneous tissues or dermis.

W. bancrofti, O. volvulus and possibly *L. loa* are the species for which man is the only definitely established host. However, strains of *B. malayi* can also be transmitted to primates and carnivores, which act as reservoirs for the infection (Mak, 1987). Fig. 1.1 shows the global distribution of "filariasis" associated with these parasites.

1.1.1 Onchocerciasis

Onchocerciasis or "river blindness" is caused by *O. volvulus,* the only species that develops to maturity and produces microfilariae in the skin of man. This filarial infection is transmitted by blackflies of the genus *Simulium* which breed in fast-flowing rivers.

Onchocerciasis is probably the most serious of the filarial diseases. Of the estimated 85.5 million people who are at risk of this disease, about 17.5 million are infected with *O. volvulus,* the majority of them living in West and Central Africa and the remainder in Yemen and in Central and South America (WHO, 1987). Approximately 340,000 people are blind and 1 million people suffer from severe visual

impairment as a result of onchocerciasis worldwide.

The adult worms develop in the subcutaneous and even connective tissues in various parts of the body where they become encapsulated in fibrous nodules. The female worms can live for up to 15 years and can release thousands of the microfilariae per day. The migrating microfilariae can induce severe damage in the skin, subcutaneous tissues and especially the eyes. The microfilariae were also found in various body fluids, e.g. blood, urine, tears, cerebrospinal fluid and sputum (WHO, 1976).

1.1.2 Lymphatic filariasis

Human lymphatic filariasis is caused mainly by *W. bancrofti*, *B. malayi* and *B. timuri* present in the lymphatic system. *W. bancrofti* is the most widespread, affecting tropical and subtropical Africa, the Far-East and North-Eastern South America; *B. malayi* occurs in Southern and Eastern Asia and *B. Timuri* is confined to a small focus in Indonesia (WHO, 1984). It is transmitted by various species of mosquitoes. *W. bancrofti*, the predominant infection, is transmitted by mosquitoes mainly of the genera *Culex*, *Anopheles* and *Aedes; B. malayi* by *Anopheles* spp. and *Mansonia* spp., and *B. timuri* by *Anopheles barbirostris* (Mak, 1987).

The World Health Organization (1992) estimated that 751 million people are living in endemic areas, and 78.6 million are infected of which more than 92% (72.8 million) have *W. bancrofti* and less than 8% (5.8 million) have *B. malayi* and *B. timuri*.

The clinical manifestations of the infection are variable, differing from one endemic area to another; the spectrum of manifestation is, ranging from asymptomatic to chronic lymphangitis and elephantiasis. The adult worms in the lymphatic system produce granulomatous tissue reactions which are responsible for obstruction, thus interfering with drainage of lymph from the tissues. This effect may be seen in the extremities, the breast, the scrotum or the labia. The released microfilariae enter the blood stream and circulate to various parts of the body (WHO, 1984).

1.1.3 Loiasis

Loa loa is responsible for chronic infections in up to 13 million people living in the rainforest areas of West and Central Africa (Sasa, 1976). This disease is transmitted by tabanid flies of the genus *Chrysops*. The adult worms live in the connective tissues just beneath the skin, migrate to the subcutaneous tissues and occasionally across the conjuctiva causing ocular damage but not affecting vision. The movement of the worm in the subcutaneous tissues forms large swellings known as "Calabar swellings" which cause pain and severe pruritus (Argyll-Robertson, 1895).

Fig. 1.1 Global distribution of filarial diseases.

(from James and Gilles, 1985a)

1.2 LIFE CYCLE OF FILARIAL PARASITES

The life cycle of the parasites is very similar in all species (Fig. 1.2). Upon biting an infected person, the insect vectors, which also serve as the intermediate host, may ingest microfilariae (first stage larvae, L_1) with their blood meal. The microfilariae enter the body cavity of the insects and migrate to the thoracic muscle where larval development occurs (L_1 and L_3). The infective third stage larvae (L_3) escape and enter the blood stream of the host during the next blood meal. The larvae develop into adult worms at selected tissues in the infected man. The male and female worms mate and the female releases microfilariae (L_1) into the surrounding tissues. These microfilariae rapidly become distributed in the blood or skin. The density of microfilariae in blood fluctuates over 24 hours; in lymphatic filariasis the density is greatest at night whereas in loiasis the greatest density is reported during the day (Hawking et al., 1966; James and Gilles, 1985b).

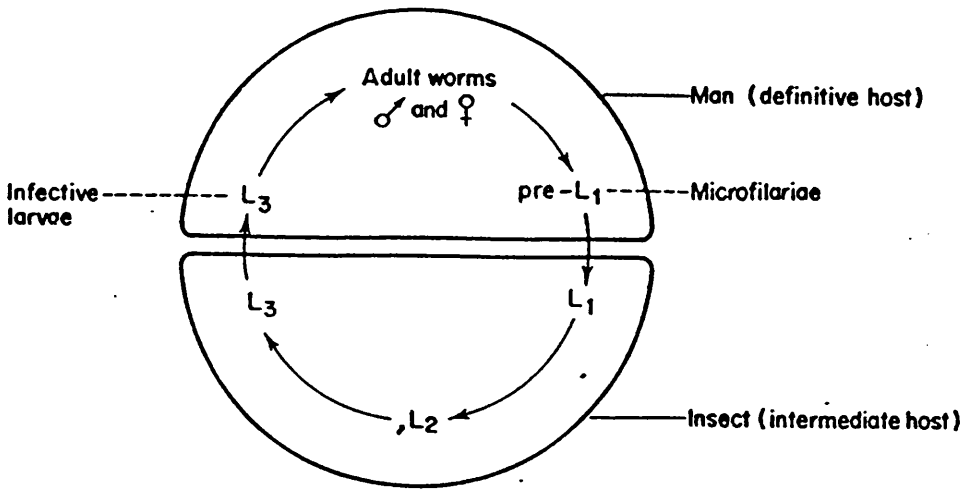


Fig. 1.2 Life cycle of the filarial parasite

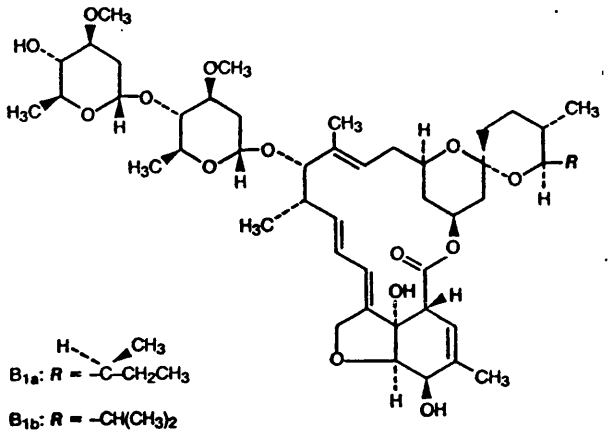
(from James and Gilles, 1985b)

1.3 CHEMOTHERAPY OF FILARIASIS

Onchocerciasis, lymphatic filariasis and loiasis are major public health problems in many countries in tropical and subtropical regions of the world. At present there is no safe and effective chemotherapeutic agent to treat these filarial infections.

Diethylcarbamazine (DEC) has been used relatively safely against lymphatic filarasis and is effective against microfilariae. However it does have side-effects. It may be macrofilaricidal when large doses are administered over a long period of time. For onchocerciasis, DEC also kills microfilariae effectively but usually causes intense reactions. Suramin has been in use for about 40 years against the adult worms in onchocerciasis, but it causes severe, occasionally fatal, toxic effects (WHO, 1984).

The World Health Organization (WHO), through the Steering Committee of the Filarisis Scientific Working Group of the Tropical Disease Research Programme (TDR) has promoted a global collaborative effort in the search for a new drug that is safe, effective and that can be used, preferably in a single dose, for all filarial infections. TDR and the Onchocerciasis Control Programme (OCP) in West Africa have been working together in attempts to improve the use of existing antifilarial drugs in Africa (WHO, 1976). Drugs currently available for filariasis include DEC, suramin, ivermectin and benzimidazole carbamates (Fig. 1.3).



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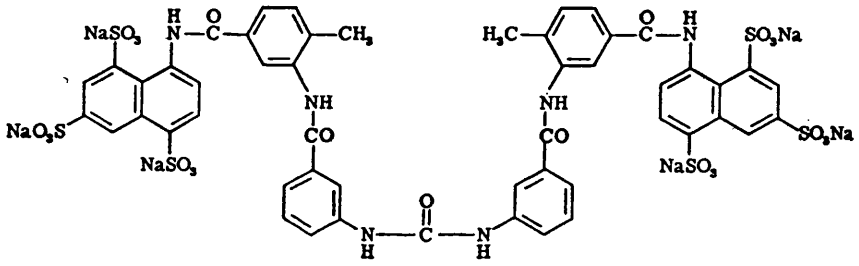

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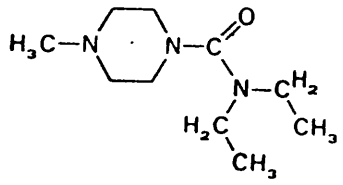

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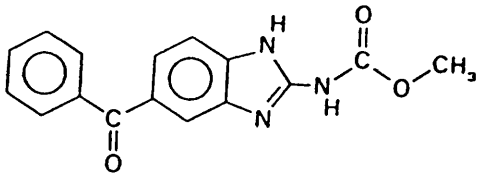
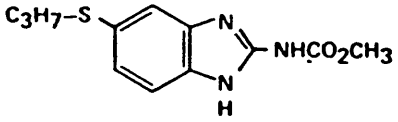


mebendazole



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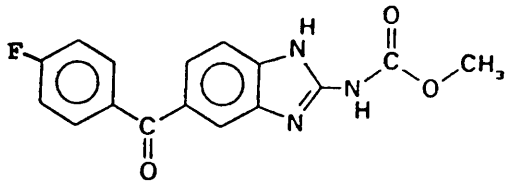


flubendazole

Fig 1.3 Drugs for the treatment of filariasis.

1.3.1 Diethylcarbamazine (DEC)

DEC is a derivative of piperazine (Fig. 1.3), active against microfilariae and adult worms of a variety of filarial species.

Several groups have reported that DEC is effective against microfilariae of *O. volvulus*. The skin microfilariae counts are reduced (>90%) within the first week, but DEC is unable to sustain the low levels of microfilaria in the skin, as the level gradually increases by the end of 6-12 months, even at high doses of 30 mg/kg/day. (Dominguez-Vazquez *et al.*, 1983; Greene *et al.*, 1985; Albeiz *et al.*, 1988). In patients suffering from onchocerciasis the use of DEC is usually accompanied by an intense reaction known as the Mazzotti reaction which is believed to be attributable by the death of the microfilariae. The severity of this reaction depends on the number of microfilaria killed, which in turn depends on the intensity of the infection and the dose of DEC (Francis *et al.*, 1985). Awadzi and Gilles (1992) have recently described a recommended dosage regimen for DEC which aims to improve the use of the drug.

DEC has also been shown to be active against both adults and microfilariae of *L. loa* (Hawking, 1979). It has been demonstrated that prophylaxis against *L. loa* can be achieved by continuous administration of DEC at 5 mg/kg/day (Sasa, 1976). Recently, Nutman *et al.* (1988) has shown that a weekly dose of 300 mg DEC provided prophylaxis against *L. loa* infection with no serious side-effects, despite administration of DEC for 2 years.

In lymphatic filariasis, DEC is an effective macro- and microfilaricidal agents (Goodwin, 1984), and it has been the drug of choice for over 35 years (WHO, 1984). In the treatment of this

infection, a standard 12-day course of DEC rapidly cleared microfilariae in the blood within the first week, but the levels of microfilaria gradually rose to 6.0% (average) of the pretreatment values 6 months after treatment (Ottesen *et el.*, 1990). The recurrence of microfilaremia is common for this treatment. In another study, a modified DEC dose regime of a single monthly dose of 6 mg/kg over 12 months was given to patients infected with *W. bancrofti*, the levels of microfilaria were reduced by 90% and the infection rates among mosquitoes dropped to zero (Nathan *et al.*, 1987). Although DEC exhibits low toxicity and is safe for large-scale treatment of lymphatic filariasis (Lin *et al.*, 1992), the dosage regimen of the drug is rather inconvenient; multiple doses from 1-2 weeks to one year.

1.3.2 Suramin

Suramin (Fig. 1.3) was first introduced in 1921 for the treatment of human trypanosomiasis and was first used as an antifilarial drug in 1947. Suramin is mainly used in the treatment of onchocerciasis; it is active against adult worms of *O. volvulus* but lacks microfilaricidal activity (Hawking, 1978).

When given orally suramin is absorbed only to a limited extent from the intestine and causes a local irritation when given by subcutaneous or intramuscular injections (Hawking, 1978). In clinical practice, suramin is administered intraveneously at weekly intervals for 6 weeks, with a total dose of 60 mg/kg. A higher dose may produce dangerous side-effects and occasionally may result in death due to its intrinsic toxicity (WHO,1987). The female worms are more susceptible to the lethal action of suramin than the males, but even

the females take 4-5 weeks to die. To achieve a parasitological cure, a minimum concentration of 0.1 mg/ml of suramin in plasma needs to be maintained for 2 weeks (Hawking, 1978). The microfilariae gradually die after the adult worms have been killed. Side-effects resembling the Mazzotti reaction accompanies the death of the parasites.

1.3.3 Ivermectin

Ivermectin (Fig. 1.3) belongs to a family of macrocyclic lactones, the avermectins. It is an 80:20 mixture of avermectin B_{1a} and avermectin B_{1b}, produced by actinimycete *Streptomyces avermitilis* (Campbell *et al.*, 1983). Ivermectin is active against a wide variety of nematodes and arthropods (Campbell *et al.*, 1985; Jackson, 1989). In humans, it is known to be effective against microfilariae of *O. volvulus* (Aziz *et al.*, 1982a, b) and *W. bancrofti* (Kumaraswami *et al.*, 1988). There are several reviews of the use of ivermectin in the treatment of filariasis, particularly in onchocerciasis (Hay and Burr, 1989; Campbell, 1991, 1993; Goa *et al.*, 1991; Mak *et al.*, 1991; Vande Waa, 1991; Hillyer and Rajan, 1992).

In onchocerciasis, ivermectin is not effective against adult *O. volvulus*, as indicated by the appearance of microfilariae in the skin a few months post-treatment (Campbell, 1991). It has been suggested that ivermectin paralyses microfilariae within the uterus of the female worms so that they are not released normally, but instead degenerate and are resorbed *in utero* (Awadzi *et al.*, 1985; Schulz-Key, 1988, 1990). In chimpanzees, ivermectin has shown a partial *in vivo* effect against the third infective larval stage (L_3) of *O. volvulus* but has no effect on the fourth larval stage (L_4) of the parasite (Taylor *et al.*, 1988). However, ivermectin appears to reduce

the uptake of *O. volvulus* microfilariae by the vectors (blackflies) as a results of its microfilaricidal activity, and therefore reduces the transmission of the infection (Cupp *et al.*, 1986, 1989; Taylor *et al.*, 1990).

Ivermectin has been shown to be efficacious against microfilariae of *W. bancrofti* (Kumaraswami *et al.*, 1988). Dose-finding studies in Senegal (Diallo *et al.*, 1987), French Polynesia (Cartel *et al.*, 1991) and Haiti (Richards *et al.*, 1991) showed that a single dose of 100-200 µg/kg ivermectin reduced the microfilaria counts more effectively than a dose of 50 µg/kg. However, studies in India (Kumaraswami *et al.*, 1988; Vijayasekaran *et al.*, 1990; Ottesen *et al.*, 1990) indicated that lower doses of 10-25 µg/kg ivermectin have a comparable efficacy to 100-200 µg/kg dose in reducing the microfilariae and were associated with significantly fewer side-effects.

Ivermectin has shown no effect against all stages of *B. malayi* infection in the leaf monkeys, *Presbytis cristata* (Mak *et al*, 1988). Only recently, the efficacy and safety of ivermectin in patients infected with *B. malayi* was reported (Shenoy *et al.*, 1992). The clearance of the microfilaria was not complete; the effect was maximal at the end of the first month and gradually rose to 20-50% of the pretreatment levels at 6 months. The patients were treated again after 6 months and the levels were 10-35% of the pretreatment levels 6 months later. The side-effects were mild to moderate and not related to the dose or the density of microfilariae. This study shows that ivermectin may be useful in Brugian filariasis but the dosage regime have to be evaluated.

A low frequency adverse reaction associated with ivermectin has been reported in many studies (Aziz *et al.*, 1982a, b; Awadzi *et al.*, 1985; Diallo *et al.*, 1986). The intense Mazzotti reactions are normally associated with the treatment of DEC which is believed to be caused by the death of microfilariae. Ivermectin however, causes paralysis of the microfilariae before they die and, therefore, a less intense response is associated with ivermectin (Ette *et al.*, 1990). The frequency and severity of adverse reactions of ivermectin therapy are directly related to the intensity of the infection (de Sole *et al.*, 1989; Rothova *et al.*, 1989).

Comparison studies of ivermectin and DEC in the treatment of onchocerciasis (Green *et al.*, 1985; Awadzi *et al.*, 1985; Diallo *et al.*, 1986) and lymphatic filariasis (Ottesen *et al.*, 1990; Vijayasekaran *et al.*, 1990) demonstrated that ivermectin is at least as effective es DEC and produced similar side-effects. The only advantage ivermectin has over DEC is that it is given less frequently and as a single dose. At present, ivermectin is the drug of choice for the treatment of onchocerciasis (British National Formulary, 1992) and it is safe and effective drug for mass treatment.

1.3.4 Benzimidazole Carbamates

In experimental studies, mebendazole and flubendazole (Fig. 1.3), broad spectrum anthelmintics of the class benzimidazole carbamate have been demonstrated to have macrofilaricidal activity (Van den Bossche *et al.*, 1982). Earlier studies in patients infected with *O. volvulus*, *W. bancrofti* and *D. perstans* treated with mebendazole did not show improvement in microfilarial counts (Van den Bossche *et al.*,

1982). However, recent studies of mebendazole in the treatment of human onchocerciasis (Rivas-Alcalá *et al.*, 1981a, b; Awadzi *et al.*, 1982) showed that mebendazole reduced the microfilaria counts both in the skin and the eye; this is thought to be an effect of embyrogenesis in the adult female worms. The low microfilaria counts were maintained for at least 12 months (Rivas-Alcalá *et al.*, 1981b) after treatment with 2 g/day mebendazole for 28 days and fewer side-effects were produced by mebendazole compared to those associated with DEC therapy. The Mazzotti reaction was not observed because of the decrease in the microfilariae release due to the disruption of embryogenesis.

Mebendazole has also been shown to have some activity in decreasing the microfilaria counts in loiasis and may also kill the adult worms when given in high doses (Van Hoegaerden *et al.*, 1987). It has also macrofilaricidal activity in patients suffering from *B. malayi* and *W. bancrofti* but it has to be given in large doses over a long period of time; 500 mg 3 times daily for 21 days (WHO, 1984). However, due to its potential teratogenicity and its erratic oral absorption, mebendazole can be toxic when given in high doses and therefore it is not recommended for the treatment of lymphatic filariasis (WHO, 1984).

Because of its low bioavailability, the use of mebendazole in the treatment of filarial infections was limited (Tropical Disease Research, 1984). Mebendazole-citrate, a derivative of mebendazole prepared by Janssen Pharmaceutica (Goodwin, 1984) has a better bioavailability and was found to be more potent against mebendazole (Awadzi *et al.*, 1990).

Flubendazole, like mebendazole is very poorly absorbed from the gastrointestinal tract, and was only active when given by parenteral administration in the treatment of onchocerciasis (Dominguez-Vázquez *et al.*, 1983). The microfilaria counts were progressively reduced and approximately 2% of the pretreatment levels were present at 12 months following 750 mg flubendazole intramuscularly for 5 weeks. No systemic side-effects attributable to the death of microfilariae were recorded. However, the intramuscular injection caused a severe reaction at the site of injection. Although flubendazole was more effective and safer than DEC, the drug has to be reformulated before undergoing further investigation (Tropical Disease Research, 1984).

Recently, another benzimidazole carbamate, albendazole (Fig. 1.3) has been studied as an antifilarial drug in the treatment of human onchocerciasis (Awadzi *et al.*, 1990; Cline *et al.*, 1992). Albendazole has a wider spectrum of anthelmintic activity, including activity against both the larvae and adult of a variety of nematodes and cestodes (Theodorides *et al.*, 1976; Cook, 1990) and it has a much better absorption than mebendazole following oral administration (Morris *et al.*, 1983). Studies in patients suffering from onchocerciasis in Ghana (Awadzi *et al.*, 1990) and in Venezuela (Cline *et al.*, 1992) indicated that albendazole was neither a microfilaricidal nor a macrofilaricidal agent at all the dose regimes given (800 mg daily for 3 days, 1200 mg daily for 3 days or 800 mg daily for 7 days in Ghana; 400 mg daily for 10 days in Venezuela). However, the skin microfilarial counts were suppressed for over one year suggesting that albendazole was active against *O. volvulus* presumably because of to its embryotoxic effect.

Albendazole has been shown to be more potent than mebendazole against *O. volvulus* (Awadzi *et al.*, 1991) and a change in formulation of this promising antifilarial drug may improve its aqueous solubility and therefore its bioavailability.

Albendazole has also been shown to have antifilarial activity against *B. malayi* in leaf monkeys, *Presbytis* spp. and *B. pahangi* in jirds (WHO, 1992). Albendazole showed a marked adulticidal activity with a gradual elimination of microfilariae from the blood when given subcutaneously, but there was no effect either on microfilariae or adults of *B. pahangi* in *Mastomys natalensis* (Reddy *et al.*, 1983). In the treatment of lymphatic filariasis, a recent study comparing albendazole (400 mg daily for 21 days) and DEC showed that albendazole has macrofilaricidal effect with less effect on the microfilariae (WHO, 1992).

1.3.5 Other antifilarial drugs

There is a small number of new potential antifilarial drugs that have been discovered during screening in the experimental studies, including CGP 6140 (amocarzine) and CGP 20376 (benzothiazole) (Mak *et al.*, 1991; Vande Waa, 1991).

CGP 6140 has been studied in patients with onchocerciasis (Soula *et al.*, 1989; Lecaillon *et al.*, 1990; Poltera *et al.*, 1991). The skin microfilarial counts were reduced within one week and remained low (14-18% of pretreatment levels) for at least 6 months following 3 mg/kg CGP 6140 twice a day after food for 3 days (Poltera *et al.*, 1991). CGP 6140 has been found ineffective against *B. malayi* in leaf monkeys, *Presbytis cristata* at oral doses of 25 and 50 mg/kg daily

for 5 days (Mak *et al.*, 1991). However, there was some adulticidal activity at higher doses.

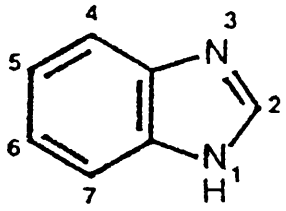
CGP 20376 has shown to have both macro- and microfilaricidal activity against *B. malayi* and *B. pahangi* in *Mastomys natalensis* (Zahner *et al.*, 1988) and in *Presbytis cristata* (Mak *et al.*, 1990). However, due to its hepatotoxicity, CGP 20376 was not recommended for the treatment of lymphatic filariasis until better formulation is developed (Mak *et al.*, 1991).

There are two other compounds; CGI 18041 (a benzothiazole) and UMF 078 (a benzimidazole) that are still under development (WHO, 1992). CGI 18041 has been shown to have antifilarial activity in rodents and active against *B. malayi* and *W. kalimantan* in leaf monkeys, and UMF 078 is active against *B. pahangi* in dogs (WHO, 1992).

1.4 HISTORY OF BENZIMIDAZOLE CARBAMATES

The discovery of thiabendazole in 1961 (Brown *et al.*, 1961) has brought about the development of other benzimidazoles, the only class of broad-spectrum anhelmintics (Van den Bossche *et al.*, 1982).

Benzimidazole is a ring system where a benzene ring is fused to the 4,5-positions of imidazole and the systemic numbering of the benzimidazole is as shown;



Some modifications to the ring have been made during development and modifications at positions at 2- and 5- of benzimidazole ring produced the most active compounds (Townsend and Wise, 1990). This 2,5-substituted benzimidazole anthelmintics may be classified into two main types: (1) - the 2-thiazolyls, (2) - the 2-carbamates.
Type (1) are well absorbed from the gastrointestinal tract whereas type (2) are poorly and erratically absorbed.

Thiabendazole is an example of the 2-thiazolyls. Although thiabendazole is an effective drug for the treatment of helminthiasis, its use in human has been limited due to its toxicity (Cook, 1990).

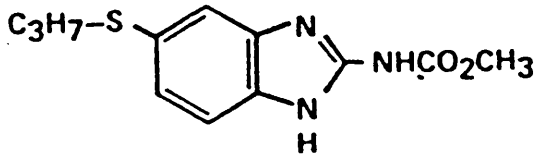
The 2-carbamates are widely used in veterinary and human medicine (Campbell, 1990; Martindale, 1988). There are three 2-carbamates currently in use in human clinical practice; mebendazole,

flubendazole and albendazole (Martindale, 1988). The use of these benzimidazole carbamates have been largely restricted to the treatment of intestinal helminth infections. Since these drugs are very effective and safe, investigations have been undertaken to explore the possible use of these drugs in the treatment of systemic infections such as filariasis (section 1.3.4). In this thesis, research has been focussed only on albendazole.

1.5 PHARMACOLOGY OF ALBENDAZOLE

1.5.1 Introduction

Albendazole (ABZ) is methyl N-[5-(propylthio)-1*H*-benzimidazol-2-yl]carbamate, molecular weight of 265.3 with melting point of 214-215^{o}C; it is insoluble in water and only slightly soluble in most organic solvents (Theodorides *et al.*, 1976). The partition coefficient of albendazole between octanol and water is 40.0 and the pK_a value is 9.4 (Jung H., personal communication).



Several studies have demonstrated the efficacy of albendazole in the management of systemic infections such as hydatid disease (Saimot *et al.*, 1983; Morris *et al.*, 1985; Guermoche *et al.*, 1988) in man and have shown the drug to have advantages over mebendazole (Todorov *et al.*, 1992a, b). Albendazole has also been reported to be effective in the treatment of cysticercosis (Jung *et al.*, 1992; Del Brutto *et al.*, 1992; Vazquez and Sotelo, 1992).

1.5.2 Pharmacokinetics

There is little data available on the pharmacokinetics of albendazole in man because it is largely undetectable in human plasma due to its low gastrointestinal absorption and rapid metabolism (Penicaut *et al.*, 1983; Marriner *et al.*, 1986). However, when an albendazole tablet (400 mg) was given orally, the maximum concentrations of the

active metabolite albendazole sulphoxide were in the range of 0.04 to 0.55 µg/ml between 0.5 and 4 hours after dosing (Marriner *et al.*, 1986; Lange *et al.*, 1988). The mean plasma elimination half-life was 8.5 hours (Penicaut *et al.*, 1983; Marriner *et al.*, 1986; Lange *et al.*, 1988).

Marriner et al. (1986) reported an inconsistent increase in albendazole sulphoxide concentration when albendazole was administered with oil and milk. A significant increase of albendazole sulphoxide concentration was observed in patients with echinococcosis when albendaozle was given with a fatty breakfast (425% relative to fasting patients (Lange *et al.*, 1988)).

1.5.3 Metabolism

Albendazole undergoes extensive metabolism. The main biotransformation observed in man (Penicaut *et al.*, 1983), cattle, sheep, rats and mice (Gyurik *et al.*, 1981) was similar; the hydrolysis of the carbamate moiety and oxidation of the sulphur atom, the alkyl side-chain and the aromatic ring (Fig. 1.4).

In all species, the major metabolites found in the urine and plasma are the sulphoxide and sulphone metabolites and the proportion varies considerably among species (Lienne *et al.*, 1989; Delatour *et al.*, 1991; Lanusse *et al.*, 1992). The parent compound however was found in trace amount in the urine (Gyurik *et al.*, 1981) and is normally undetectable in plasma of human (Penicaut *et al.*, 1983; Marriner *et al.*, 1986), rats (Delatour *et al.*, 1984) and sheep (Marriner *et al.*, 1981).

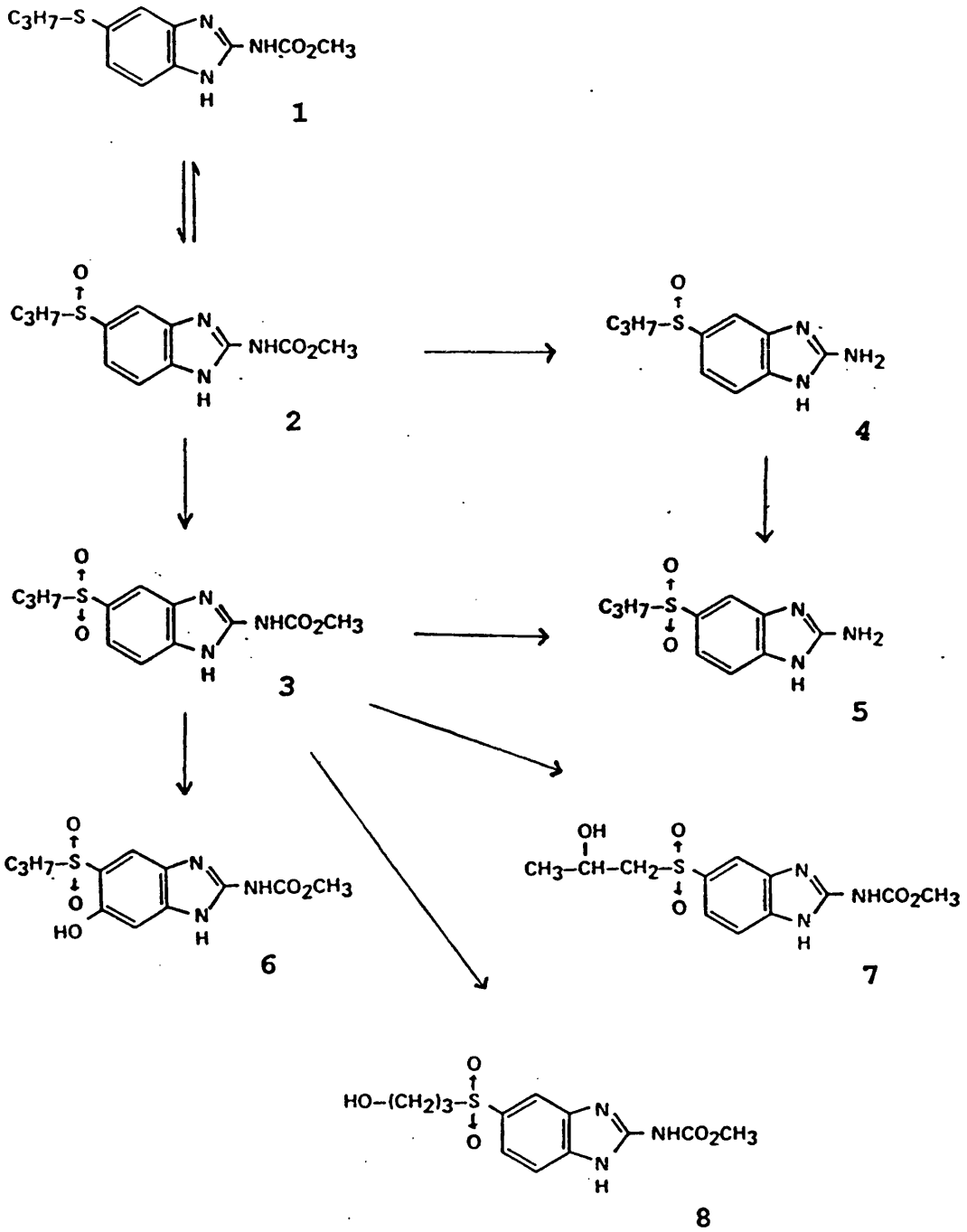


Fig 1.4 Metabolic pathway of albendazole.

1. Methyl N-[5-(propylthio)-1*H*-benzimidazol-2-yl]carbamate
2. Methyl N-[5-(propylsulphinyl)-1*H*-benzimidazol-2-yl]-carbamate
3. Methyl N-[5-(propylsulphonyl)-1*H*-benzimidazol-2-yl]-carbamate
4. 5-(propylsulphinyl)-1*H*-benzimidazol-2-amine
5. 5-(propylsulphonyl)-1*H*-benzimidazol-2-amine
6. Methyl N-[6-hydroxy-5-(propylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate
7. Methyl N-{5-[(2-hydroxypropyl)sulphonyl]-1*H*-benzimidazol-2-yl}carbamate
8. Methyl N-{5-[(3-hydroxypropyl)sulphonyl]-1*H*-benzimidazol-2-yl}carbamate

In *in vitro* studies, albendazole was converted to sulphoxide by liver microsomes (Fargetton *et al.*, 1986; Souhaili-El Amri *et al.*, 1987; Rolin *et al.*, 1989) an effect mediated by cytochrome P-450 and/or FAD-containing monooxygenase depending on the system used; rat (Fargetton *et al.*, 1986), sheep (Galtier *et al.*, 1986), pig (Souhaili-El Amri *et al.*, 1987) or human (Rolin *et al.*, 1989) liver microsomes. Albendazole sulphonation however has been exclusively attributable to cytochrome P-450c in perfused rat liver and it has also been shown that albendazole induced its own metabolism (Souhaili-El Amri *et al.*, 1988).

1.5.4 Mode of action

The biochemical mode of action of benzimidazoles has not been fully explained. Although benzimidazoles cause many effects such as inhibition of the fumarate reductase system and glucose uptake (Van den Bossche *et al.*, 1982), studies have indicated that the primary mode of action of benzimidazole is binding to tubulin and inhibiting tubulin polymerization to microtubules (Lacey, 1988, 1990).

Benzimidazoles have been shown to inhibit [^{3}H]-colchicine binding to parasite tubulin (Barrowman *et al.*, 1984). Mebendazole was 400 times and febendazole was 250 times more potent as inhibitor to the tubulin of embryonic *Ascaris suum* than of mammalian tubulin (Friedman and Platzer, 1978). A recent study supports the finding that benzimidazole specifically binds nematode tubulin with high affinity (Lubega and Prichard, 1991).

1.6 IMPROVED BIOAVAILABILITY OF POORLY ABSORBED DRUGS USING LIPID-BASED DOSAGE FORM

Several reports have indicated that bioavailability of poorly absorbed drugs such as griseofulvin (Carrigan and Bates, 1973; Bates and Sequeira, 1975; Bloedow and Hayton, 1976), probucol (Palin and Wilson, 1984) and phenytoin (Shinkuma *et al.*, 1985) were improved following administration of these drugs incorporated with a lipid.

Drug absorption from a lipid vehicle is considered to proceed mainly via diffusion into aqueous luminal fluid, followed by absorption through the mucosal membrane (Kakemi *et al.*, 1972). Partitioning of the drug and transport from one phase to the other are therefore of importance. A critical factor which governs the degree of absorption of drugs in oil-water emulsions, when the drug partition coefficients are greater than unity, is the amount of drug in the aqueous phase rather their overall concentration (Kakemi *et al.*, 1972).

The oral absorption of a variety of drugs has been shown to be altered in the presence of lipids and emulsions (Armstrong and James, 1980). Various physiological mechanisms have been proposed to explain this effect including inhibition of gastric emptying (Bates and Sequeira 1975; Shinkuma *et al.*, 1985), increased bile flow leading to drug solubilization (Bates and Sequeira, 1975), increased membrane permeability (Muranushi *et al.*, 1980), facilitated lymphatic absorption (Palin *et al.*, 1982) and hydrolysis by lipase (Borgström and Erlanson, 1973).

1.6.1 Mechanism of Lipid Absorption

1.6.1.1 Inhibition of Gastric Emptying

Gastric emptying time is a very important factor affecting the absorption of drugs. The increased bioavailability of griseofulvin in rats when administered in suspensions and emulsions of corn-oil could be due to the long gastric transit caused by the fatty acids liberated during digestion of corn-oil (Carrigan and Bates, 1973; Bates and Sequeira, 1975). Inhibition of gastric emptying and motility of the upper small intestine were reported by Shinkuma *et al.* (1985) when phenytoin was given in sesame-oil and oleic acids. Corn-oil and sesame oil consist of long-chain triglycerides which are metabolised quite slowly (Alvarez and Stella, 1992). The saturated, low-chain and medium-chain triglycerides however, did not cause gastric retention although digested *in vivo* in the small intestine as observed by Yamahira *et al.* (1979). Rapid metabolism (Alvarez and Stella, 1992) and rapid dispersion (Yamahira *et al.*, 1979) of medium-chain triglycerides contributed to the uniformity of gastric emptying rates of the lipids.

1.6.1.2 Stimulation of Bile Flow

It is known that triglycerides and fatty materials stimulate flow of bile into the small intestine, which may be a contributory factor in partitioning and absorption of poorly water-soluble drugs (Bates and Sequeira, 1975; Miyazaki *et al.*, 1980). During intestinal digestion of dietary fat, oil was found dispersed in a micellar bile salt solution in the lumen of the small intestine (Hofmann and Borgström, 1964). Therefore, increase in drug absorption by bile salts probably involves micellar solubilization and/or by lowering the surface tension of the gastrointestinal fluid (Miyazaki *et al.*, 1979). The

partition of fatty acids from emulsified oil phase to micellar phase was found to be strongly dependent on the chain length of the triglycerides and the pH of the dispersion (Borgström, 1967). At low pH values, the long-chain fatty acids were distributed in favour of the emulsified oil phase, but between pH 6 and 8 a marked increase in favour of the micellar phase occurs. As the chain-length decreased, the distribution of the fatty acids to the micellar phase was increased. Medium- and short-chain fatty acids are probably present mainly in the form of soaps in a weakly acid solutions. This can be the important determinant for their mechanism of transport and absorption; either via the portal or lymphatic pathways. Absorption of medium-chain triglyceride was however not affected by the presence of bile salts.

Bile salts micelles readily incorporate with biliary cholesterol and phospolipids to form mixed micelles which will remove the hydrophobic products of lipolysis from oil-water interface and subsequently transport the fatty acids and monoglycerides through the aqueous phase to the enterocyte membrane where they are absorbed (Poelma *et al.*, 1990).

1.6.1.3 Increase Membrane Permeability

Enhanced absorption of drug coadministered with oil has been proposed to increase mucosal permeability (Muranushi *et al.*, 1980). Palin *et al.* (1982) suggested that formation of mixed bile salt micelles containing unsaturated fatty acids was responsible for this increase in mucosal membrane permeability. According to Sallee and Dietschy (1973) the rate of tissue uptake of fatty acids can be described using apparent permeability coefficient, *P and in general increases

with increase in the chain length. However, several reports demonstrated that membrane absorption is more effective for medium-chain than long-chain fatty acids (Greenberger *et al.*, 1966; Palin *et al.*, 1986; Yoshitomi *et al.*, 1987; Ichihashi *et al.*, 1992).

1.6.1.4 Increase in Lymphatic Absorption

The absorption pathway of a drug in the gastrointestinal tract can be altered by the products of triglycerides digestion (Palin *et al.*, 1982, 1984; Ichihashi *et al.*, 1992). The class of lipid vehicle can affect the rate and extent of lymphatic transport of a lipophilic drug (Muranishi *et al.*, 1980; Charman and Stella, 1986); short- and medium-chain fatty acids are transported unesterified into the systemic circulation via the portal vein whereas long-chain fatty acids are considered to form chylomicrons and are transported by the lymphatic system as summarised in Fig. 1.5 (Myers and Stella, 1992). Lymphatic absorption varies in the GI tract being lowest in the stomach and large intestine and greatest in the small intestine (Noguchi *et al.*, 1977).

DDT and Probucol in arachis oil, Miglyol 812 and liquid paraffin were studied by Palin *et al.* (1982, 1984). Both drugs were absorbed via a lymphatic pathway in all formulations with the greatest absorption from arachis oil. Long unsaturated and saturated fatty acids liberated from arachis oil digestion, incorporated with mixed bile salt micelles and the formation of chylomicrons within the enterocytes, facilitated the transport of DDT and probucol by the lymphatic system. It was suggested that lymphatic drug transport from the small intestine is dependent upon concurrent chylomicron synthesis by the epithelial cells (Sieber, 1976; Charman *et al.*,

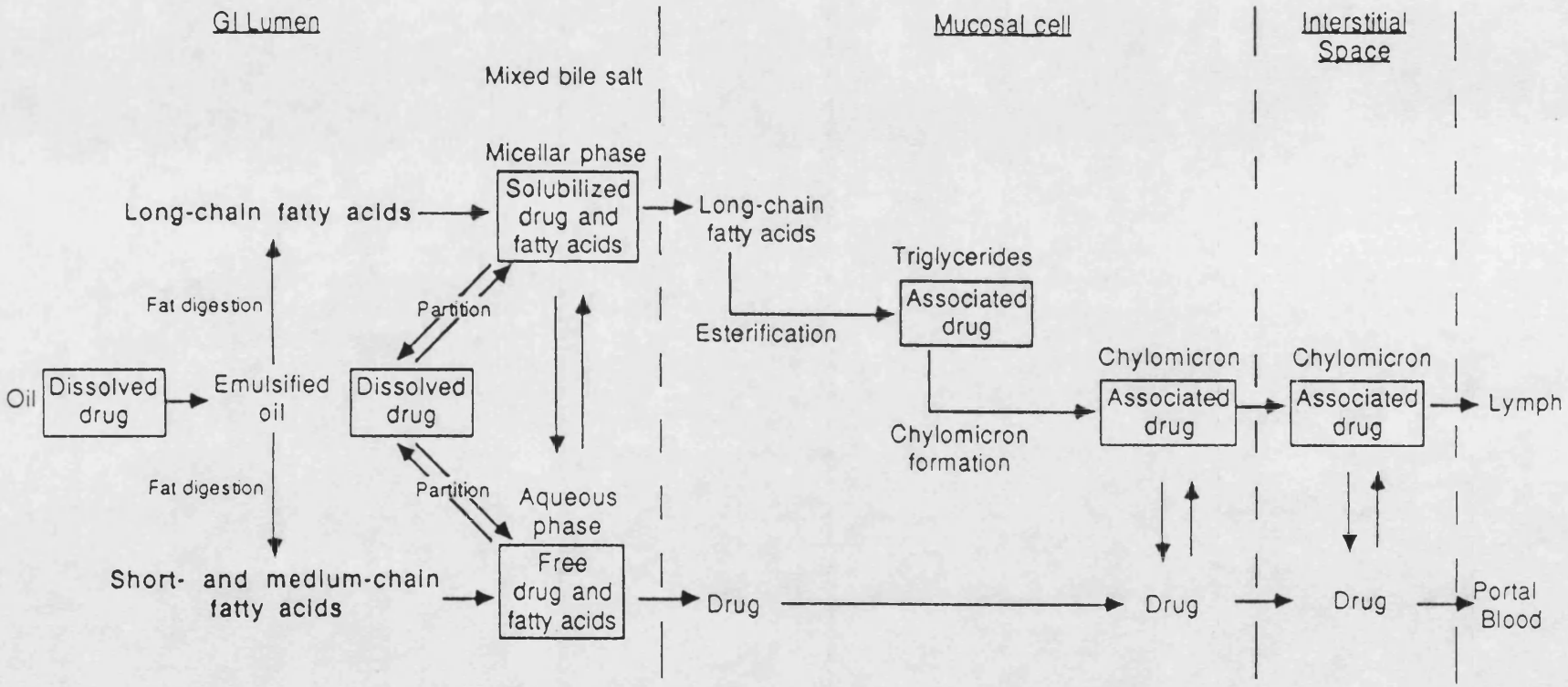


Fig 1.5 Pathways for drugs absorption into venous and lymphatic system (from Myers and Stella, 1992).

1986).

The effect of lymph flow is unclear. According to DeMarco and Levine (1969), when coadministered with lipids the amount of drug transported to lymph increases as the lymph flow increases. However, the distribution of mepitiostane (Ichihashi *et al.*, 1992) and DDT (Charman and Stella, 1986) in the chylomicrons and the transportation in the lymphatic system were not affected by the lymph flow.

1.6.1.5 Hydrolysis by Lipase

Hydrolysis of oil by lipase is an important factor in drug release from the vehicle, and may influence the absorption of poorly water-soluble drugs from lipid-containing dosage forms in man (Palin, 1982). The products of triglyceride hydrolysis, the corresponding monoglycerides and fatty acids which are more hydrophilic than the parent triglycerides (Wiseman, 1964), will combine with bile salt forming mixed micelles and provide aid in solubilizing the drug in the aqueous environment of the intestinal lumen (Poelma *et al.*, 1990). It has been demonstrated that the short- and medium-chain triglyceride are hydrolysed more rapidly by lipase than the long-chain triglycerides (Greenberger *et al.*, 1966; Playoust and Isselbacher, 1964).

Digestion of dietary fat in mammals occurs in the stomach but is initiated by the lingual and gastric lipase (Borgström *et al.*, 1957; Hamosh and Scow, 1973; Hamosh *et al.*, 1975). Studies on digestion of milk showed that fat was partially hydrolyzed by lingual lipase (originates from the back of the tongue) at pH 5.4, resulting in

fatty acid and diacylglycerol existing as an oil phase in aqueous phase (Patton *et al.*, 1982). Fatty acid has been shown to increase the colipase binding and subsequent hydrolysis by lipase (Borgström, 1980). The product of lingual lipase therefore makes the fat droplets better substrates for pancreatic lipase for more efficient digestion in the small intestine (Patton *et al.*, 1982).

Bile salt may have direct effects on the pancreatic lipolysis. One function of bile in the intestine is to protect the hydrophobic surface of the substrates from the pancreatic lipase (Brockerhoff, 1971; Borgström and Erlanson, 1973) by building up a negatively charged detergent monolayer on the substrate surface and by electrostatic repulsion preventing the lipase from reaching the substrate. The maximum inhibition by bile salt is when the critical micelle concentration is reached (Borgström and Erlanson, 1973). The inhibition may also be seen as competition between lipase and bile salts for the interface or reduction of interfacial tension to hinder lipase adsorption to the interface (Nano and Savary, 1976).

It has been shown that colipase, a protein co-factor of lipase, overcomes the inhibition of lipase caused by bile salt (Borgström and Erlanson, 1973; Brockerhoff, 1971; Vandermeers, 1974; Borgström, 1975) by interacting with the lipase to form a less-charged and more hydrophobic complex than the layer built by the bile salt on the substrate. The colipase-lipase complex penetrates the bile salt layer to reach the substrate allowing lipolysis to occur.

1.6.2 Self-Emulsifying System

Appropriate mixtures of oil and surfactant are known as self-emulsifying systems due to its capability to emulsify under gentle agitation (Groves and de Galindez, 1976). When given orally, the digestive movement of the gastrointestinal tract will emulsify this oily solution. The system rapidly produces a fine dispersion with droplet size of approximately 0.1-10 μm (Pouton, 1985b; Stout *et al.*, 1988; Wakerly, 1989; Challis, 1991). Formation of these small oil droplets results in a much larger interfacial area and therefore the rate of transfer in the gastrointestinal tract by diffusion from the oily phase to the aqueous phase (luminal fluid) is increased (Lostritto *et al.*, 1987).

The absorption profile of a lipophilic drug in a self-emulsifying system depends greatly on the choice of oil and surfactant. It was proposed that surfactant may modify drug absorption by an number of mechanisms (Attwood and Florence, 1983), for example, non-ionic surfactant such as Tween 80 has been reported to modify gastrointestinal absorption by partitioning drug into micelles and changing the mucosal barrier (Dermer, 1967). Compounds that possess surfactant properties such as lauric acid may solubilize lipophilic constituents of membrane e.g. cholesterol (Palin *et al.*, 1986). The absorption and digestibility of an oil and/or the products may also affect the absorption of a drug when administered in oily solution by a number of mechanisms which have been discussed in section 1.6.1.

A great deal of work has been done using oil e.g. Miglyol 812 and non-ionic surfactant e.g. Tween 80, Tween 85 and Tagat TO (Groves and de Galindez, 1976; Palin *et al.*, 1982, 1986; Palin and Wilson, 1984;

Pouton, 1984, 1985a, b; Pouton *et al.*,1988; Reymond *et al.*, 1988; Woolfrey *et al.*, 1989; Challis, 1991; Ichihashi *et al.*, 1992; Charman *et al.*, 1992). It was suggested that the mechanism of self-emulsification involves formation of specific liquid crystalline (LC_a) phase at the o/w interface (Wakerly, 1989) which aid the penetration of water into the emulsifying mixture by creating aqueous channels, thereby increasing the surface pressure close to the interface which results in interfacial instability.

Palin *et al.* (1986) demonstrated that Miglyol 812 did not promote the absorption of cefoxitin by increased lymphatic absorption of the drug nor by a reduction of gastrointestinal motility, but by protecting the molecule from degrading in the acid environment of the stomach. It was proposed that the improvement of the plasma profile following the oral administration of the self-emulsifying system is a function of solubilisation and dispersion of drug in the gastrointestinal tract (Charman *et al.*, 1992).

1.7 AIM OF STUDY

The aim of this study was primarily to investigate the possible role of formulation using triglyceride oil in overcoming the low bioavailability of albendazole associated with the oral administration for the treatment of filarial infections. This study involved three phases:

(1) Analytical studies

Published analytical methods were available for albendazole and its metabolites. However no suitable assay method was available for simultaneous analysis of albendazole with its two major metabolites, the sulphoxide and sulphone. The published methods were tedious often involving more than one chromatographic system. Similarly there were no published methods for chemical degradates. Development of selective, sensitive and reproducible HPLC methods for albendazole and its two metabolites was therefore required.

(2) Pharmaceutical aspects

This phase of the study involved formulation of albendazole in an oily system suitable for *in vivo* administration. Additionally, since there is no information available on the chemical stability of albendazole, a study was necessary to establish any degradation products.

(3) *In vitro* and *in vivo* biological studies

These studies were aimed at the assessment of drug absorption and bioavailability, metabolism and pharmacokinetic profiling. *In vivo* animal studies involved dosing rats with the various albendazole formulations and following the plasma/serum profiles. Following the

rat studies, the formulations were selected for bioavailability evaluation in a higher animal (monkey). Additionally, the site of albendazole metabolism, either pre- or post-gastrointestinal tract absorption was to be studied *in vitro*.

CHAPTER 2

MATERIALS

2.1 REAGENTS AND SOLVENTS

Albendazole (ABZ) and mebendazole (MBZ) were obtained from Sigma Chemical Co. (Poole). The micronised standards of ABZ and its sulphoxide (ABZSO) and sulphone ($ABZSO_2$) metabolites were supplied by Lancaster Synthesis (Morecambe).

Methanol (MeOH), acetonitrile (ACN), hexane and ethanol (EtOH) of HPLC grade were purchased from Fisons (Loughborough). Analytical grade dimethylsulphoxide (DMSO), potassium dihydrogen phosphate, sodium hydroxide, glacial acetic acid and hydrochloric acid (HCl) were also obtained from Fisons (Loughborough). Ammonium acetate used was a GPR grade obtained from BDH Chemicals Ltd. (Poole).

HPLC grade MeOH and ACN, and analytical grade potassium dihydrogensulphate used at USM were obtained from E. Merck (Darmstadt, Germany). Analytical grade ammonium acetate (Ajax Chemical Ltd., Auburn, NSW, Australia), HCl Analar (BDH Chemicals Ltd., Poole) and glacial acetic acid, 91.8% (Riedel-de-Haen, Seelze, Germany) were also used.

Polyethylene glycol (PEG) 400 and gluconic acid (50% aqueous solution) were purchased from Aldrich Chemical Co. Ltd. (Gillingham). Tween 80 and 85 were obtained from Sigma Chemical Co. (Poole) and used as received.

Miglyol 812 is a medium-chain saturated triglyceride oil produced by esterification of glycerol with fatty acid fraction obtained by distillation of hydrolysed coconut oil and it was supplied by Dynamit Nobel (UK) Ltd. (Slough). It is refer as Miglyol throughout this

thesis.

2.2 EQUIPMENT

Three HPLC systems were used;

(1) Spectra Physics HPLC system (St. Albans) consisting of a SP8100 liquid chromatograph, SP8440 UV/VIS detector and SP4200 computing integrator.

(2) An LC pump model 330 (SSI, State College, PA, USA), a Pye-Unicam variable wavelength UV/VIS detector and a Hewlett-Packard HP3390A integrator.

(3) Waters-Millipore 2-solvent delivery system model 510, a Waters-Millipore automated gradient controller (Petaling Jaya, Malaysia), a Gilson UV detector model 116 (Petaling Jaya, Malaysia) and a Hewlett-Packard HP3392A integrator (Petaling Jaya, MAlaysia).

A Perkin-Elmer Lambda3 UV/VIS spectrophotometer (Beaconsfield) or a Milton Roy Spectronic 601 spectrophotometer (Stone) was used for measuring the UV absorbance in the solubility studies.

Gilson Pipetman adjustable pipettes (200 , 1000 and 5000 µl) with disposable polypropylene tips were obtained from Anachem House (Luton). The calibration of these pipettes was checked to ensure accurate and reproducibile sample delivery.

An Analytichem International Vac Elut SPS 24 (Jones Chromatography, Mid Glamorgan) or Supelco SPE Vacuum manifold was used for the solid phase extraction (SPE) of plasma/serum samples.

An Eppendorf Centrifuge 5414 (Hamburg, Germany) or Hettich Universal Centrifuge (A.R. Horwell Ltd., London) was used for centrifugation of blood samples to give either serum or plasma.

A Segma interchangeable precision syringe (Italy) and a syringe pump model 341A (Sage Intruments, Cambridge) were used for solvent delivery in the solubility studies.

Samples from the stability studies of albendazole in solvents were heated in a heating block obtained from Grant, model BT3 (Cambridge).

A student microscope (M5) obtained from Vickers (York) was used to examine samples from solubility studies.

2.3 OTHER MATERIALS

Nucleosil C_{18} columns, 250 x 4.6 mm , 5 µm particle size were obtained from Fisons Scientific Apparatus (Loughborough) or packed by Capital HPLC Specialists (West Lothian, Scotland). These columns were employed in the reversed-phase HPLC method. The normal-phase Hypersil C_{18} silica column, 100 x 4.6 mm, 5 µm particle size was obtained from Shandon Scientific (Cheshire).

Bonded octadecyl C_{18} packing material (200mg), 40 µm particle size (J.T. Baker, Hayes) was packed in a Bakerbond SPE glass column fitted with PTFE frit (J.T. Baker, Hayes) or in a disposable syringe packed with glass wool and used as SPE cartridges.

Disposable syringes (1, 5, 10, 20 ml) and needles used in the animal and human studies were obtained from various companies. Blood was collected in lithium heparin tubes or heparinised vacutainers (FBG Trident Ltd. Bristol).

2.4 PREPARATION OF STANDARD SOLUTIONS FOR HPLC

A stock solution containing either 500 µg/ml ABZ, ABZSO or $ABZSO_2$ was prepared in MeOH for reversed-phase HPLC. The "working standard solutions" were prepared as a mixture of the three compounds by diluting the stock solutions to give final concentrations of 1, 10 or 100 µg/ml of ABZ, ABZSO and $ABZSO_2$. The internal standard, MBZ stock solution was prepared in MeOH (50 µg/ml). The solution was then diluted to give a concentration of 10 µg/ml and used as the working internal standard solution.

For normal-phase HPLC, stock solutions containing either 500 µg/ml ABZ or ABZSO, or 100 µg/ml $ABZSO_2$ were prepared in hexane:EtOH (90:10%, v/v). These stock solutions were diluted to give a final concentration of 10 µg/ml ABZ, ABZSO and $ABZSO_2$ (prepared as a mixture) and used as the "working standard solution". The stock solution of the internal standard, MBZ was also prepared in hexane:EtOH (100 µg/ml) and diluted to give a "working internal standard solution" of 10 µg/ml.

2.5 PREPARATION OF BUFFERS

2.5.1 Buffer for HPLC

0.05M ammonium acetate solution was prepared and the pH was adjusted to 4.7 with glacial acetic acid. The buffer was filtered through a cellulose nitrate membrane filter, 0.45 µm pore size (Whatman Ltd.,

Kent) prior to mixing with organic modifier (ACN) and the mixture was then degassed by sparging with helium.

2.5.2 Buffer for Extraction

0.01M and 0.017M potassium dihydrogenphosphate solutions were prepared and the solutions were buffered at pH 5.7 and 7.4, respectively with 0.8M sodium hydroxide.

The pH of these buffers were adjusted using a Kent EIL 7020 pH meter (Kent Industrial Measurement Ltd., Chertsey) which was calibrated with 0.05M potassium hydrogen phthalate buffer (pH 4.0).

2.5.3 Buffer for solubility studies

0.1M and 0.01M HCl solutions were prepared with the same ionic strength of 0.154M by addition of 0.054M and 0.144M of sodium chloride, respectively. These solutions were used in the solubility studies of albendazole using Method 1.

CHAPTER 3

ANALYTICAL STUDIES

3.1 INTRODUCTION

There have been many reports on the determination of ABZ and its major metabolites, ABZSO and $ABZSO_2$ in various body fluids. One of the first reports for detecting benzimidazole anthelmintics including ABZ in plasma and gastrointestinal fluids using HPLC was developed by Bogan and Marriner (1980). However, this method required a large volume of plasma/gastrointestinal fluid (4 ml) and repeated extraction with large volumes of diethyl ether. The two metabolites were eluted at similar retention times but could be assayed using this method. The levels of detection for the individual metabolites were not reported. The same authors (Marriner *et al.*, 1981) later developed an improved HPLC method to monitor ABZ, ABZSO and $ABZSO_2$ simultaneously using a different solvent system. However this method was not sensitive enough for ABZSO as the limit of detection for ABZSO was 300 ng/ml whereas 50 ng/ml for $ABZSO_2$.

A normal-phase HPLC method was described by Alvinerie and Galtier (1984) for the simultaneous analysis of ABZ, ABZSO and $ABZSO_2$. The separation was performed on a silica column with hexane-ethanol (445:55 v/v) as the eluent and detection at 225 nm. The average recoveries were 78-84%. However the method was too sensitive to interferences from the endogenous compounds extracted from plasma. There are certain practical considerations that need to be taken into account which limit the use of normal-phase HPLC for routine analysis. For example , the need to control the water content of mobile phase to obtain reproducible results.

Meulemans *et al.* (1984) reported a reversed-phase HPLC method for the determination of ABZ and ABZSO in various human organs and fluids

used to investigate ABZ concentration in patients with hydatid disease. Although the method was sensitive with limit of detection of 10 ng/ml for both ABZ and ABZSO, it was not convenient as separate procedures was required for each compound because isocratic separation was difficult to achieve on a column. The analysis of ABZ was therefore performed on μBondapak C_{18} column and μPorasil C_{18} column for ABZSO.

Recently, simultaneous HPLC methods for ABZ and ABZSO were developed. Hurtado *et al.*(1989) developed a sensitive method to detect ABZ and ABZSO in plasma with limit of sensitivity of 20 ng/ml and 15 ng/ml, respectively. Hoaksey *et al.* (1991) also developed a method for ABZ and ABZSO in plasma but the limit of detection of the compounds were not reported. However these methods were not suitable for the present study as $ABZSO_2$ was not included.

Due to its rapid metabolism and poor absorption, ABZ was largely undetectable in man. Consequently, HPLC methods were developed to determine only the major and active metabolite, ABZSO (Guermoche *et al.*, 1986; Lange *et al.*, 1988) or together with $ABZSO_2$ (Prichard *et al.*, 1985; Guan *et al.*, 1990; Zeugin *et al.*, 1990).

Zeugin *et al.* (1990) developed a procedure for ABZ, ABZSO and $ABZSO_2$ in human plasma using HPLC with UV detection at 290 nm to monitor the drugs in the treatment of human echinococcosis. However only ABZSO was determined quantitatively whereas ABZ and $ABZSO_2$ were determined qualitatively since $ABZSO_2$ has no activity against the parasite and ABZ normally is not detectable in man. Although this method is sufficiently sensitive, it is inconvenient due to the long retention

time of ABZ (~33 min.) and a lengthy liquid-liquid extraction procedure.

The detection of ABZ and metabolites in the HPLC methods described above were monitored by UV detection. ABZ can be detected as low as 10 ng/ml at 254 nm (Meulemans *et al.*, 1984) and the limit of detection of 2 ng/ml was reported for ABZSO and $ABZSO_2$ measured at 292 nm (Prichard *et al.*, 1985). Separate HPLC methods with fluorescence detection for ABZ, ABZSO and $ABZSO_2$ were described by Glänzer *et al.* (1988). The limits of detection were 2 ng/ml for ABZ and $ABZSO_2$, and 10 ng/ml for ABZSO. Although fluorescence methods gain sensitivity compared with UV detection (in the case of ABZ), because each compound requires its own excitation and emission wavelength for maximum sensitivity, the method is suitable for the determination of one compound only in one chromatographic analysis. In general, the use of fluorescence detection is limited due to the limited number of strongly fluorescing compounds, although this problem can be overcome by conversion of non-fluorescent compounds into fluorescent products.

A summary of HPLC methods for the determination of ABZ, ABZSO and $ABZSO_2$ is given in Table 1.1.

ABZ was also investigated in animal tissues (Prieto *et al.*, 1988; Barker *et al.*, 1990; Long *et al.*, 1990), meat (Marti *et al.*, 1990) and milk (Long *et al.*, 1989) using HPLC. Other analytical techniques have been used to determine ABZ and its metabolites. The metabolic studies of ABZ (Gyurik *et al*, 1981) were assessed using radiolabelled compounds in cattle, sheep, rats and mice. The products were assayed

by scintillation counting and identified by MS and NMR. Methods for detection of ABZ and other benzimidazoles by gas chromatography (GC) and verification of results by GC-MS have also been described (Marti *et al.*, 1990; Markus and Sherman, 1992). However an additional derivatization procedure was needed as the benzimidazoles are basic and show low volatility. Quantitative analysis of the derivatives was not possible by GC because the derivatives decomposed during the process of injection and chromatography due to the high temperatures used.

The HPLC methods described in this chapter were developed for the detection of ABZ and its two major metabolites simultaneously in plasma/serum and oil.

3.1.1 Sample preparation

Liquid-liquid extraction is the most commonly reported sample preparation procedure in the determination of ABZ and its metabolites in body fluids, but these methods are frequently tedious, time-consuming (Zeugin *et al.*, 1990) and require large volume of solvent (Bogan and Marriner, 1980; Prieto *et al.*, 1988). The blank extracts of body fluids were generally not clean (Meulemans *et al.*, 1984). These problems may be reduced by solid phase extraction (SPE) procedures. This procedure involves a partitioning of compounds between a solid and liquid phase rather than between two immiscible solvents as in liquid-liquid extraction.

SPE generally consists of a column or cartridge packed with a small quantity of sorbent, similar to those used in reversed-phase HPLC columns. The particle size of SPE sorbent is however, much larger

(40-50 μm) than the corresponding HPLC materials. This is to allow flow-rates to be obtained without applying high vacuum. To separate a compound from the impurities, the first step involves conditioning the sorbent by wetting surface silanols and bonded functional groups with an organic solvent such as methanol or acetonitrile and followed by water or buffer to prepare the sorbent for the sample. Once the conditioning step has commenced, the top of the sorbent should not be allowed to dry out because excessive drying can alter retention characteristics of the sorbent and may result in variable recoveries. The sample is then introduced to the cartridge and the components either become adsorbed onto the sorbent or pass through unretained. Impurities may be removed from the cartridge by washing the cartridge with suitable aqueous buffers and solvents of varying strength. Finally the compounds of interest are eluted with solvent such as methanol, acetonitrile or a mixture of organic solvent with water or buffer.

An SPE procedure for ABZ and ABZSO was described by Hurtado *et al.* (1989) using C_{18} Sep Pak cartridges. Methanol (3 ml) was used to elute the compounds producing cleaner extracts for chromatographic analysis with recoveries of >95%.

The advantages of using SPE procedures are selectivity and rapid extraction, small volumes of solvent give complete recovery and clean extracts of body fluids (Allan *et al.*, 1980; Hurtado *et al.*, 1989). The disadvantage of this procedure is potential lack of reproducibility due to slight variation in batches of sorbent.

Table 3.1 Summary of HPLC methods for analysis of ABZ, ABZSO and $ABZSO_2$ in plasma or serum. (LLE: liquid-liquid extraction; SPE: solid phase extraction)

References	Column	Mobile phase	Flow-rate (ml/min)	Detector and limit of detection	Sample treatment and recovery
Bogan & Marriner, 1980	ODS Hypersil 10 µm	MeOH-ammonium carbonate (0.05M) (65:35%, v/v)	1.5	UV 292nm ABZ :20 ng/ml	LLE ABZ :73-103%
Marriner *et al.*, 1981	ODS Hypersil 10 µm	MeOH-H_2O-perchloric acid(80:20: (1.1%w/v))	1.5	UV 292nm ABZSO :300 ng/ml ABZSO2: 50 ng/ml	LLE
Meulemans *et al.*, 1984	µBondapak C_{18}	MeOH-H_2O (65:35%,v/v)	1.5	UV 254nm ABZ :10 ng/ml	LLE ABZ : 94-102%
	µPorasil C_{18}	ACN-MeOH-H_2O (259:12:15, v/v/v)	1.5	ABZSO:10 ng/ml	ABZSO : 93-101%
Alvenerie & Galtier, 1984	normal-phase Partisil	hexane-EtOH (445:55,v/v)	1.5	UV 225nm ABZ :20 ng/ml ABZSO :50 ng/ml $ABZSO_2$:40 ng/ml	LLE ABZ :78.2% ABZSO :84.2% $ABZSO_2$:81.2%

Prichard *et al.*, 1985	C_{18} Radial-Pak A, 10 µm	ACN- 0.025M ammonium carbonate pH 7.5 (32:68%)	1.0	UV 292nm ABZSO : 2 ng/ml $ABZSO_2$: 2 ng/ml	SPE ABZSO & $ABZSO_2$ >90%
Guermoche *et al.*, 1986	µBondapak C_{18}	0.05M acetate buffer pH 4.7 -ACN (78:22%)	1.5	UV 220 or 313nm ABZSO :20 ng/ml	LLE ABZSO: 95-97%
Prieto *et al.*, 1988	Novapak C_{18}	ACN-TEA-H_2O (60:15:35%, v/v/v) pH 3.0	0.3	UV 292 nm	LLE ABZ: >80%
Glänzer *et al.*, 1988	µBondapak C_{18}	ACN-0.01M phosphate buffer (30:70%,v/v)	1.0-1.3	Fluorescence excitation:300nm emmision :320nm ABZSO :10 ng/ml $ABZSO_2$: 2 ng/ml	SPE ABZSO :98±7.5% $ABZSO_2$:96±6.3%
	as above	ACN-0.01M phosphate buffer (35:65%,v/v)	as above	excitation:304nm emmision :350nm ABZ : 2 ng/ml	ABZ :85±18.2%
Lange *et al.*, 1988	LiChroSorb Si-60 5 µm	MeOH-ACN-H_2O (93:5:2,v/v/v)	2.0	UV 290 nm ABZSO:56.2 ng/ml	LLE

Hurtado *et al.*, 1989	ODS C_{18} 5 µm	MeOH-0.05M phosphate buffer pH 5.7 (70:30%, v/v)	0.8	UV 295 nm ABZ :20 ng/ml ABZSO:15 ng/ml	SPE ABZ & ABZSO 95-105%
Guan *et al.*, 1990	µBondapak C_{18}	MeOH + 0.4% acetic acid	2.0	UV 290 nm ABZSO :3.05 ng/ml $ABZSO_2$:1.24 ng/ml	LLE ABZSO :96% $ABZSO_2$:91%
Zeugin *et al.*, 1990	Nucleosil 100, C_{18}	phosphate buffer-ACN/MeOH(560/90,v/v) (75:25%)	1.1	UV 290 nm ABZSO :14 ng/ml $ABZSO_2$:30 ng/ml	LLE ABZSO :65±2% $ABZSO_2$:82±7%
Hoaksey *et al.*, 1991	µBondapak phenyl 10 µm	H_2O(1%TEA)-MeOH-ACN (70:10:20%)	2.5	UV 254 nm	LLE ABZ :97% ABZSO:75%

3.2 METHODS

3.2.1 Simultaneous Determination of Albendazole, Albendazole Sulphoxide and Albendazole Sulphone in Biological Fluids.

3.2.1.1 Reversed-phase HPLC

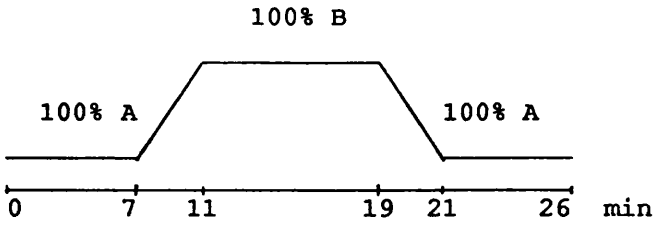
(a) A Spectra Physics Liquid Chromatograph equipped with a pump with a tertiary gradient generator and automatic injector, a variable wavelength UV detector and a computing integrator were used. The samples were separated using a Nucleosil ODS C_{18} column (250 mm x 4.6 mm, 5 µm particle size). The mobile phase consisted of acetonitrile and 0.05M ammonium acetate buffer (pH 4.7) based on the work of Guermoche *et al.* (1986). The flow-rate was kept constant at 1 ml/min and detection was carried out at 292 nm with sensitivity of 0.01 AUFS.

Initially, isocratic separations of ABZ, ABZSO, $ABZSO_2$ and internal standard, mebendazole (MBZ) were evaluated with different compositions of acetonitrile ranging from 22 to 60% v/v acetonitrile. Because of their wide k' range ($1<k'<20$), a gradient elution was developed using the same mobile phase.

Gradient elution analysis commenced with solvent reservoir A (30:70% v/v acetonitrile-acetate buffer) supplying 100% of the mobile phase. A linear gradient began at 7 min which brought solvent reservoir B (40:60% v/v acetonitrile-acetate buffer) mobile phase to 100% at 11 min and was maintained up to 19 min. The mobile phase was then returned to 100% A at 21 min. This condition was held for 5 min for equilibration to occur before the next analysis was begun. These conditions were used to determine ABZ, ABZSO and $ABZSO_2$ in most of the rat studies described in Chapter 5.

A - 30% acetonitrile

B - 60% acetonitrile

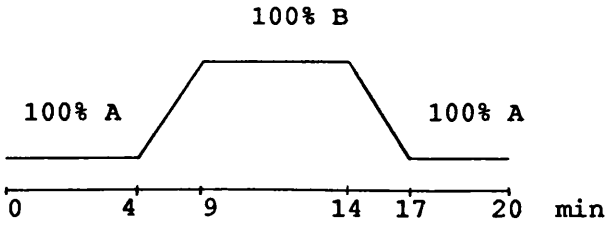


(b) The gradient elution analysis was modified when the HPLC method was used in Universiti Sains Malaysia (USM), Penang, Malaysia. The instrument used for this analysis was a two-solvent delivery system and an automated gradient controller (Waters-Millipore). The absorbance was recorded at 292 nm with sensitivity of 0.01 AUFS using a variable UV detector (Gilson).

The analysis started with 100% mobile phase from reservoir A (25:75%v/v acetonitrile-acetate buffer). A linear gradient began at 4 min until at 9 min solvent reservoir B (60:40% v/v acetonitrile:acetate buffer) was supplying 100% mobile phase and this was maintained until 14 min. The mobile phase was brought to 100% A at 17 min and 3 min was allowed for equilibration time.

A - 25% acetonitrile

B - 60% acetonitrile



3.2.1.2 Calibration Curves for Standard Solutions

Standard solutions containing all three compounds ABZ, ABZSO and $ABZSO_2$ at concentrations of 10, 20, 50, 100, 200, 400, 800 and 1600 ng/ml were prepared in mobile phase for HPLC. These solutions, (injection volume of 50 µl) were used to measure the linearity and sensitivity of the detector within the range of the concentrations used.

3.2.1.3 Preparation of Plasma Samples

Sample preparation was carried out based on an SPE procedure reported in the literature (Hurtado *et al.*, 1989) with some modifications.

50 µl internal standard solution (MBZ, 500 ng/ml), and appropriate volumes of standard solutions were evaporated in test-tubes. Plasma (1 ml) was added to the test-tube followed by 1 ml of 0.01M phosphate buffer (pH 7.4) and vortex-mixed for 30 sec. The SPE column was conditioned by flushing with 5 ml MeOH and 5 ml 0.017M phosphate buffer (pH 5.5), followed by the plasma sample. The column was washed with 20 ml phosphate buffer (pH 7.4) and 1 ml water-MeOH (80:20% v/v) and the compounds eluted with 3 ml acidic MeOH (0.033M HCl in MeOH) into tapered test-tubes, and evaporated to dryness under a gentle stream of nitrogen gas in a water-bath at 40°C. The residues were redissolved in 500 µl of mobile phase and aliquots of 50 µl were injected into HPLC (in duplicate).

3.2.1.4 Calibration Curves for Plasma Samples

Spiked plasma samples containing ABZ, ABZSO and $ABZSO_2$ at concentrations of 50-1600 ng/ml and a fixed concentration of internal standard, MBZ (50 µl of 10 µg/ml) were extracted using SPE procedure

described previously (3.2.1.3) and analysed by HPLC (3.2.1.1(a)).

The peak area ratio of ABZ, ABZSO or $ABZSO_2$ internal standard were calculated and plotted against the corresponding concentrations of ABZ, ABZSO and $ABZSO_2$. All unknown plasma samples were treated the same way and concentrations were determined from the calibration curves. The calibration curves were prepared on four different days.

3.2.1.5 Recoveries from Plasma

The recoveries of ABZ, ABZSO and BAZSO2 were determined by comparison of peak areas of spiked plasma samples (3.2.1.4) with the peak areas of the standard solution of the corresponding concentrations (3.2.1.2). The recovery of the internal standard, MBZ was determined similarly at a fixed concentration (500 ng/ml) to assure good and consistent recovery.

3.2.1.6 Within Day and Day to Day Reproducibility

The within day variations were determined by analysing a range of concentrations (50-1600 ng/ml) of ABZ, ABZSO and $ABZSO_2$ spiked in plasma. Three replicates of each concentration were extracted and analysed by HPLC on the same day.

Three concentrations of ABZ, ABZSO and $ABZSO_2$ (low, medium and high) were used to measure the day to day variations. The spiked plasma samples were analysed as described previously on six different days.

3.2.1.7 Limits of Detection

The limit of detection of the method used was assessed with spiked plasma samples at concentrations of 50, 40, 20 and 10 ng/ml of ABZ, ABZSO and $ABZSO_2$. The "concentrations" were determined from the calibration curves.

3.2.2 Simultaneous Determination of Albendazole, Albendazole Sulphoxide and Albendazole Sulphone in Oil

3.2.2.1 Normal-phase HPLC

The normal-phase HPLC method was developed for the analysis of ABZ, ABZSO and $ABZSO_2$ in Miglyol (oil) as part of the solubility studies. A preliminary separation of ABZ and Miglyol was carried out using a TLC method. A good separation was obtained using a mobile phase system of hexane-ethanol (90:10% v/v). This mobile phase system was subsequently used in the normal-phase HPLC method.

The HPLC system consisted of a SSI LC pump (model 330), a Pye-Unicam variable wavelength UV/Vis detector and a Hewlett-Packard integrator (HP3390A). Analysis was performed on a normal-phase Hypersil C_{18} silica column (100 mm x 4.6 mm, 5 µm particle size) with hexane-ethanol (90:10% v/v) as mobile phase at a flow-rate of 1 ml/min. The detection was measured at 292 nm with sensitivity of 0.01 AUFS.

3.2.2.2 Calibration Curves for Standard Solutions

Standard solutions of ABZ, ABZSO and $ABZSO_2$ were prepared in mobile phase. The concentration range was 20-5000 ng/ml and aliquots of 20 µl were injected into the HPLC (3.2.2.1).

3.2.2.3 Calibration Curves for Oil Samples

Standard calibration curves of ABZ , ABZSO and $ABZSO_2$ were generated by adding known concentrations of the three compounds (50-5000 ng/ml) to a fixed concentration of internal standard, MBZ (2000 ng/ml) in Miglyol and diluted with mobile phase. The peak area ratio of each compound to internal standard was calculated and plotted against the corresponding concentration. The peak area ratio of the unknown samples were treated similarly and the concentrations were determined from the calibration curves using linear regression method. The calibration curves were prepared on four separate days.

3.2.2.4 Within Day and Day to Day Reproducibility

The within day variation was studied by injecting solutions of 100 to 5000 ng/ml of ABZ, ABZSO and $ABZSO_2$ in oil. Three replicates of each concentrations were analysed on the same day.

The day to day variation was determined on solutions of 200, 500 and 5000 ng/ml of ABZ, ABZSO and $ABZSO_2$ of the three compounds. The analysis was carried out for several days.

3.2.3 Determination of Albendazole in DMSO and 95% EtOH by UV Spectrophotometry.

The UV absorbance of ABZ in DMSO and 95%EtOH was measured in a 10 mm quartz cell using a Perkin-Elmer Lambda3 UV/VIS spectrophotometer at the required maximum absorbances of 298 and 296 nm in DMSO and 95% EtOH, respectively. The determination of ABZ in these solvents was part of the solubility studies.

Stock solutions of ABZ in DMSO and 95%EtOH of 100 µg/ml were prepared and diluted to give ABZ concentrations of 1-30 µg/ml in DMSO and 1-40 µg/ml in 95%EtOH. Calibration curves for ABZ in these solvents were generated by measuring the absorbances and the data were analysed by linear regression analysis. The unknown samples of ABZ in DMSO and 95%EtOH obtained from the solubility studies (4.2.2.1) were diluted with the respective solvent and absorbance measured. The concentration of these samples was determined from the calibration curves. Three replicate calibration curves were prepared for each solvent.

3.2.4 Analysis of data

Least-square regression statistical analysis was used for calibration data of ABZ, ABZSO and $ABZSO_2$ to determine the slope, intercept and correlation coefficient (r). The equation obtained was used to determine the concentrations of the unknown samples.

The Bartlett test (Appendix 1) and analysis of variance (ANOVA) were used appropriately for comparison of data.

3.3 RESULTS

3.3.1 Analysis of Albendazole, Albendazole Sulphoxide and Albendazole Sulphone in Plasma

Isocratic analysis of ABZ, ABZSO and $ABZSO_2$ simultaneously was not possible due to the long retention time of ABZ (>50 min) at low concentrations of acetonitrile. At 40% and 50% acetonitrile there was no baseline resolution between ABZSO and $ABZSO_2$, R_s= 1.03 and 0.90, respectively. At 60% acetonitrile ABZSO and $ABZSO_2$ were eluted together. Capacity factor, k' for all the compounds were calculated for a range of acetonitrile concentrations as shown in Table 3.1.

Table 3.1 Capacity factors and retention times for ABZ, ABZSO and $ABZSO_2$ in reversed-phase HPLC isocratic analysis.

% of acetonitrile	Retention times (min.)			k'		
	ABZ	ABZSO	$ABZSO_2$	ABZ	ABZSO	$ABZSO_2$
22		11.7	16.6		3.41	5.30
25		8.01	11.1		2.02	3.20
30	50.3	6.38	8.77	18.0	1.42	2.31
35	26.7	4.97	6.37	9.12	0.88	1.40
40	15.7	4.29	5.07	4.93	0.62	0.91
50	8.50	3.55	3.95	2.20	0.34	0.49
60	5.81	3.28	3.28	1.19	0.24	0.24

A gradient elution system was developed for the separation of ABZ, ABZSO and $ABZSO_2$ by trial and error based on the isocratic analysis. The retention times of ABZ, ABZSO and $ABZSO_2$ and internal standard MBZ were 19.24, 5.54, 8.06 and 16.61 minutes, respectively. with an analysis time of 26 minutes. The mean k' for the four compounds are given in Table 3.2 and all the peaks were symmetrical (Table 3.2). A representative chromatograms of a standard solution is shown in Fig. 3.2(a).

Table 3.2 Retention data of gradient analysis for ABZ, ABZSO, $ABZSO_2$ and MBZ. (n = 6)

Compounds	k'		A_s
	Mean	RSD(%)	
ABZSO	1.06	1.56	1.07
$ABZSO_2$	2.00	1.77	1.13
MBZ	5.22	0.77	1.13
ABZ	6.26	1.68	1.07

Linear calibration curves for standard solutions were obtained in the range of 0-1600 ng/ml (r = 0.999). The mean slopes were $2.08x10^{-3}$ (4.20% RSD) for ABZ, $2.81x10^{-3}$ (1.56% RSD) for ABZSO and $3.03x10^{-3}$ (2.95% RSD) for $ABZSO_2$.

Similarly the calibration curves for plasma extracts were linear over the range 0-1600 ng/ml for the three compounds (r = 0.997-0.999). Table 3.3 shows the linear regression data for the calibration curves with mean slopes (±SD) of $2.12x10^{-3}\pm3.30x10^{-5}$ (χ^2 = 1.67), $2.80x10^{-3}\pm6.40x10^{-5}$ (χ^2 = 4.41) and $2.91x10^{-3}\pm1.00x10^{-5}$ (χ^2 = 5.02) for ABZ, ABZSO and ABZSO2, respectively. The replicate slopes for each compound were not significantly different (χ^2_{tab} = 9.46 at P = 0.05) and the intercepts for all the curves were not significantly different from zero (P<0.05). Fig. 3.2 shows typical calibration curves for ABZ and the 2 metabolites.

Extraction of plasma samples produced clean extracts as shown in Fig. 3.3(b)-(d). The mean analytical recovery (±SD) was 95.5%±3.28 for ABZ, 94.1%±2.70 for ABZSO and 95.7%±2.00 for $ABZSO_2$. Table 3.4 shows the recoveries of the three compounds at different concentrations. The recovery of MBZ was 99.2±3.87 with RSD of 3.90%.

The within day and day to day variations were between 0.67 and 8.82% as shown in Tables 3.5 and 3.6. The results from the day to day variation were not significantly different when compared using the analysis of variance ($P<0.01$). The detection limits of the method, taking a signal-to-noise ratio of two as the criterion were found to be 10 ng/ml (1 ng on-column) for ABZSO and 20 ng/ml (2 ng on-column) for ABZ and $ABZSO_2$.

Table 3.3 Linear regression data for ABZ, ABZSO and $ABZSO_2$ calibration curves using the reversed-phase HPLC method.

Replicate	Slope ($x10^3$)	SD ($x10^5$)	Intercept	SD	r
ABZ					
1	2.10	2.04	0.013	0.014	0.999
2	2.08	6.42	0.032	0.045	0.998
3	2.14	3.02	-0.003	0.021	0.999
4	2.15	4.88	-0.011	0.034	0.999
Mean	2.12*	4.09	0.008	0.028	
ABZSO					
1	2.75	5.54	-0.043	0.039	0.999
2	2.85	6.74	-0.054	0.047	0.999
3	2.85	4.12	-0.021	0.029	0.999
4	2.73	4.29	-0.031	0.030	0.999
Mean	2.80*	5.17	-0.037	0.036	
$ABZSO_2$					
1	2.90	7.31	-0.031	0.051	0.998
2	3.09	0.10	-0.058	0.071	0.997
3	2.87	3.27	-0.034	0.023	0.999
4	3.00	8.46	-0.034	0.059	0.999
Mean	2.96*	7.31	-0.046	0.051	

* (not significantly different at P = 0.05)

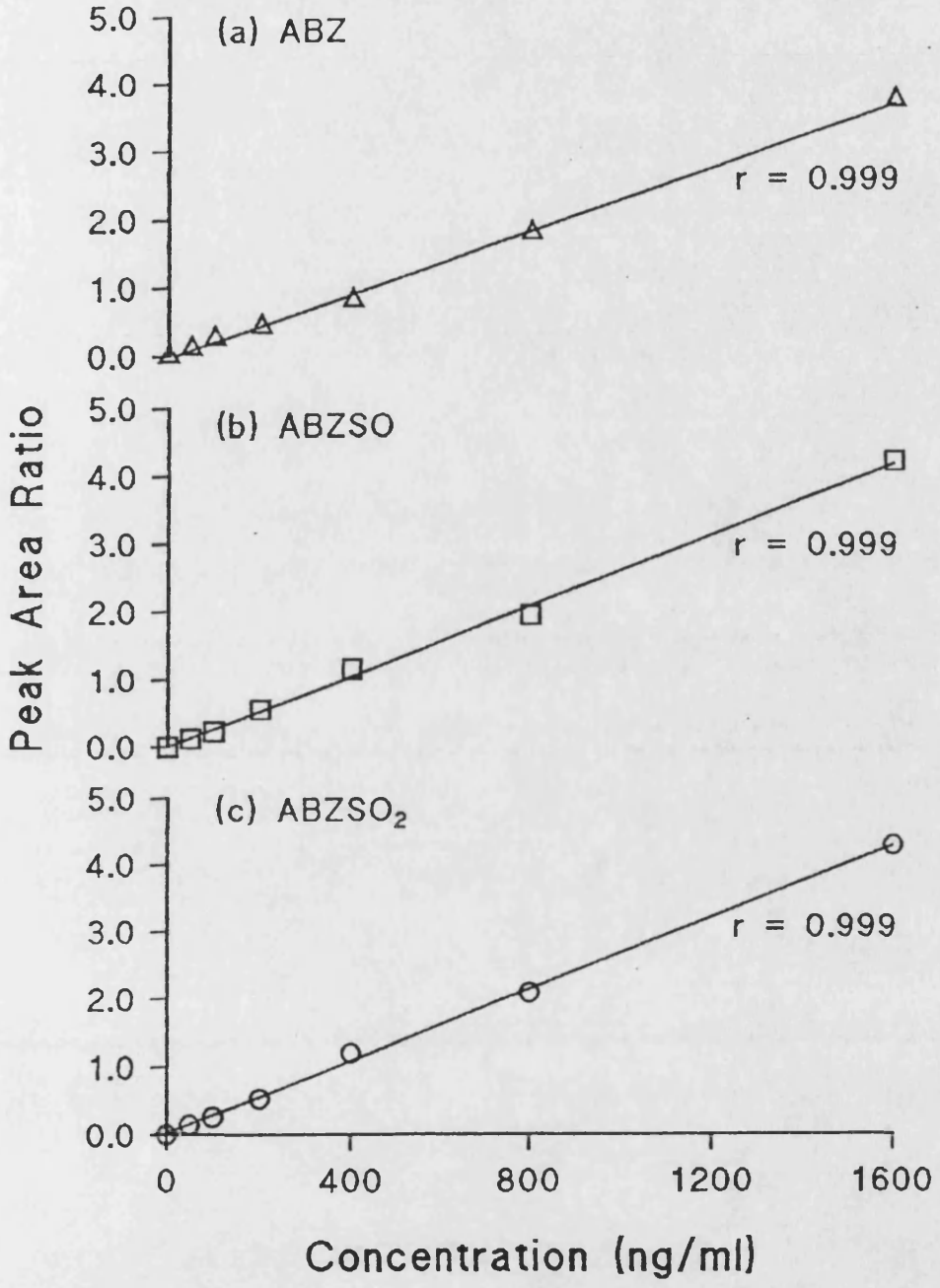


Fig. 3.2 Typical calibration curves for (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ using reversed-phase HPLC method described in 3.2.1.1(a).

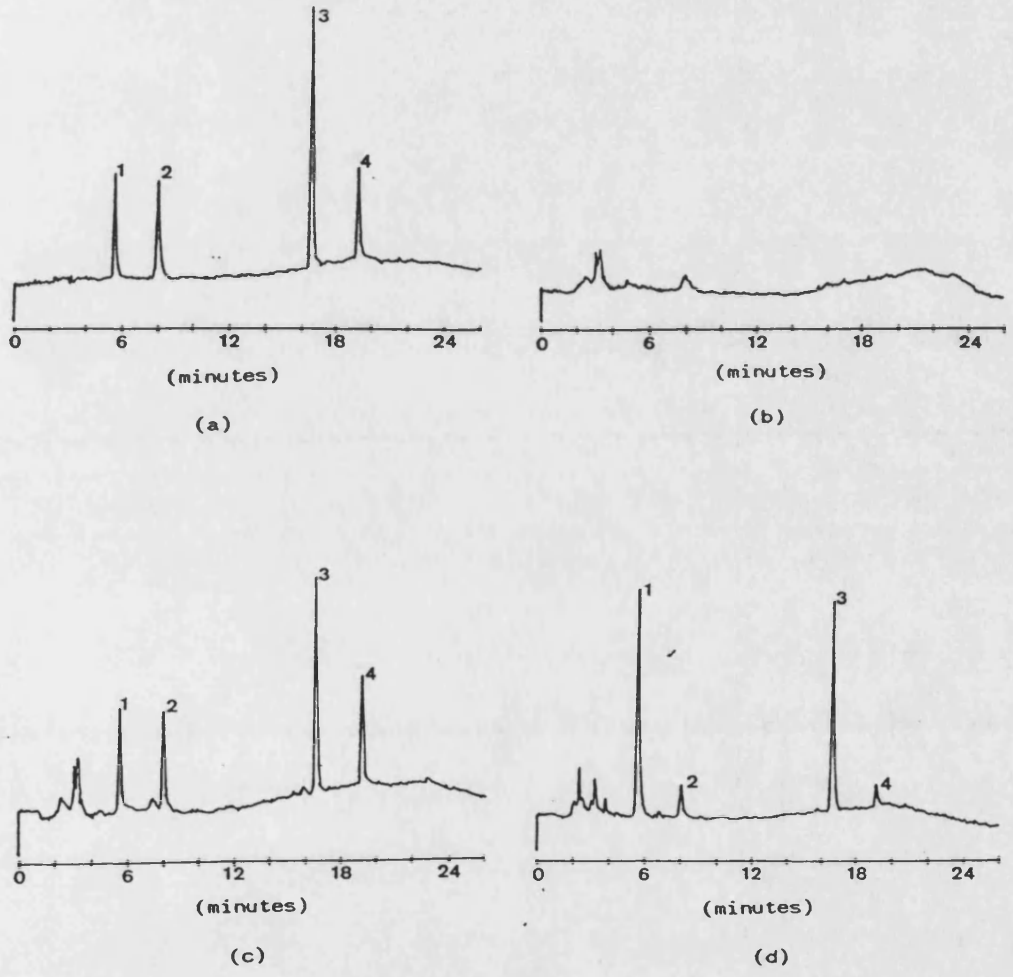


Fig. 3.3 Representative chromatograms of reversed-phase HPLC of (a) standard solution of 800 ng/ml of ABZSO (1), $ABZSO_2$ (2), MBZ (3) and ABZ (4); (b) blank plasma extract; (c) spiked standards of 1600 ng/ml in plasma; (d) plasma extract obtained after oral administration of 5 mg/kg ABZ in rat.

Table 3.4 Recoveries of ABZ, ABZSO and $ABZSO_2$ from plasma using reversed-phase HPLC method. (n =3)

Concentration (ng/ml)	Mean recovery (%)	SD	RSD (%)
ABZ			
50	90.5	6.10	7.58
100	92.7	5.03	5.43
200	99.1	4.37	4.41
400	96.8	4.51	4.66
800	97.9	3.43	3.50
1600	95.9	1.87	1.95
ABZSO			
50	90.8	5.36	5.90
100	93.2	5.86	6.27
200	95.3	5.30	5.56
400	91.5	3.45	3.77
800	97.7	3.26	3.34
1600	96.0	1.61	1.68
$ABZSO_2$			
50	93.1	7.40	7.95
100	97.8	5.97	6.14
200	96.7	5.08	5.25
400	93.4	4.28	4.58
800	96.7	2.55	2.64
1600	96.5	1.88	1.94

Table 3.5 The within day variation for ABZ, ABZSO and $ABZSO_2$ using the reversed-phase HPLC method. (n = 3)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
50	47.4	4.18	8.82
100	97.8	5.10	5.21
200	202.6	5.08	2.51
800	797.2	9.75	1.22
1600	1596.4	10.70	0.67
ABZSO			
50	50.0	3.98	7.98
100	102.3	6.79	6.79
200	203.3	8.79	4.32
800	801.9	13.27	1.65
1600	1596.9	12.00	0.75
$ABZSO_2$			
50	49.6	3.67	7.40
100	98.0	5.65	5.77
200	198.0	7.12	3.60
800	797.2	8.68	1.09
1600	1598.0	14.87	0.93

Table 3.6 The day to day variation for ABZ, ABZSO and $ABZSO_2$ using the reversed-phase HPLC method. (n = 6)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
50	49.2(F = 4.16)*	4.02	8.17
200	200.7(F = 2.64)*	7.02	3.50
800	798.9(F = 4.19)*	6.22	0.78
ABZSO			
50	50.8(F = 5.45)*	3.91	7.70
200	199.1(F = 5.40)*	6.27	3.15
800	797.6(F = 2.61)*	6.23	0.78
$ABZSO_2$			
50	47.9(F = 5.06)*	3.54	7.39
200	201.6(F = 4.60)*	6.69	3.32
800	799.9(F = 2.75)*	6.27	0.78

* not significantly different at P = 0.01 (F_{tab} = 6.07)

3.3.2 Analysis of Albendazole, Albendazole Sulphoxide and Albendazole Sulphone in Oil

A preliminary TLC analysis showed a good separation between ABZ and the Miglyol with R_f of 0.11 for ABZ and 0.20, 0.45 for the Miglyol.

The normal-phase HPLC was developed for separation of Miglyol, ABZ, ABZSO and $ABZSO_2$ with MBZ as the internal standard. The retention times for ABZ, ABZSO, $ABZSO_2$ and MBZ were 2.15, 6.86, 4.86 and 3.05 minutes, respectively. The mean k' values calculated from 6 samples were found to be 0.58 (0.71% RSD) for ABZ, 1.24 (0.44% RSD) for MBZ, 2.58 (0.56% RSD) for $ABZSO_2$ and 4.05 (0.51% RSD) for ABZSO. The peak shapes were symmetrical with A_s = 1.0, 1.11, 1.15 and 1.37 for ABZ, MBZ, $ABZSO_2$ and ABZSO. The oil was eluted with the solvent front. Representative chromatograms of oil in mobile phase and standard solution in oil diluted in mobile phase are shown in Fig. 3.4.

The standard calibration curves were linear over the range 0 to 5000 ng/ml for all the three compounds (r = 0.998-0.999) and the linear regression data are presented in Table 3.7. The mean slopes were $1.08x10^{-3}\pm1.26x10^{-5}$ (χ^2 = 0.64) for ABZ, $1.48x10^{-3}\pm3.87x10^{-5}$ (χ^2 = 6.10) for ABZSO and $1.45x10^{-3}\pm2.50x10^{-5}$ (χ^2 = 1.27) for $ABZSO_2$. The replicate slopes for each compound were not significantly different (χ^2_{tab} = 9.46 at P = 0.05). The intercepts for the curves passed through the origin within ±2 SD. Fig. 3.5 shows a typical calibration curve for ABZ, ABZSO and $ABZSO_2$.

The limits of detection were 50 ng/ml (2.5 ng on-column) for ABZ and 100 ng/ml (5 ng on-column) for ABZSO and $ABZSO_2$. The within day and day to day variations were 0.32-6.66% as tabulated in Tables 3.8 and

3.9. When compared by analysis of variance the data from the day to day variation were not significantly different within and between days ($P<0.01$).

Table 3.7 Linear regression data for ABZ, ABZSO and $ABZSO_2$ calibration curves using the normal-phase HPLC method.

Replicate	Slope ($x10^3$)	SD ($x10^5$)	Intercept	SD	r
ABZ					
1	1.06	1.11	0.026	0.027	0.999
2	1.08	3.54	-0.054	0.086	0.998
3	1.08	3.98	0.065	0.096	0.998
4	1.09	0.40	0.015	0.010	0.998
Mean	1.08*	2.26	0.013	0.055	
ABZSO					
1	1.50	2.78	-0.058	0.067	0.999
2	1.44	1.50	-0.041	0.036	0.999
3	1.47	3.31	-0.010	0.074	0.999
4	1.53	2.78	-0.022	0.058	0.999
Mean	1.48*	2.59	-0.033	0.059	
$ABZSO_2$					
1	1.46	1.63	-0.050	0.039	0.999
2	1.48	3.04	-0.018	0.074	0.998
3	1.42	1.28	0.006	0.031	0.999
4	1.45	3.14	0.064	0.071	0.998
Mean	1.45*	2.27	0.001	0.054	

* not significantly different at P = 0.05

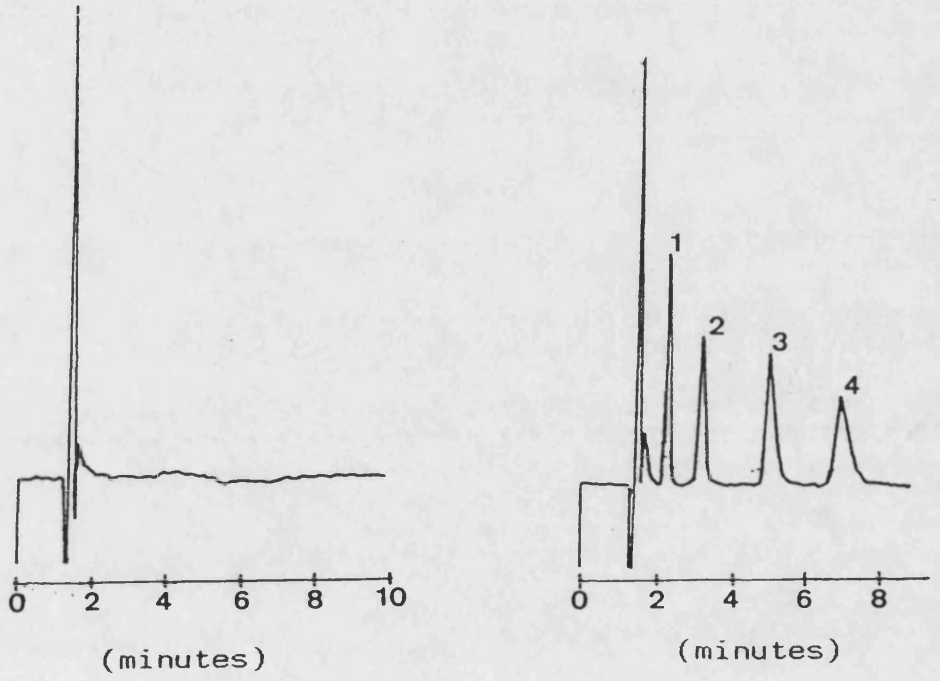


Fig. 3.4 Representative chromatograms of normal-phase HPLC of (a) Miglyol in mobile phase; (b) standard solution of 500 ng/ml of ABZ (1), $ABZSO_2$ (3), ABZSO (4) and MBZ (2).

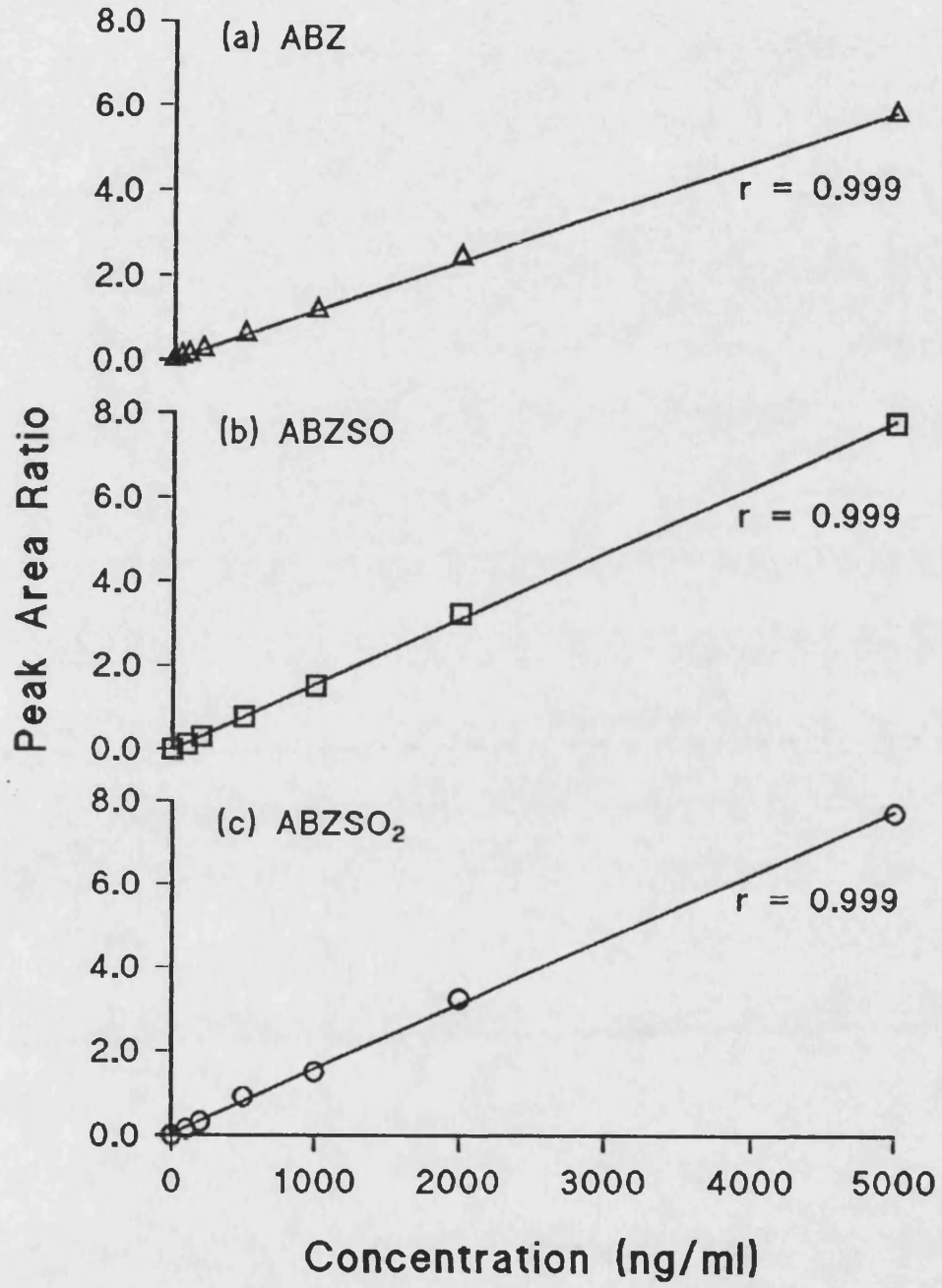


Fig. 3.5 Typical calibration curves for (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ using the normal-phase HPLC method described in 3.2.1.2.

Table 3.8 The within day variation for ABZ, ABZSO and $ABZSO_2$ using the normal-phase HPLC method. (n = 3)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
50	47.5	2.85	6.00
100	99.4	4.37	4.40
200	197.1	7.55	3.82
500	499.8	6.83	1.37
2000	2002.0	24.2	1.20
5000	4999.8	29.0	0.58
ABZSO			
100	98.9	6.57	6.68
200	198.4	8.80	4.44
500	498.6	8.40	1.68
2000	2007.1	23.7	1.18
5000	4999.5	22.4	0.45
$ABZSO_2$			
100	99.0	6.15	6.21
200	200.0	8.24	4.12
500	501.9	9.44	1.88
2000	1998.1	27.8	1.39
5000	4997.2	27.0	0.54

Table 3.9 The day to day variation for ABZ, ABZSO and $ABZSO_2$ using the normal-phase HPLC method. (n = 5)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
200	207.4 (F = 3.39)*	13.0	6.26
500	502.3 (F = 4.43)*	22.2	4.42
5000	4999.7 (F = 7.12)*	47.7	0.95
ABZSO			
200	197.1 (F = 5.57)*	9.99	5.07
500	498.8 (F = 3.14)*	8.35	1.67
5000	4991.5 (F = 7.04)*	16.0	0.32
$ABZSO_2$			
200	195.2 (F = 3.74)*	9.84	5.04
500	497.9 (F = 6.78)*	20.0	4.02
5000	4997.6 (F = 5.54)*	18.0	0.36

* not significantly different at P = 0.01 (F_{tab} = 7.34)

3.3.3 Validation of Reversed-phase HPLC Method

The reversed-phase HPLC method was validated before it was used in USM, Malaysia. Some modifications were made as described previously (3.2.1.1(b)). The retention times of ABZ, ABZSO, $ABZSO_2$ and internal standard, MBZ were 14.99, 8.11, 11.15 and 13.45 minutes, respectively. Representative chromatograms of standard solution, a blank plasma extract and a spiked plasma extract are shown in Fig. 3.6 and plasma sample extracts obtained after oral administration of ABZ tablet (400 mg) in man in Fig. 3.7.

The calibration curves for standard solution were linear over the range of 0-1600 ng/ml ($r = 0.999$). The mean slopes were 2.20×10^{-3} (3.27% RSD) for ABZ, 2.86×10^{-3} (1.34% RSD) for ABZSO and 22.82×10^{-3} (1.88% RSD) for $ABZSO_2$.

The calibration curves for plasma extracts for ABZ and the two metabolites were also linear over the range of 0-1600 ng/ml ($r = 0.997$-0.999). Table 3.10 shows the linear regression data with mean slopes of $2.20 \times 10^{-3} \pm 2.45 \times 10^{-5}$ ($\chi^2 = 0.65$) for ABZ, $2.90 \times 10^{-3} \pm 5.68 \times 10^{-5}$ ($\chi^2 = 1.89$) for ABZSO and $2.83 \times 10^{-3} \pm 6.60 \times 10^{-5}$ ($\chi^2 = 7.21$) for $ABZSO_2$. The replicate slopes for all the compounds were not significantly different ($\chi^2_{tab} = 9.46$ at $P = 0.05$) and the intercepts were not significantly different from zero ($P<0.05$). Fig. 3.8 shows typical calibration curve of ABZ, ABZSO and $ABZSO_2$.

Plasma samples were extracted using the SPE procedure described in 3.2.1.4 with mean recoveries (±SD) of 97.6%±2.19 for ABZ, 92.3%±3.24 for ABZSO and 96.3%±1.55 for $ABZSO_2$. The recoveries of the three compounds at different concentrations are shown in Table 3.11. The

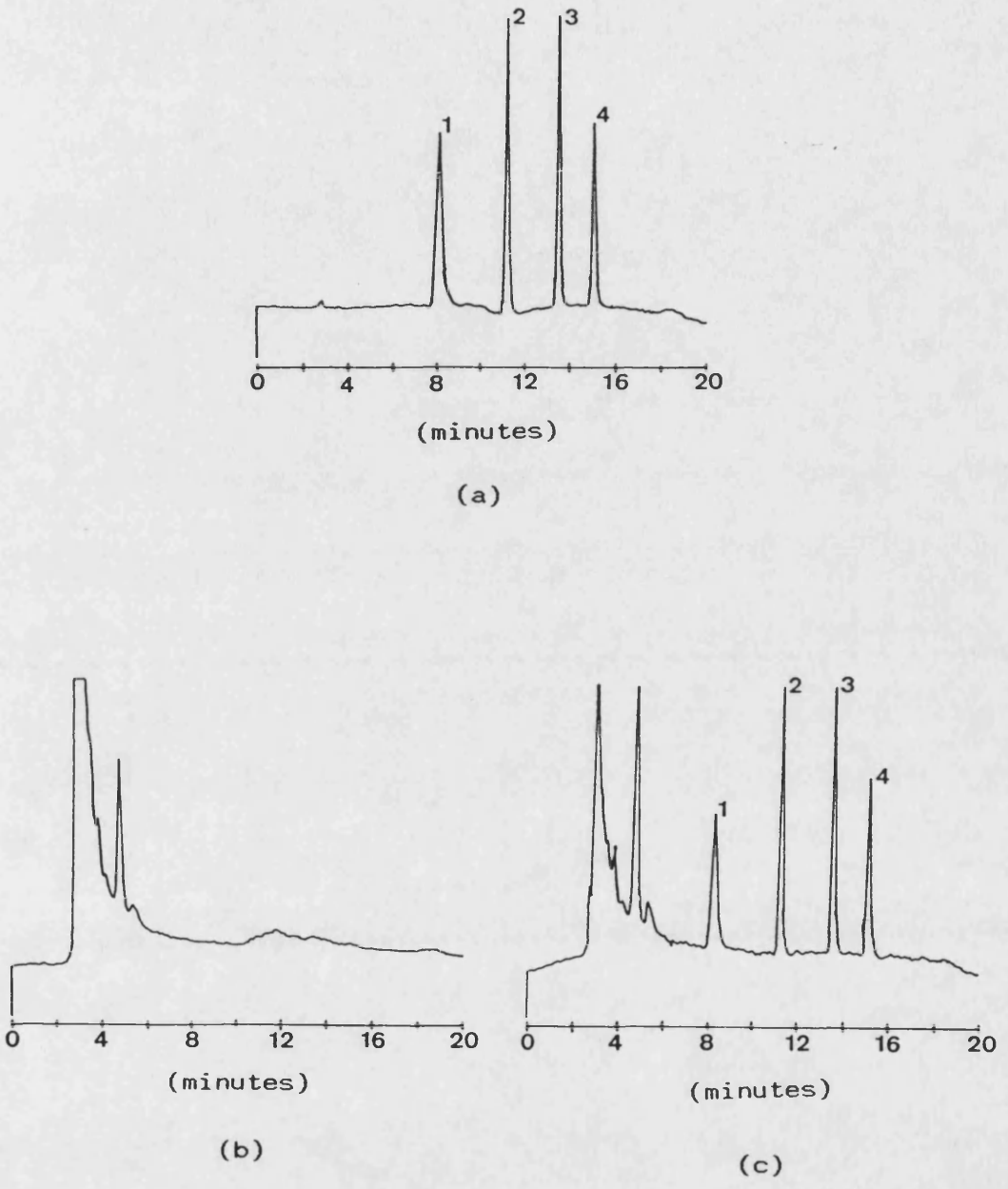
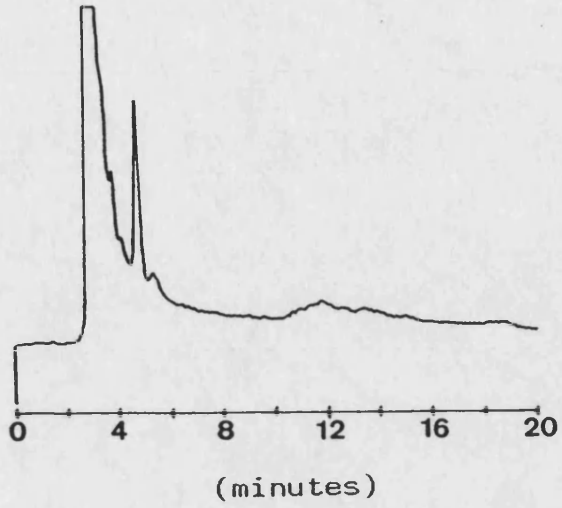
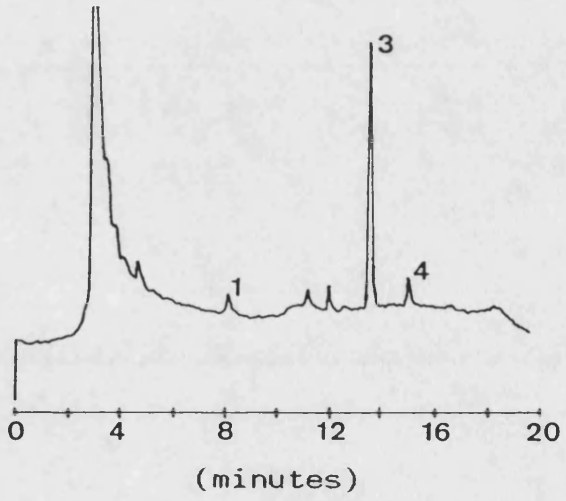


Fig. 3.6 Representative chromatograms of reversed-phase HPLC (USM) of (a) standard solution of 400 ng/ml of ABZSO (1), $ABZSO_2$ (2), ABZ (4) and MBZ (3); (b) blank plasma extract; (c) spiked plasma extract of 400 ng/ml.



(a)



(b)

Fig. 3.7 Representative chromatograms of extracted plasma obtained from human study after oral administration of 400 mg albendazole tablet at (a) pre-dose and (b) 2.5 hr after dosing (1 = ABZSO, 3 = MBZ and 4 = ABZ).

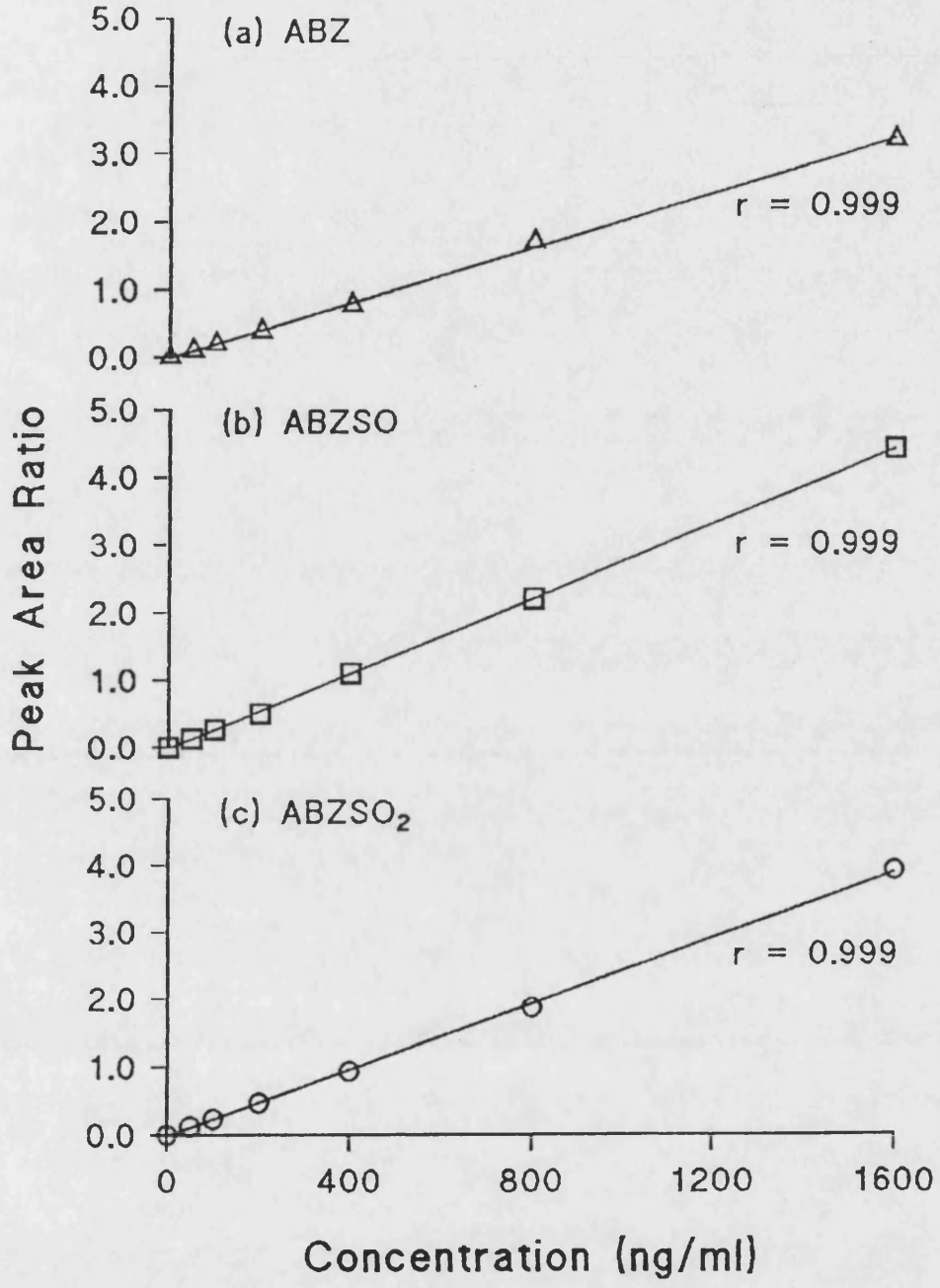


Fig. 3.8 Typical calibration curves for (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ using the reversed-phase HPLC method described in 3.2.1.1(b).

recovery of internal standard MBZ was 95.1%±0.34 with 3.78% RSD for extraction carried out on 6 samples.

The within day and day to day variations were in the range of 0.15 and 8.72% as tabulated in Tables 3.12 and 3.13. The results from the day to day variation were not significantly different within and between days ($P<0.01$). The limit of detection taking into account a signal-to-noise ratio of 2, were found to be 10 ng/ml for ABZSO and 20 ng/ml for ABZ and $ABZSO_2$.

Table 3.10 Linear regression data for ABZ, ABZSO and $ABZSO_2$ calibration curves using the reversed-phase HPLC method (USM).

Replicate	Slope ($x10^3$)	SD ($x10^5$)	Intercept	SD	r
ABZ					
1	2.19	4.60	-0.020	0.032	0.999
2	2.22	4.34	-0.044	0.030	0.999
3	2.22	5.72	-0.062	0.040	0.999
4	2.17	6.19	-0.025	0.043	0.998
Mean	2.20*	1.58	-0.040	0.036	
ABZSO					
1	2.89	10.0	0.016	0.070	0.997
2	2.98	4.83	-0.004	0.034	0.999
3	2.86	7.24	-0.013	0.051	0.999
4	2.86	5.35	-0.085	0.037	0.998
Mean	2.90*	6.86	-0.022	0.048	
$ABZSO_2$					
1	2.93	5.45	-0.033	0.038	0.999
2	2.82	3.17	-0.027	0.022	0.999
3	2.79	5.11	-0.066	0.036	0.999
4	2.79	2.79	-0.076	0.066	0.997
Mean	2.83*	4.13	-0.051	0.040	

* not significantly different at P = 0.05

Table 3.11 Recoveries of ABZ, ABZSO and $ABZSO_2$ from plasma using the reversed-phase HPLC method (USM).
(n = 3)

Concentration (ng/ml)	Mean recovery (%)	SD	RSD (%)
ABZ			
50	97.9	8.85	9.04
100	96.2	6.50	6.75
200	99.1	6.45	6.51
400	101.0	5.85	5.79
800	96.2	3.26	4.43
1600	95.1	1.92	2.06
ABZSO			
50	89.9	8.65	9.63
100	90.5	6.91	7.64
200	92.9	6.45	6.62
400	90.5	5.92	6.54
800	94.7	3.62	3.82
1600	95.3	2.65	2.78
$ABZSO_2$			
50	95.2	7.91	8.74
100	94.8	6.97	7.35
200	95.9	6.77	7.06
400	98.8	5.45	5.52
800	97.1	4.43	4.56
1600	94.9	2.30	2.42

Table 3.12 The within day variation for ABZ, ABZSO and $ABZSO_2$ using the reversed-phase HPLC method (USM).
(n = 3)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
50	47.6	4.15	8.72
100	101.0	6.09	6.03
200	201.0	11.6	5.76
800	795.3	11.5	1.45
1600	1598.0	18.1	1.13
ABZSO			
50	47.7	3.58	7.50
100	97.4	4.50	4.62
200	199.5	7.52	3.77
800	797.0	10.1	1.26
1600	1599.9	12.5	0.78
$ABZSO_2$			
50	48.2	3.51	7.28
100	96.5	3.98	4.12
200	199.2	6.67	3.35
800	798.4	8.99	1.12
1600	1597.6	10.8	0.63

Table 3.13 The day to day variation for ABZ, ABZSO and $ABZSO_2$ using the reversed-phase HPLC method (USM).
(n = 6)

Concentration (ng/ml)	Mean concentration determined (ng/ml)	SD	RSD (%)
ABZ			
50	47.8 (F = 5.80)*	4.03	8.43
200	199.8 (F = 6.01)*	6.98	3.49
1600	1600.1 (F = 3.02)*	5.60	0.35
ABZSO			
50	49.2 (F = 4.34)*	3.98	8.09
200	200.1 (F = 5.66)*	6.10	3.05
1600	1600.2 (F = 2.20)*	4.45	0.28
$ABZSO_2$			
50	48.2 (F = 4.64)*	3.96	8.22
200	199.8 (F = 4.45)*	4.52	2.26
1600	1601.4 (F = 0.60)*	2.43	0.15

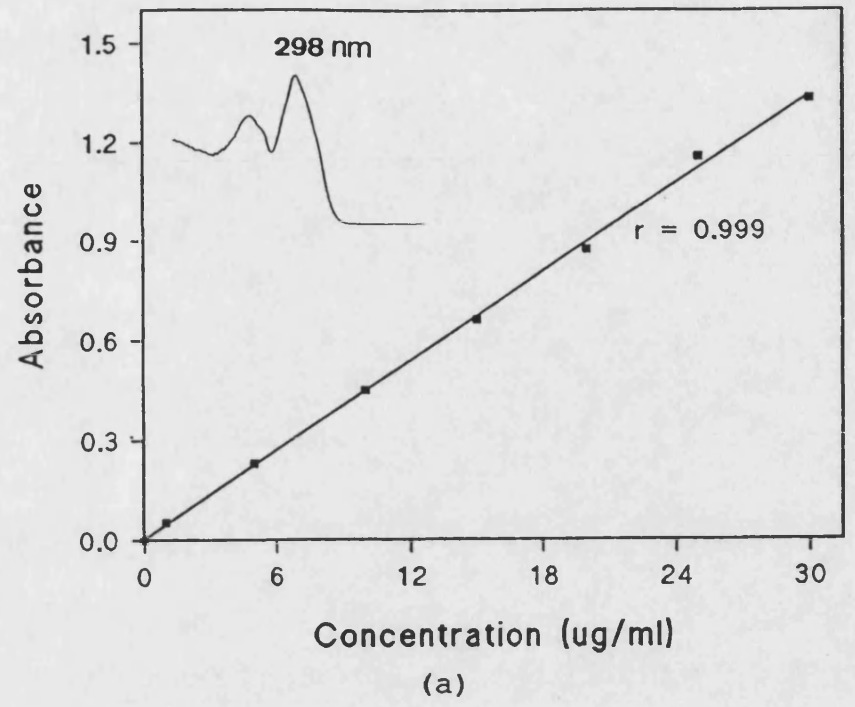
* not significantly different at P = 0.01 (F_{tab} = 6.07)

3.3.4 Calibration curves for albendazole in DMSO and 95% EtOH

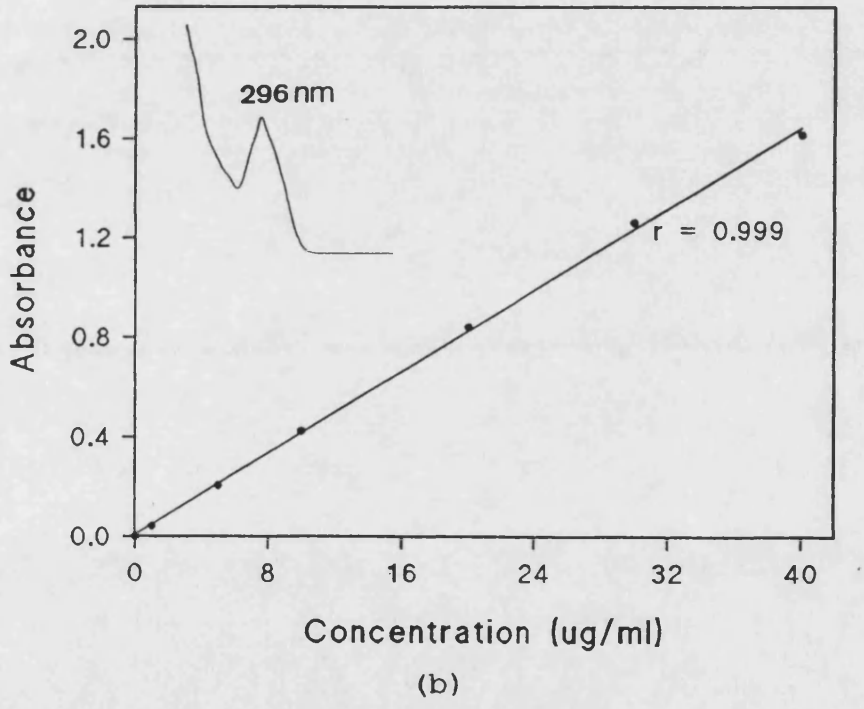
The calibration curves for ABZ in DMSO and 95% EtOH were linear over the range 0-30 µg/ml and 0-40 µg/ml, respectively. Table 3.14 shows the linear regression data for ABZ in these solvents. The replicate slopes for each solvents were not significantly different with mean slope of 0.044±0.0006 (χ^2 = 1.64) for ABZ in DMSO and 0.041 in 95%EtOH (χ^2_{tab} =5.99 at P = 0.05). All the intercepts passed through the origin within ±2 SD. Fig. 3.9 shows a typical calibration curve for ABZ in DMSO and 95% EtOH.

Table 3.14 Linear regression data for ABZ in DMSO and 95% EtOH.

Replicate	Slope	SD	Intercept	SD	r
In DMSO					
1	0.044	0.0007	0.0007	0.010	0.999
2	0.045	0.0006	0.003	0.012	0.999
3	0.044	0.0006	0.006	0.010	0.999
Mean	0.044	0.0006	0.003	0.011	
In 95%EtOH					
1	0.041	0.0007	0.010	0.015	0.999
2	0.041	0.0004	0.005	0.009	0.999
3	0.041	0.005	0.008	0.041	0.999
Mean	0.041	0.002	0.005	0.022	



(a)



(b)

Fig. 3.9 Typical calibration curves for ABZ in (a) DMSO and (b) 95% EtOH. Inserts are the UV absorbance of ABZ in the respective solvent.

3.4 DISCUSSION

The separation of ABZ and its metabolites was not achieved by reversed-phase HPLC isocratic analysis using 0.05M ammonium acetate buffer pH 4.7 and acetonitrile. The capacity factor, k' values of the three compounds fell outside the desired range of $1<k'<20$ when low acetonitrile concentration was used (22-35%). When the acetonitrile concentration was varied from 40 to 60% v/v, no baseline resolution was observed ($R_s<1.5$) between ABZSO and $ABZSO_2$. A gradient elution analysis was therefore developed based on these retention data with initial concentration of acetonitrile of 30% v/v to avoid any interference from plasma peaks. A good separation was achieved with k' value ranged between 1 and 7. The reproducibility of the retention times of all the compounds were good (0.77-1.77% RSD) considering it was a gradient analysis system and the shape of the peaks were sharp and symmetrical (A_s = 1.07-1.13). This is probably due to the excellent masking of residual silanol groups by ammonium acetate (Lim *et al.*, 1984) and therefore minimized the silanol effects of the column.

The advantage of employing gradient elution analysis is that the late eluting compounds still produce sharp peak shapes which helps to improve quantitation. The retention time of ABZ was considerably shorter (19.24 min.) using the gradient run compared to the isocratic analysis (~33 min.) developed by Zeugin *et al.* (1990).

Sample preparation using SPE was employed with modifications from the work reported by Hurtado *et al.* (1989). 1 ml of plasma was used in this extraction procedure and the compounds were eluted with acidic methanol since ABZ is more soluble in hydrochloric acid.

Pre-preparation of plasma prior to the SPE stage was carried out by diluting the plasma with buffer at pH 7.4 which may improve the selectivity of the extraction and reduce drug-protein binding. The extraction produced clean extracts as shown in Fig. 3.1(b) and there were no interfering peaks from the plasma. The recoveries were found to be comparable with those reported by Hurtado *et al.* (1989) although the initial volume of plasma used was reduced by 50%. The detection limits for ABZ (20 ng/ml) was comparable and were lower for ABZSO (10 ng/ml) and $ABZSO_2$ (20 ng/ml) compared to those detected at 225 nm (Alvinerie and Galtier, 1984) when the three compounds were analysed simultaneously. A lower ng/ml sample concentration may be determined by dissolving the final residue for analysis in smaller volume. Volume of 0.5 ml was used because the HPLC system (Spectra Physics auto-injector) needs a larger volume for duplicate injections of 50 µl sample. The within day and day to day reproducibility of the assay were within the acceptable limits for the analysis of biological samples for pharmacokinetics studies (<10% RSD).

Some adjustments were made to the gradient elution run when a different HPLC system was used in USM, Malaysia. The method was validated in the same manner as in Bath. The recoveries of all the three compounds were comparable to those found in Bath and the precisions were within the acceptable limits. Initially, there were problems in getting reproducible recoveries when the extracts were dried using a Speedvac Concentrator (where extracts were centrifuged under vacuum). It was realised that these problems probably arise owing to the drugs sticking strongly to the test-tube wall caused by the centrifugal force. The amount of drugs dissolved during the reconstitution step were therefore not consistent. This method was

tried as an alternative way to reduce the time taken to dry the extracts. However the extracts were dried by using the nitrogen gas. The validated method was then used to determine ABZ and the two metabolites in rat serum and human plasma.

As mentioned earlier, ABZ normally is not detectable in human plasma. A peak at a retention time of 14.94 minutes was observed in the extracted plasma obtained from a human volunteer who received 400 mg ABZ tablet which was not present in the pre-dose sample (Fig. 3.7). This retention time corresponded to the ABZ peak. There was no chromatographic interference from the any endogenous plasma compounds and therefore this peak could be ABZ. However this could not be confirmed by any other methods (eg. diode-array detector, LC-MS) because of limited volume of plasma sample. Nevertheless it was taken as ABZ peak in the determination of ABZ concentration in human plasma described in Chapter 5.

Although the previous HPLC methods developed to determine ABZ have acceptable sensitivity; the limits of detection of ABZ were low (<20 ng/ml) and the recoveries of ABZ from plasma were generally high (>90%), ABZ has not previously been detected in human plasma (Marriner *et al.*, 1986; Hurtado *et al.*, 1989; Hoaksey *et al.*, 1991). ABZ was only detected in some echinococcosis patients; in one study ABZ was detected in one out of 13 patients (Zeugin *et al.*, 1990). However the patient suffered from severe cholestasis and due to this obstruction ABZ was not recycled and therefore present in a higher concentration. In another study ABZ was detected <45 ng/ml in plasma of a few echinococcosis patients who received 5 to 7 mg/kg ABZ for two 39 consecutive days (Meulemans *et al.*, 1984). These patients

suffered from cirrhosis and therefore probably had difficulty in metabolising ABZ.

Possible explanations of this finding of an ABZ peak are (i) the use of gradient elution analysis which produces a sharp ABZ peak, (ii) the SPE procedure employed which gives clean plasma extracts and (iii) the detection wavelength used which matches the wavelength of maximum absorption of ABZ.

In the preliminary analysis, Miglyol and ABZ were well separated on the TLC plate. The same mobile phase system was then transferred to the HPLC system without any problems. The order of elution of ABZ, ABZSO and $ABZSO_2$ was reversed from the reversed-phase method as expected. Miglyol was eluted rapidly by the column and therefore did not interfere with the quantitation of ABZ. All the compounds were baseline resolved and the retention times were reproducible. The analysis time was <10 minutes. However this system was not as sensitive as the reversed-phase HPLC; the limits of detection for ABZ were 50 ng/ml and 100 ng/ml for ABZSO and $ABZSO_2$ using the normal-phase HPLC method whereas the limits of detection of ABZ and $ABZSO_2$ were 20 ng/ml and 10 ng/ml for ABZSO using the reversed-phase HPLC method.

The maximum absorbance of ABZ in DMSO and 95% EtOH was observed at 298 and 296 nm, respectively. The calibration curves of ABZ in these solvents were linear and reproducible and therefore can be used to determine ABZ concentration in the samples from the solubility studies.

In conclusion, the gradient elution reversed-phase HPLC system is sensitive, precise and reproducible for the determination of ABZ and it two metabolites, ABZSO and $ABZSO_2$ in plasma and this method can therefore be used in pharmacokinetic studies of ABZ.

Normal-phase HPLC method is simple, fast and reproducible for the analysis of ABZ and degradation products in the Miglyol and can be used as a method of quantitation for ABZ in the solubility studies.

CHAPTER 4

PHARMACEUTICAL ASPECTS

4.1 INTRODUCTION

The formulation aspects of this work are largely concerned with the aspects of stability and solubility of ABZ.

4.1.1 Stability

As demonstrated by the metabolism pathways described by Gyurik *et al.* (1981) and Penicaut *et al.* (1983) discussed in section 1.5.3, ABZ can undergo oxidative and hydrolytic degradation. It was necessary therefore to establish whether these processes could occur *in vitro* via simple thermal mechanism and to obtain some idea of the stability of drug in the various solvent systems of interest.

4.1.1.1 Hydrolysis

Hydrolytic reactions are pseudo-first order reactions and involve nucleophilic attack of the labile carbonyl centres such as those of lactones and esters by water. The rate of hydrolysis increases as temperature increases and generally follows an Arrhenius-type relationship:

$$k = Ae^{-EaRT} \qquad \text{Eqn 4.1}$$

or

$$\log k = \log A - \frac{E_a}{2.303\ RT} \qquad \text{Eqn 4.2}$$

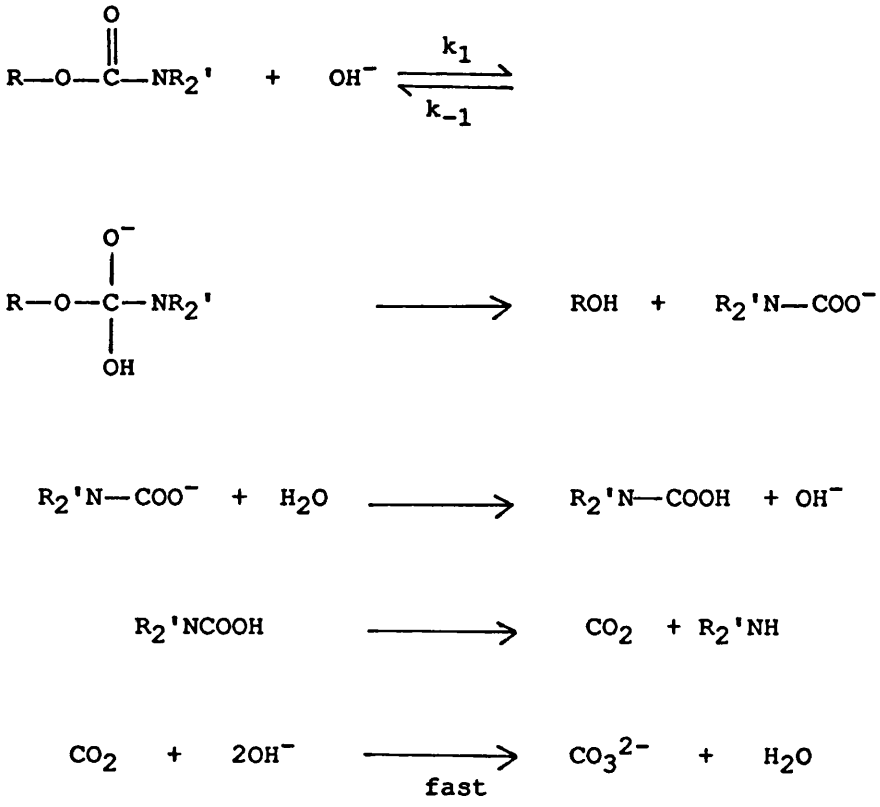
where k is the specific reaction rate, A is a constant termed as frequency factor, E_a is the activation energy, R is the gas constant and T is the temperature. Accelarated stability tests are carried out at higher temperatures to understand the rate and mechanism of the reactions as at low temperature when the rate is slow it may not be possible to study the process within a reasonable period of time. The rate constant at any temperatures may then be obtained by extrapolating from the plot of log k against 1/T.

The hydrolysis of many drugs is catalysed by pH (Connors *et al.*, 1968). In solution of high pH, the primary attacking species are the hydroxide ions and the rate of reaction depends on $[OH^-]$. At low pH solution, the hydrolysis rate depends on $[H^+]$ as the primary attacking species are the hydrogen ions.

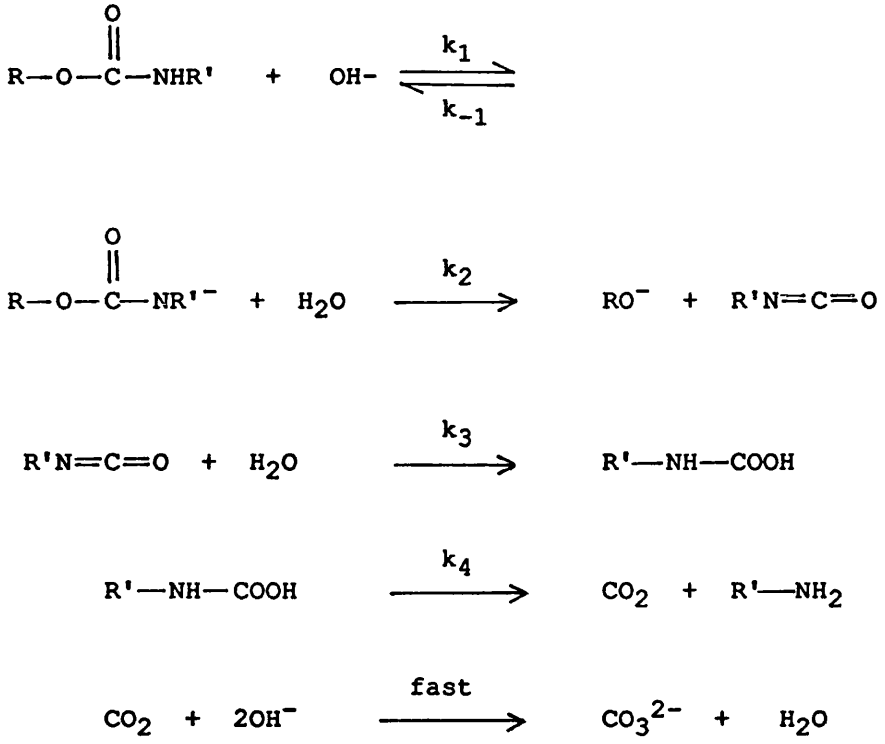
According to Dittert and Higuchi (1963), alkaline hydrolysis of carbamate esters is a pseudo-first order reaction and that two possible mechanisms are responsible for this hydrolysis; via hydroxide ion followed by a carbamate intermediate or via hydroxide ion attack followed by an isocyanate intermadiate (Fig. 4.1).

Fig. 4.1 The possible mechanisms of alkaline hydrolysis of carbamate.

(1) via hydroxide ion followed by a carbamate intermediate



(2) via hydroxide ion attack followed by an isocyanate intermediate



The second mechanism appears to be predominate in the reactions of N-substituted and N-monosubstituted aromatic compounds.

4.1.1.2 Oxidation

Oxidative reactions involve the removal of electrons from an atom or a molecule. The occurrence of these reactions does not necessarily involve molecular oxygen. However, pharmaceutical oxidations, often involve reactions with molecular oxygen and are termed as autoxidation. In an other type of autoxidation, the process is initiated by free radicals that are formed by actions of light, heat or trace metals.

Pharmaceutical compounds that are susceptible to oxidation such as antibiotics, vitamins and steroids are listed individually by Connors

et al. (1986) and the oxidation process may involve very complex pathways.

4.1.1.3 Degradation of Drugs in Suspensions

In the degradation of a drug in suspension, the amount of drug in the solution remains constant despite its degradation with time due to replenishment from the solid drug reservoir. The degradation process is therefore expected to follow apparent zero-order kinetics:

$$-dc/dt = k_o \qquad \text{Eqn 4.3}$$

where k_o is the apparent zero-order rate constant.

4.1.2 Solubility

Solubility of a drug is an important factor in its formulation since in whatever dosage form a drug is administered, the drug has to dissolve aqueous fluids of the gastrointestinal tract before absorption across the membrane can proceed.

Poorly water-soluble drugs such as griseofulvin (Carrigan and Bates, 1973) and cefoxitin (Schrogie *et al.*,1978) frequently have problems of low bioavailability primarily caused by the slow dissolution rate of the drug in the gastrointestinal tract. The dissolution rate of a drug is described by the Noyes-Whitney equation:

$$dm/dt = k_s A(S - C_t) \qquad \text{Eqn 4.4}$$

where dm/dt is the dissolution rate, k_s is the dissolution rate constant, A is the surface area of the dissolving solid, S is the solubility and C_t is the concentration of the drug in the medium at time t.

According to this equation, the solubility of a drug is one of the main factors that determine its dissolution rate. Solubility is also influence by temperature and pH of the solution as described in 4.2.2.1 and 4.2.2.2, respectively. The dissolution rate of a drug may be increased by reducing the particle size e.g. micronised form.

It was suggested that to avoid any potential problems associated with dissolution in gastrointestinal absorption, a drug should have an aqueous solubility greater than 10 mg/ml with the lower limit of 1 mg/ml (dissolution rate of 0.1 $mg/cm^2/min$). This indicates the need for a more soluble salt in these cases (Kaplan, 1972).

One approach to remove the dissolution step in the gastrointestinal tract is to incorporate drug into lipid (Palin *et al.*, 1986; Serajuddin *et al.*, 1988). Oil droplets will be formed in gastrointestinal aqueous fluids and the drug in its molecular state will partition from the oil droplets into the aqueous phase. The drug is thus available for absorption in molecular form.

4.1.2.1 Effect of Temperature

The solubility of a drug in a liquid depends on temperature. The effect can be described by a form of the Van't Hoff equation (4.5) which defines the relationship between absolute temperature and solubility, S.

$$\frac{d\log_e S}{dT} = \frac{\Delta H}{RT^2} \qquad \text{Eqn 4.5}$$

where ΔH is the standard heat of solution and R is the gas constant.

Integration of Eqn 4.5 gives

$$\log_e S = -\frac{\Delta H}{RT} + \text{constant} \qquad \text{Eqn 4.6}$$

According to Eqn 4.6, when the standard heat of solution is exothermic, solubility decreases with increases in temperature. On the other hand, when the standard heat of solution is endothermic, as temperature increases, the solubility increases.

4.1.2.2 Effect of pH

Many important drugs belong to the class of weak electrolytes; weak acids and bases. The solubility of these drugs is strongly influenced by the pH of the solution. For simple drug dissociation systems comprising a weak acid and its ionised conjugate base or a weak base and its protonated conjugate acid, pH solubility effects can be predicted from equations 4.7 and 4.8.

Weak acid-ionised base form:

$$pH - pK_a = \log\left(\frac{S - S_o}{S_o}\right) \qquad \text{Eqn 4.7}$$

Weak base-protonated acid form:

$$pH - pK_a = \log\left(\frac{S_o}{S - S_o}\right) \qquad \text{Eqn 4.8}$$

where S is the saturation solubility and S_o is the solubility of the undissociated form.

For amphoteric drugs which possess both acidic and basic groups, the dissociation process comprises of a cation (at low pH), a zwitterion and an anion (at high pH). The equations for predicting the pH solubility for amphoteric drugs are given by equations 4.9 and 4.10.

At pH values below the isoelectric point:

$$pH - pK_{a1} = \log \left(\frac{S_z}{S - S_z} \right) \qquad \text{Eqn 6.9}$$

At pH values above the isoelectric point:

$$pH - pK_{a2} = \log \left(\frac{S - S_z}{S_z} \right) \qquad \text{Eqn 4.10}$$

where S is the saturation solubility and S_z is the lowest solubility of the zwitterion.

4.1.2.3 Solvent and Solute Polarity

Solubility of a drug in a solvent depends on the polarity of the solvent and the solute, often simplicitically expressed as "like dissolves like". Polarity represents the ability of a solvent to interact with solute molecules and can be scaled using the electrostatic properties; dielectric constant and dipole moment.

Dielectric constant indicates the ability of a solvent to separate the oppositely charged ions whereas dipole moment largely determines the orientation of a solvent around the solute molecules in the absence of specific solute-solvent interactions. In general the greater the dielectric constant and dipole moment values, the more polar the solvent.

Table 4.0 shows the dielectric constants and dipole moments of some solvents. Ionic compounds such as sodium chloride will dissolve in polar solvent such as water but is insoluble in solvents having low dielectric constant such as benzene (Martin *et al.*, 1983). Having a high dielectric constant of 78, water will lower the attraction forces between the oppositely charged sodium and chloride ions.

Solubility of many drugs e.g. salicylic acid, xanthines, parabens and some barbiturates have been studied in a range of solvents of various dielectric constants (Paruta *et al.*, 1965; Paruta, 1969; Laprade *et al.*, 1976; Alexander *et al.*, 1977).

Solubility parameter is a measurement of intermolecular forces within the solvent that indicates the ability of a liquid to act as a solvent. This parameter was first introduced by Hildebrand and Scott (1964) to explain the behaviour of solvents of little or no polarity and those unable to hydrogen bond. The solubility of one components in another is predicted by calculating the difference in the solubility parameters, $(\delta_1 - \delta_2)$; the smaller the difference, the greater the solubility. Solubility of many drugs e.g. halobenzenes, benzoates, alcohols in water have been succesfully estimated using their solubility parameters (Yalkowsky and Valvani, 1980). This concept has been extended by introduction of partial solubility parameters that have improved the measurement of the systems in which polar interactions and effects occur (Beerbower *et al.*, 1984; Martin *et al.*, 1984; Bustamante *et al.*, 1989; Regosz *et al.*, 1992).

Intermolecular forces such as hydrogen bonding, dipole-dipole interaction (Keesom) and induced dipole-induced dipole interaction (London) contribute to the interaction between the solvent and solute molecules.

Table 4.0 Dielectric constant, dipole moment and solubility parameter of some solvents.

Solvent	Dielectric Constant[a] (ε)	Dipole Moment[a,c] (μ) (Debye unit)	Solubility Parameter[b] (δ)
Water	78.25(20)[d]	1.84	23.4
DMSO	46.6 (25)	4.49(vap)	12.0
PEG 300 or 400	35.0[b]		9.9
Methanol	32.63(25)	1.66	14.7
95% Ethanol	27.0[e]		
Ethanol	24.30(25)	1.68	12.7
Chloroform	21.81(20)	1.15	9.3
Benzyl alcohol	13.1 (20)	1.66	12.1
Benzene	2.28(25)	0	9.1
Liquid paraffin			7.1

[a] from Dack (1976)
[b] from Wells (1988)
[c] dipole moment determined in solution unless otherwise stated
[d] temperature in ^{o}C
[e] calculated using:
$\varepsilon_{95\%EtOH} = 0.95 \times (\varepsilon_{ethanol}) + 0.05 \times (\varepsilon_{water})$

Another important scale of polarity for solutes is its partition coefficient, P which can be expressed as

$$P = \frac{C_o}{C_w} \qquad \text{Eqn 4.11}$$

where C_o is the concentration of solute in oil and C_w is the concentration in aqueous phase. The partition coefficient measures the partition of a compound between aqueous phase and oil or an immiscible solvent. The greater the P value, the higher lipid

solubility of the compound. Octanol is generally used to generate this coefficient because of its solubility parameter, $\delta = 10.24$ which is in the middle of the range of most drugs (δ = 8-12). Due to its partial polarity, water is solubilised to some extent in the octanol phase, which is a feature of biological lipid membrane since the solubility parameter for the whole lipid membrane derived from erythrocyte ghost is δ = 10.3±0.4 (which is similar to that of octanol) and dehydration of the membrane to lipid constituent only decreases the polarity to δ = 8.7±1.03 (Bennett and Miller, 1974).

4.2 METHODS

4.2.1 Stability Studies

Stability of ABZ was studied in aqueous suspension and solvents of interest.

4.2.1.1 Stability of Albendazole Aqueous Suspension

When suspensions of ABZ were prepared in glycine buffer, ABZ was not readily wetted. Therefore, sodium dodecyl sulphate (SDS) was added to the suspension to aid wetting.

100 ml lots of suspensions of 0.1% ABZ in glycine buffer at pH 9.00 and 10.00 were prepared with and without the addition of 0.011% of SDS and transferred into a 3-necked 250 ml flask and was boiled under reflux at 100^{o}C. 5 ml of the solution was sampled at the appropriate sampling times using a pipette. Samples were assayed for ABZ by diluting with water (1 in 100) and followed by 1 in 10 dilution with mobile phase prior to HPLC analysis as described in Chapter 3 (3.2.1.1(a)). The zero time sample was obtained by pipetting 5 ml of the suspension before it was boiled, and was diluted in the same manner as the other samples before HPLC analysis.

In all the studies, the suspended drug dissolved after a period of time. After completing the experiment at pH 10.00 with SDS, crystals were noted in the cooled solution remaining in the flask. The crystals were collected by filtration, washed with distilled water and dried. Sample was dissolved in mobile phase and injected into HPLC. The sample was identified by NMR and MS (see Appendix 2). Sample was also analysed by TLC using solvent system of chloroform/ethyl ether/acetic acid (6:1:1) as reported by Gyurik et

al. (1981).

4.2.1.2 Stability of Albendazole Sulphoxide Aqueous Suspension

A suspension of 0.1% ABZSO with 0.011% SDS was prepared in glycine buffer at pH 10.00. 100 ml of suspension was boiled under reflux at 100°C as previously. 5 ml aliquots were sample at zero time and at the appropriate sampling times and samples were diluted 1 in 1000 (as described for ABZ samples in 4.2.1.1)) before samples were assayed for ABZSO by HPLC (3.2.1.1(a)).

4.2.1.3 Stability of Albendazole in Solvents

(a) DMSO

1 mg/ml solution of ABZ was prepared in DMSO. 1 ml of the solution was pipetted using a micropipette (Gilson Pipetman) into a series of plastic vials with plastic caps. 5 vials were kept in the freezer (-18°C) as control samples. Vials in group of 5 were placed at room temperature (22°C), in the heating block at 37°C and 55°C for 48 hours. The reaction was stopped by putting the vials in ice and stored in the freezer up to 2 weeks until time of analysis. 0.2 ml of sample was pipetted into a 1 ml volumetric flask and the volume was made up to the mark with mobile phase, and injected into HPLC (3.2.1.1(a)).

(b) 0.1M HCl

1 ml aliquots of 10 µg/ml ABZ prepared in 0.1M HCl were pipetted into plastic vials and heated at room temperature (22°C), or placed in the heating block at 37°C and 55°C for 48 hours. Vials were also heated at 55°C for 3, 7 and 24 hours. Control samples were kept in the freezer (-18°C). 0.1 ml samples were pipetted into a 1 ml volumetric

flask and was made up to volume with mobile phase and analysed by HPLC (3.2.1.1(a)).

4.2.2 Solubility Studies

Solubility of ABZ in solvents of interest was determined using four different methods. Methods 1-3 are related techniques:

Method 1

100 ml of solvent was transferred into a jacketed-beaker. Excess of ABZ was added and stirred. Nitrogen gas was bubbled into the solvent to create anoxic conditions; the vessel was covered with "para-film". The apparatus used is shown in Fig. 4.1. The solution was sampled through a tube fitted with a number 3 glass sinter filter to ensure no particles could pass through. 0.1 ml aliquots of the clear supernatant obtained were removed and assayed for ABZ spectrophotometrically (3.2.3) and by HPLC (3.2.1.1(a) and (b)). Plots of concentration against time were constructed and the solubility of ABZ was determined when the concentration was constant, indicating saturating concentration of ABZ.

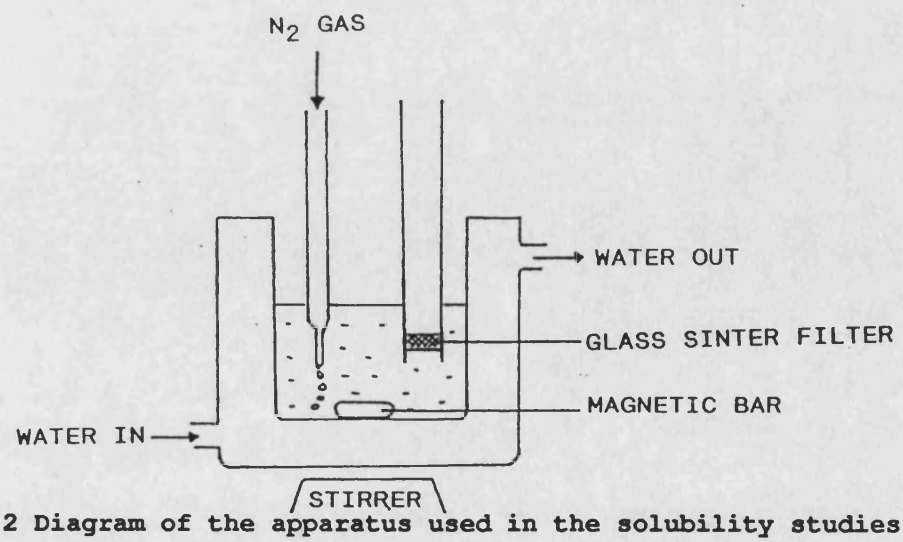


Fig. 4.2 Diagram of the apparatus used in the solubility studies of ABZ using Method 1.

Method 2

20-100 mg amounts of ABZ were accurately weighed and transferred into a mortar and ground with 10 g of solvent. The solvent was added in small amount whilst mixing to form a homogeneous slurry. The slurry was transferred into a test-tube, heated in the water-bath (70°C) until it turned clear and cooled. The solubility of ABZ was assessed by visual inspection.

Method 3

5-100 mg quantities of ABZ were accurately weighed into a series of test-tubes. 10 g of solvent was added to give ABZ concentration of 0.5-10 mg/g. The test-tubes were then placed in a water-bath at 25°C or 37°C and shaken. The solubility of ABZ was assessed visually. The test-tubes were kept at room temperature for another 2 weeks with occasional shaking.

Method 4 (Turbidity Method)

The turbidity of the system was measured using UV/VIS spectrophotometer in the visible region, 450 nm.

(a) A 100 ml volumetric flask was filled up to the mark with water or 0.1M HCl at room temperature (~17°C) or placed in a water-bath at 37°C. 50 µl aliquots of "ABZ solution" (solution in DMSO) was added to the water or HCl using a micropipette and mixed thoroughly. After each addition the absorbance of the system was measured. 2 ml was removed for this measurement and was returned to the flask after the absorbance was recorded.

(b) 100 ml of 0.1M HCl or water was pipetted into jacketed-beaker at 37°C. The "ABZ solution" was added to HCl solution or water using a 10 ml Segma Interchangeable precision syringe placed on a syringe pump. The rate of delivery was calibrated using water (10 replicates); rate (1): 1.12±0.01 ml/min. and rate (2): 0.102±0.001 ml/min. Aliquots of 0.56 ml (30 seconds at rate (1)) or 8.5 µl (5 seconds at rate (2)) of "ABZ solution " was added to HCl solution or water. 2 ml of the system was removed to measure the absorbance and was returned to the beaker before the next addition of "ABZ solution".

(c) 2 ml of "ABZ solution" was transferred into a test-tube using the syringe and the test-tubes was then placed in a water-bath at 37°C. Cumulative volumes of 1 ml 0.1M HCl were added and mixed thoroughly. Absorbance was measured after each addition of HCl solution.

4.2.2.1 Solubility of Albendazole in Solvents

The solubilities of ABZ in various solvents; DMSO, 95% EtOH, Miglyol, water, 0.1M and 0.01M HCl and at different temperatures were determined using Method 1 (4.2.2).

4.2.2.2 Solubility of Albendazole in Miglyol, Tween 80 & 85 and Polyethylene Glycol 400

The solubilities of ABZ in polyethylene glycol (PEG) 400, Miglyol, Tween 80 or mixtures of Miglyol and Tween 80 (60:40%, 58:42%, 61:39%, w/w) were determined using Method 3.

The solubility of ABZ was also determined in mixtures of Tween 80 or 85 and Miglyol (60:40%, w/w) and in PEG 400 using Method 2.

4.2.2.3 Solubility of "albendazole solutions"

Studies were carried out to establish the solubility of "ABZ solutions" in water and simulated gastrointestinal fluid.

(a) ABZ-DMSO Solution

5 mg/g of ABZ in DMSO was added to water or 0.1M HCl using Method 4(a) (4.2.2). The solubility of "ABZ-DMSO solution" in 0.1M HCl was also determined using Method 4(b) (4.2.2). 6 replicate determinations were carried out. Results are shown in Tables 4.12 and 4.13.

(b) ABZ-Miglyol:Tween 80 Solution

2 mg/g of ABZ was prepared in Miglyol:Tween 80, 60:40%, w/w (Mig:Twn). The ABZ-Mig:Twn solution was added to 0.1M HCl and the solubility was determined using Method 4(b) (4.2.2) at a rate of delivery of 0.102 ml/min. This experiment was repeated using Mig:Twn solution without ABZ (blank). 6 replicate determinations were carried out. Results are shown in Table 4.14

The system resulting from the addition of "ABZ Mig:Twn solution" to 0.1M HCl and a suspension of ABZ in water were examined under microscope (Vickers, York) with x40 magnification.

The density of Mig:Twn 80 solution was determined in duplicate at 22^{o}C using a pycnometer as described in the British Pharmacopoeia (1988).

The density of Mig:Twn solution at 22^{o}C was 0.994 g/ml.

(c) ABZ-PEG 400 Solution

The solubility of ABZ-PEG 400 solution in 0.1M HCl was determined using Method 4(c). 5 mg/g ABZ was prepared in PEG 400 was used. 5 replicate determinations were carried out.

4.2.2.4 Analysis of Samples

Samples from studies in DMSO and 95%EtOH were assayed by UV spectrophotometry as described in Chapter 3 (3.2.3). The samples were diluted with the respective solvent; 1 in 250 for DMSO and 1 in 50 for 95%EtOH. Samples from these studies were analysed for presence of any degradation products by HPLC (3.2.1.1(a)).

Samples dissolved in Miglyol were assayed for ABZ using the normal-phase HPLC method (3.2.1.1 (b)). Samples were diluted with mobile phase (1 in 50) prior to analysis.

The reversed-phase HPLC method (3.2.1.1(a)) was used to assay for ABZ in samples obtained from studies in water and HCl (0.1M and 0.01M). Samples were diluted prior to injection into HPLC.

4.2.3 Triangular Phase Solubility Diagram

A solution of ABZ in Miglyol is highly desirable. The possibility of increasing solubility of ABZ in Miglyol by addition of Tween 80 and/or PEG was considered. The miscibility of the 3 components (Miglyol, PEG 400 and Tween 80) was therefore studied since a one-phase system is required for potential drug-vehicle. Data for the construction of a triangular phase diagram were obtained by weighing the components into vials, mixing thoroughly and examining after the vials were left standing overnight.

4.2.4 Preparation of Gluconate Salt

5 mg/g ABZ was prepared in mixture of Tween 80:gluconic acid (20:80%, w/w) by heating. The clear solution was evaporated on a rotary evaporator at 40°C and a sticky solid was formed. The solubility of the solid in some solvents was studied. The solid product (0.5 g) was dispersed in Miglyol (5-10 g), water (1-5 ml) and PEG 400 (1-5 g) in test-tubes, heated and cooled.

4.2.5 Statistical Analysis

The data from the solubility studies of "ABZ solutions" in water and 0.1M HCl (4.3.2.3) were subjected to least-square regression analysis to determine the slope and the intercept of the curves.

The Student's t-test, analysis of variance (ANOVA) and Bartlett test (Appendix 1) were used appropriately for comparison of data.

4.3 RESULTS

4.3.1 Stability Studies

4.3.1.1 Results from Stability Study of ABZ Aqueous Suspension

The results from the studies of 0.1% ABZ with and without 0.011% SDS in glycine buffer suspension at pH 9.00 and 10.00 heated at 100°C are presented in Tables 4.1 and 4.2.

Chromatograms in Figs. 4.3 and 4.4 illustrate the loss of ABZ and the appearance of degradation compounds; ABZSO (R_T=5.70 min.), Unknown 1 (R_T=7.54 min.) and Unknown 2 (R_T=12.84 min.) in systems with and without SDS at pH 9.00. The "dissolution" of ABZ was higher in the presence of SDS (Fig. 4.5). ABZSO was present in both zero time samples and in higher concentration in suspension with no SDS (Fig. 4.6). Table 4.3 shows the rate of formation of the degradation compounds which was calculated from the peak area ratio against time plot (Fig. 4.6).

Other than for the crystalline materials of ABZ, ABZSO and $ABZSO_2$ used as the standard solutions, no attempts were made to identify the degradation compounds and therefore standards are not available. For this reason peak area ratios are used in Figs. 4.5 and 4.6 to compared the changes in the concentration of ABZ and its degradation compounds.

Table 4.1 Results from the stability study of 0.1% ABZ suspension with and without 0.011% SDS at pH 9.00.

Time (hr)	Peak Area Ratio ABZ	ABZSO	Unknown 1	Unknown 2
With SDS				
0	1.002	0.033	0.000	0.000
24	1.077	0.029	0.000	0.000
48	0.515	0.027	0.000	0.000
120	0.330	0.052	0.059	0.146
144	0.072	0.036	0.082	0.259
Without SDS				
0	0.216	0.128	0.000	0.000
24	0.251	0.120	0.000	0.000
48	0.183	0.116	0.039	0.126
115	0.124	0.120	0.089	0.425

Table 4.2 Results from the stability study of 0.1% ABZ suspension with and without 0.011% SDS at pH 10.00.

Time (hr)	Peak Area Ratio ABZ	Unknown 1	Unknown 2
With SDS			
0	1.585	0.000	0.000
6	1.771	0.000	0.285
9	1.242	0.000	0.470
12	0.836	0.052	0.604
24	0.000	0.125	1.510
30	0.000	0.150	1.484
Without SDS			
0	0.197		0.000
6	0.294		0.131
12	0.141		0.172
24	0.055		0.447

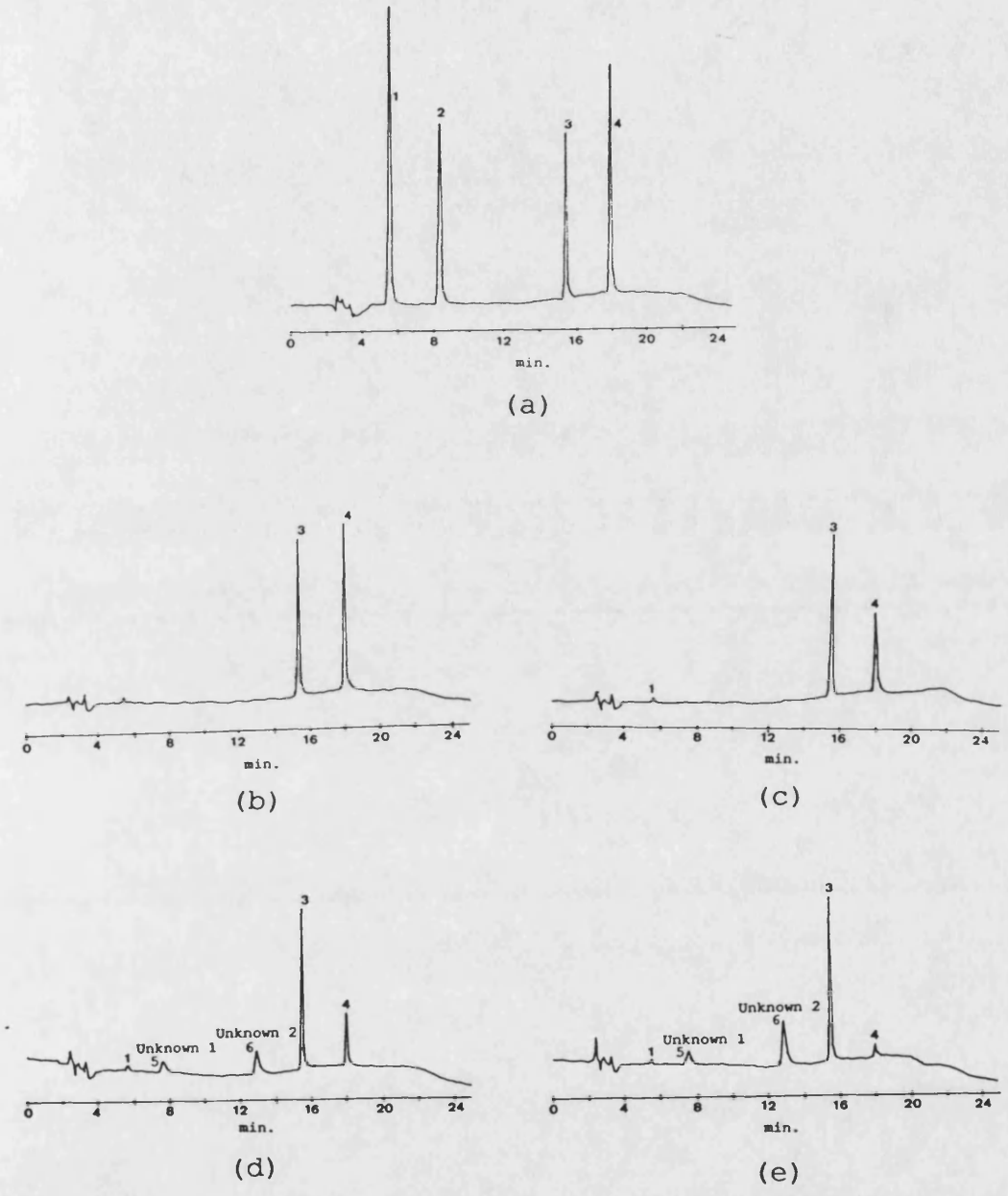


Fig.4.3 Chromatograms of (a) std. solution of 0.8 µg/ml each of ABZSO (1), $ABZSO_2$ (2), MBZ (3) and ABZ (4), and 1/1000 dilutions of 0.1% ABZ suspension with 0.011% SDS in glycine buffer at pH 9.00 heated at 100°C for (b) 24hrs, (c) 48hrs, (d) 129 hrs and (e) 144 hrs.

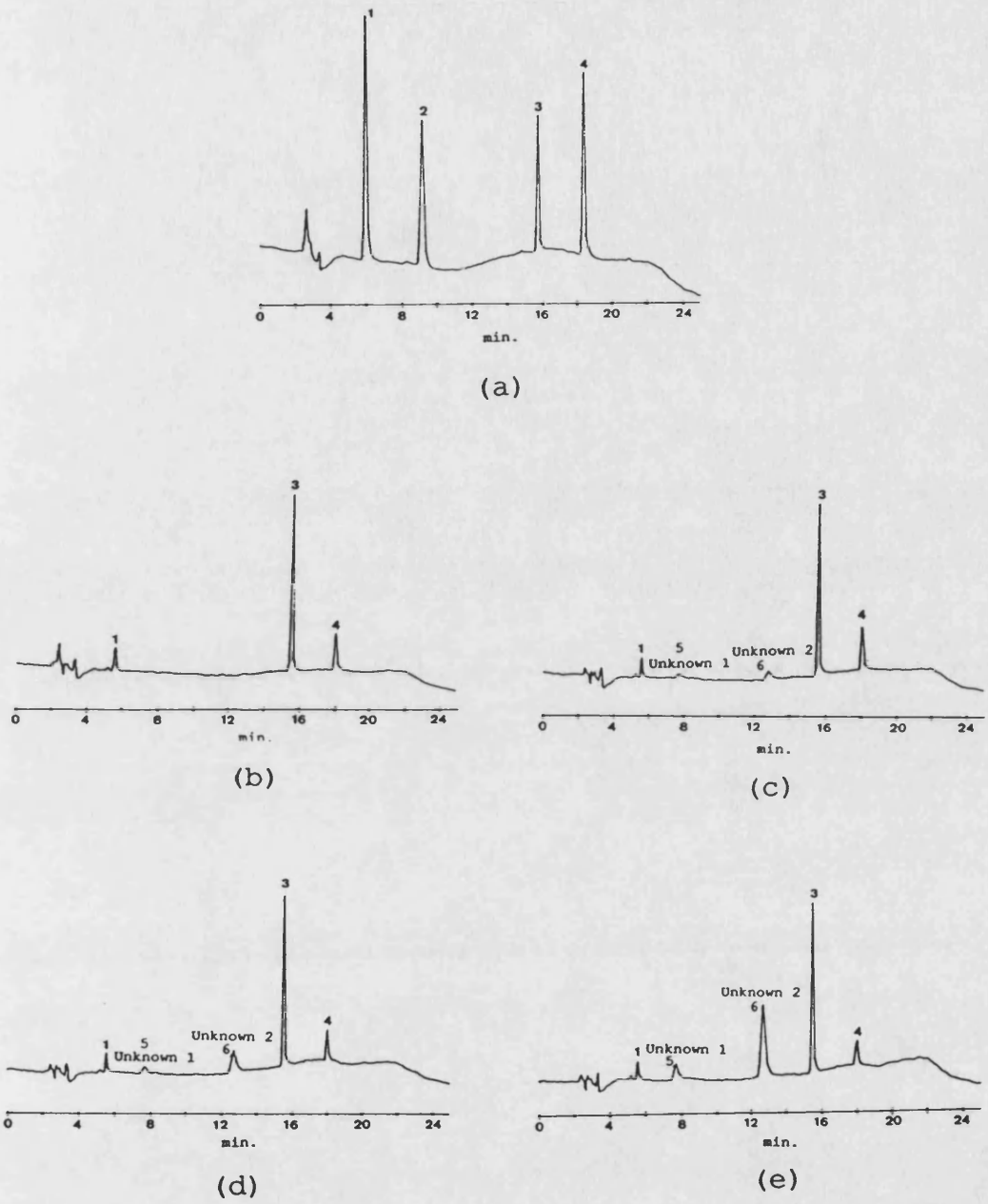


Fig. 4.4 Chromatograms of (a) std. solution of 0.8 µg/ml each of ABZSO (1), $ABZSO_2$ (2), MBZ (3) and ABZ (4),and 1/1000 dilutions of 0.1% ABZ suspension in glycine buffer (no SDS) at pH 9.00 heated at 100°C for (b) 0hr, (c) 24hrs, (d) 48hrs and (e) 115hrs.

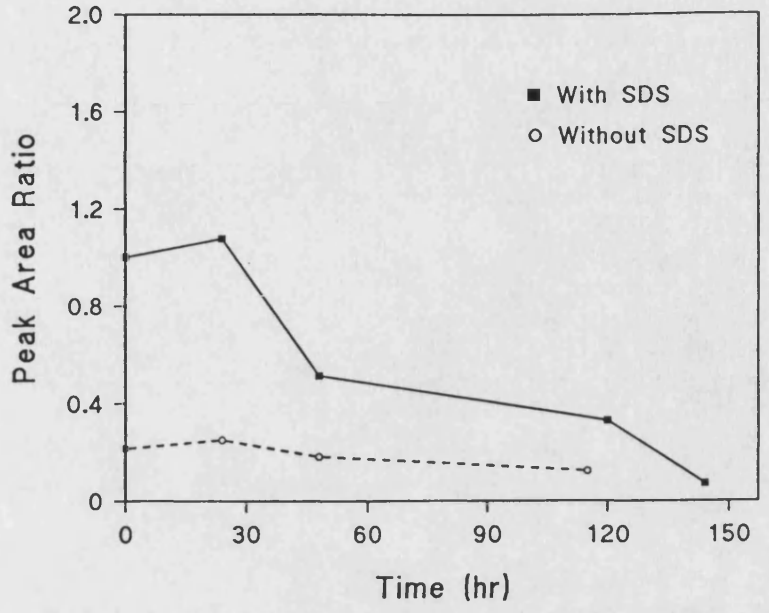


Fig.4.5 Degradation profile of ABZ after heating 0.1% ABZ suspension at 100°C with and without 0.011% SDS in glycine buffer at pH 9.00. Data are shown as peak area ratio against time.

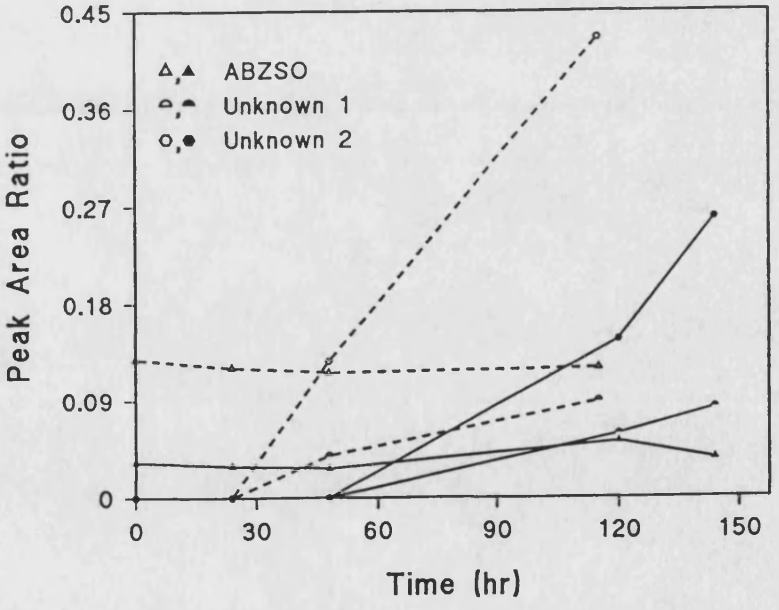


Fig. 4.6 Peak area ratio-time profile showing the formation of the degradation compounds after heating 0.1% ABZ suspension at 100°C with (●) and without (o) 0.011% SDS in glycine buffer at pH 9.00.

Table 4.3 Rate of formation of degradation compounds after heating 0.1% ABZ suspension with and without SDS in glycine buffer at pH 9.00 at 100°C.

Degradation compounds	Rate of formation (peak area ratio/hr) x 10^3
With SDS	
ABZSO	*
Unknown 1	0.85
Unknown 2	2.55
Without SDS	
ABZSO	*
Unknown 1	0.93
Unknown 2	4.63

* Remains constant at initial sample value.

The chromatograms in Figs. 4.7 and 4.8 show degradation compounds, Unknown 1 and 2 were formed when ABZ suspension with and without SDS at pH 10.00 were heated at 100°C. "Dissolution" of ABZ was again higher in suspension prepared with SDS and it was completely degraded at 24 hours (Fig. 4.9). It appears that degradation of ABZ was faster at pH 10.00 than pH 9.00 when ABZ suspensions with and without SDS were heated at 100°C (Fig. 4.6 and 4.9). Only Unknown 2 was formed in suspension with no SDS (Fig. 4.10) but both Unknowns 1 and 2 were detected in suspensions with SDS. ABZSO was not present in any of the system at pH 10.00. The rate of formation of the degradation compounds is given in Table 4.4, was calculated from the peak area ratio against time plot (Fig. 4.10).

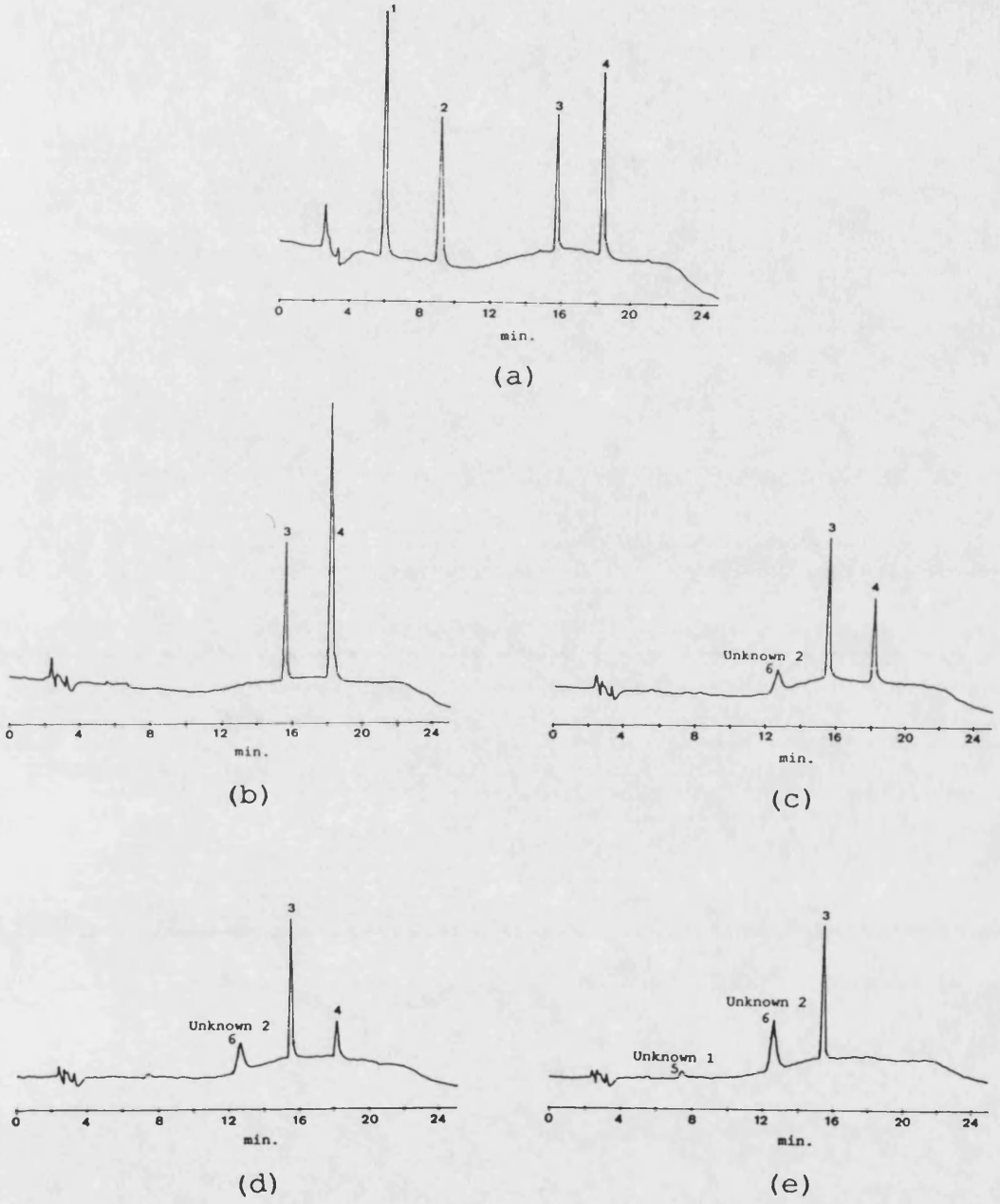


Fig. 4.7 Chromatograms of (a) std. solution of 0.8 µg/ml each of ABZSO (1), $ABZSO_2$ (2), MBZ (3) and ABZ (4), and 1/1000 dilutions of 0.1% ABZ suspension with 0.011% SDS in glycine buffer at pH 10.00 heated at 100°C for (b) 0hr, (c) 6hrs, (d) 12hrs and (e) 24hrs.

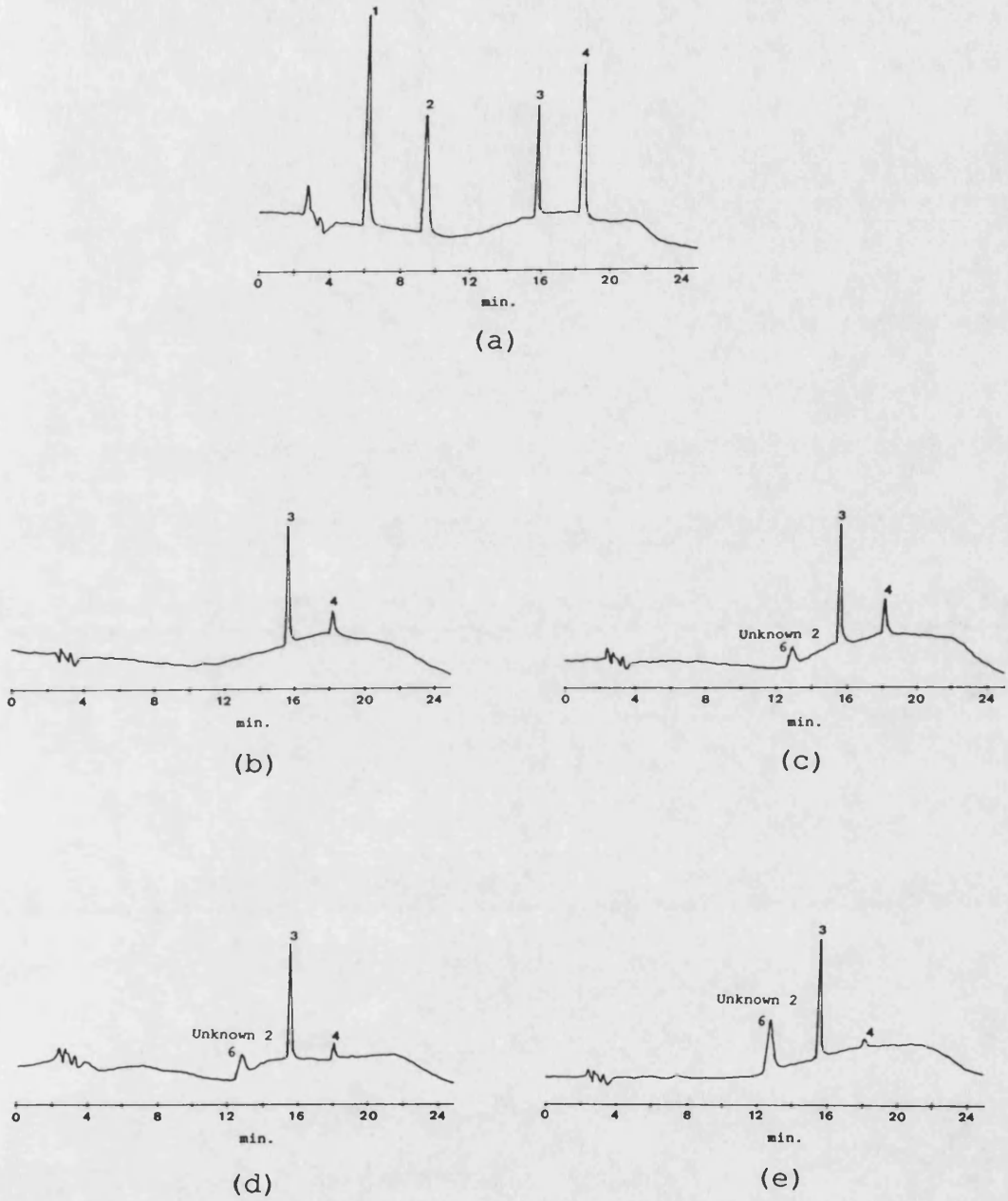


Fig. 4.8 Chromatograms of (a) std. solution of 0.8 µg/ml each of ABZSO (1), $ABZSO_2$, MBZ (3) and ABZ (4), and 1/1000 dilutions of 0.1% ABZ suspension in glycine buffer at pH 10.00 heated at 100°C for (b) 0hr, (c) 6hrs, (d) 12hrs and (e) 24hrs.

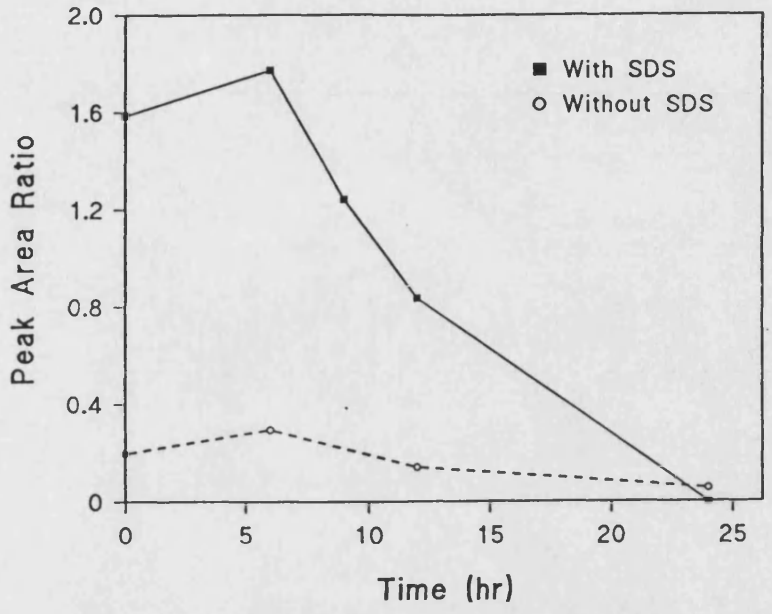


Fig.4.9 Degradation profile of ABZ after heating 0.1% ABZ suspension at 100°C with and without 0.011% SDS in glycine buffer at pH 10.00. Data are shown as peak area ratio agianst time.

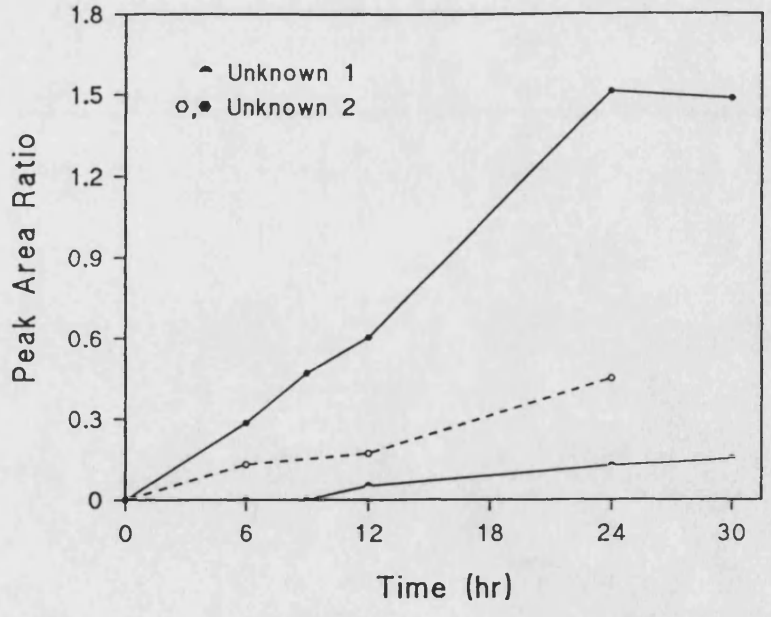


Fig. 4.10 Peak area ratio-time profile showing the formation of the degradation compounds after heating 0.1% ABZ suspension at 100°C with (●) and without (o) 0.0115% SDS in glycine buffer at pH 10.00.

The crystals found in the degraded ABZ solution (pH 10.0) gave a peak at 13.2 min when injected into the HPLC which corresponds to Unknown 2. The crystals were identified by NMR and confirmed by MS (Tables 4.5 and 4.6) to be the 5-(propylthio)-1*H*-benzimidazol-2-amine (ABZ 2-amine). The NMR and MS spectra are given in Appendix 2. This characterization was also confirmed by TLC analysis. The R_f of Unknown 2 (R_f = 0.10) corresponded to the R_f of the ABZ 2-amine compound (R_f = 0.09) as reported by Gyurik *et al.* (1981).

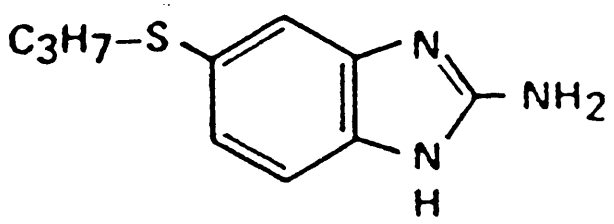


Table 4.4 Rate of formation of degradation compounds after heating 0.1% ABZ suspension with and without SDS in glycine buffer at pH 10.00 at 100°C.

Degradation compounds	Rate of formation (peak area ratio/hr) x 10^3
With SDS	
Unknown 1	6.76
Unknown 2	63.6
Without SDS	
Unknown 2	18.1

Table 4.5 NMR data (δ, multiplicity) for ABZ, ABZSO and Unknown 2.

Compound	H-aromatic	CH_3-O	H-α	H-β	H-γ	N-H
ABZ	7.1-7.4,m	3.7,s	2.8,t	1.5,m	0.9,t	11.6,s
ABZSO	7.3-7.7,m	3.7,s	2.8,m	1.5,m	0.9,t	11.9,s
Unknown 2	7.6-7.2,m		2.8,t	1.5,m	0.9,t	10.7,s 6.2,s

Table 4.6 Mass-spectral data for ABZ, ABZSO and Unknown 2.

Compound	m/z(relative intensity and attribution)			
ABZ	265(87) [M]	234(10) [M-CH_3O]	233(58) [M-CH_3OH]	223(35) [M-C_2H_5]
	204(23) [233-C_2H_5]	191(100) [M-C_3H_6S]	190(30) [M-C_3H_7S]	178(7) [233-CO-HCN]
ABZSO	281(18) [M]	265(8) [M-O]	264(4) [M-OH]	249(12) [M-CH_3OH]
	239(30) [M-C_3H_6]	238(100) [M-C_3H_7]	207(42) [239-CH_3OH]	206(87) [238-CH_3OH]
	191(25) [M-C_3H_6SO]	159(58) [191-CH_3OH]		
Unknown 2	207(100) [M]	178(15) [M-C_2H_5]	165(97) [M-C_3H_6]	149(32) [165-NH_2]
	132(7) [M-C_3H_7S]	105(18) [132-HCN]		

4.3.1.2 Results from Stability Study of ABZSO Aqueous Suspension

Table 4.7 shows the results from the 0.1% ABZSO suspension with 0.011% SDS at pH 10.00 when heated at 100°C. ABZSO was completely degraded at 9 hours and a third degradation compound, Unknown 3 can be seen in the chromatograms in Fig. 4.11 having a retention time of 3.29 minutes. The loss of ABZSO and the formation of Unknown 3 are illustrated in Fig. 4.12. The rate of formation of Unknown 3 was calculated to be 0.34±0.07.

Table 4.7 Results from the stability study of 0.1% ABZSO with .011% SDS in glycine buffer at pH 10.00, heated at 100°C.

Time	Peak Area Ratio	
	ABZSO	Unknown 3
0.0	3.67	0.00
1.5	4.77	0.72
3.0	0.86	1.60
6.0	0.10	2.06
9.0	0.00	2.08
12.0	0.00	2.13
24.0	0.00	1.93

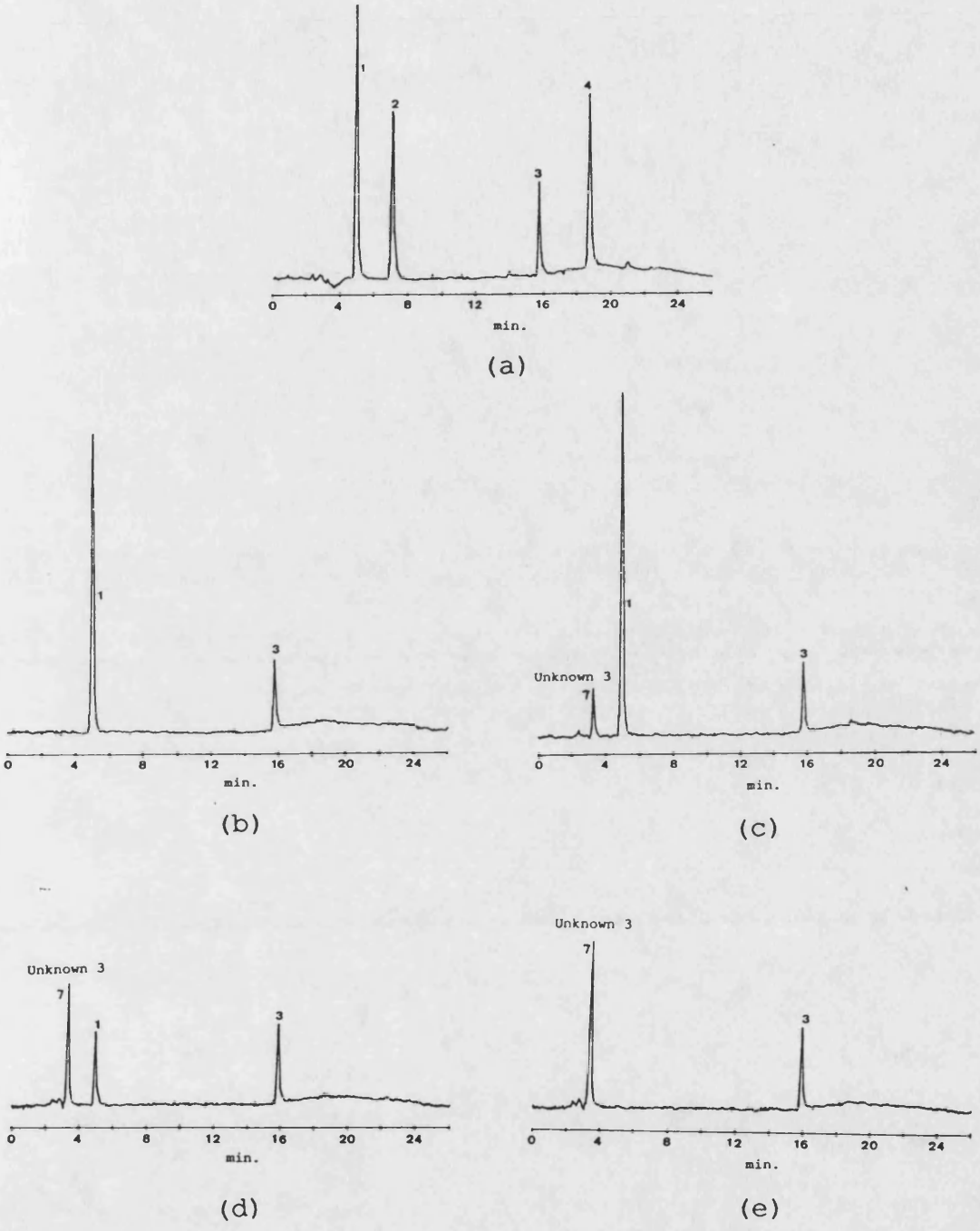


Fig. 4.11 Chromatograms of (a) std. solution of 0.8 µg/ml each of ABZSO (1), $ABZSO_2$ (2), MBZ (3) and ABZ (4), and 1/1000 dilutions of 0.1% ABZSO suspension with 0.011% SDS in glycine buffer at pH 10.00 heated at 100°C for (b) 0hr, (c) 1.5hrs, (d) 3.0hrs and (e) 9.0hrs.

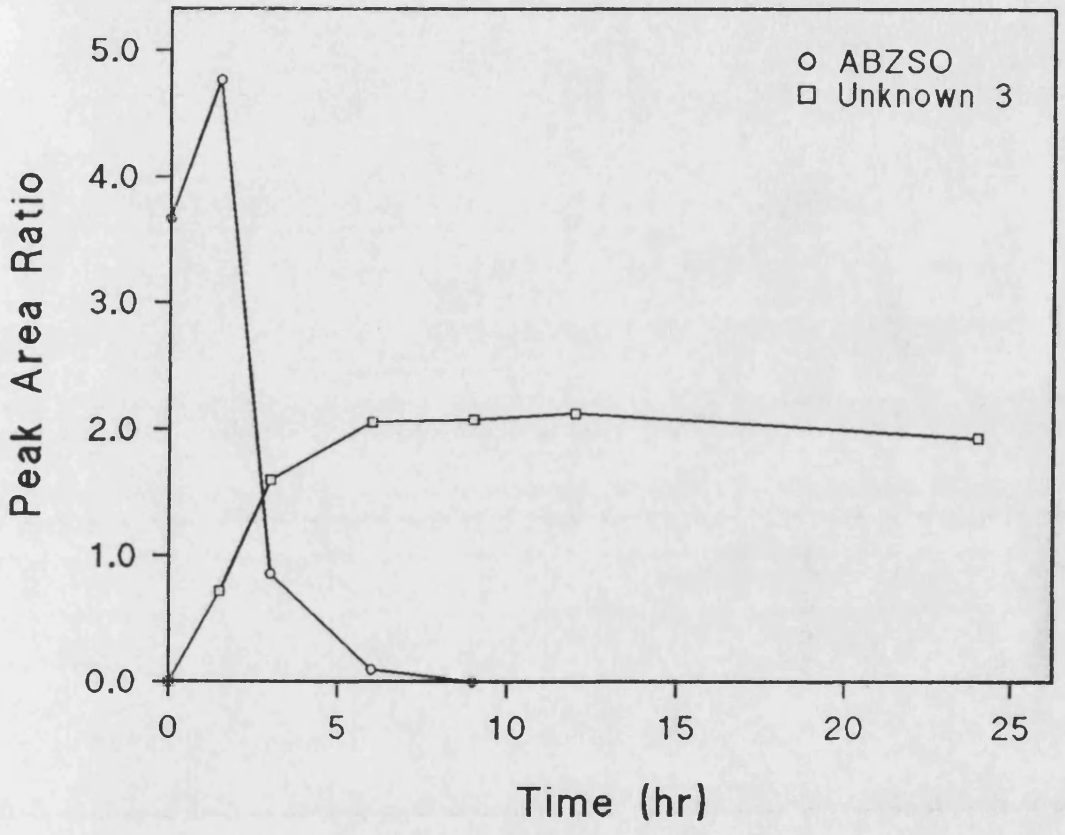


Fig. 4.12 Peak area ratio-time profile showing the degradation of ABZSO and the formation of the degradation compound after heating 0.1% ABZSO suspension at 100°C with 0.011% SDS in glycine buffer at pH 10.00.

4.3.1.3 Results From Stability Studies of ABZ in Solvents

(a) DMSO

ABZ was found to be degrading in all the samples at all temperatures studied, including control samples which were kept in the freezer (-18°C). Fig 4.13 shows typical chromatogram patterns for ABZ solutions (1 mg/ml) in DMSO at all temperatures. There were 9 peaks detected by HPLC. Peak 3 is the internal standard, MBZ spiked prior to the analysis and peak 4 is ABZ. Peaks 1, 2, 6, 8-11 are the degradation compounds. When compared with standard solutions, peaks 1, 2, and 6 correspond to ABZSO, $ABZSO_2$ and ABZ 2-amino compound (Unknown 2), respectively. Peaks 8-11 are unknown and no attempts were made to identify them. Percentage total of peak area ratio of all the peaks are summarised in Table 4.8 and they were comparable at all temperatures except for peak 6. Peak 6 makes a significant contribution (~4.3%) to the %total of peak area ratio compared to control sample and samples at 22°C and 37°C. Correspondingly ABZ degraded faster at 55°C in DMSO (lowest %total of peak area ratio).

Table 4.8 Percentage total of peak area ratio (against MBZ) of ABZ and degradation compounds in the stability study of ABZ in DMSO for 48 hr. Results represent mean of 5 samples. (mean±SD)

Peak	% Total Peak Area Ratio			
	Control	22°C	37°C	55°C
1	0.08±0.04	0.10±0.04	0.26±0.05	0.10±0.07
2	0.09±0.02	0.09±0.06	0.12±0.01	0.13±0.12
4	98.46±2.67	98.44±1.01	97.96±1.52	94.23±2.28
6	0.11±0.02	0.09±0.03	0.26±0.04	4.30±0.34
8	0.05±0.01	0.04±0.01	0.12±0.01	0.07±0.04
9	0.17±0.05	0.17±0.04	0.14±0.03	0.15±0.04
10	0.34±0.08	0.35±0.09	0.43±0.03	0.37±0.03
11	0.70±0.03	0.71±0.01	0.72±0.06	0.65±0.05

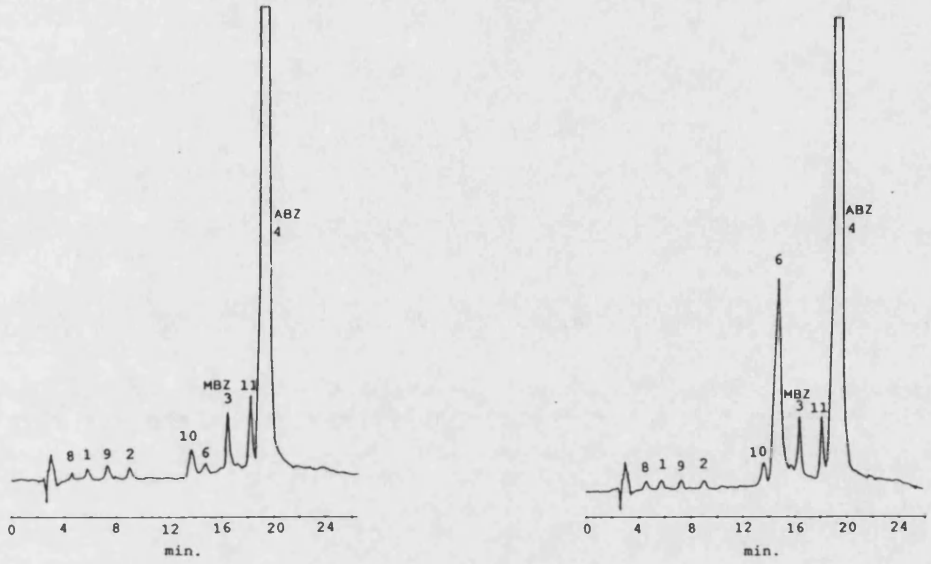


Fig. 4.13 Typical chromatogram pattern for ABZ (4) solutions (1 mg/ml) in DMSO. (a) sample heated at 22°C and (b) sample heated at 55°C for 48 hours.

(b) 0.1M HCl

Table 4.9 shows the results of ABZ stability in 0.1M HCl at different temperatures studied; -18°C (control), 22°C, 37°C and 55°C for 48 hours. The mean of these 4 values was 9.79±0.45 (RSD = 4.6%) and comparison by analysis of variance at P = 0.05 showed there was no significant difference ($F = 3.77$, $F_{tab} = 4.47$) between them; no degradation products were detected by HPLC (Fig. 4.14).

Samples heated in 0.1M HCl at 55°C were also analysed at 3, 7 and 24 hours. Although a decreasing trend in concentration of ABZ was observed with time of heating (Table 4.9) these values were not significantly different when compared using analysis of variance at P=0.05. ($F = 3.77$, $F_{tab} = 4.47$; $t_{3/48} = 1.92$, $t_{tab} = 2.31$). The mean of these 4 values at 55°C was 9.65±0.24 (RSD = 2.5).

Table 4.9 Concentration (mean±SD) of ABZ in the stability study of ABZ in 0.1M HCl. (n = 5)

Temperature (°C)		Concentration (µg/ml)
Control	(-18°C)	9.86±0.75[a]
22°	48hr	10.40±0.37[a]
37°	48hr	9.51±0.43[a]
55°	3hr	9.96±0.49[b,*]
55°	7hr	9.70±0.91[b]
55°	12hr	9.55±1.22[b]
55°	48hr	9.39±0.45[a,b,*]
	General mean:	9.77±0.34
		RSD = 3.5%

[a] not significantly different F = 3.77
[b] not significantly different F = 3.77
[*] not significantly different $t_{3/48} = 1.92$

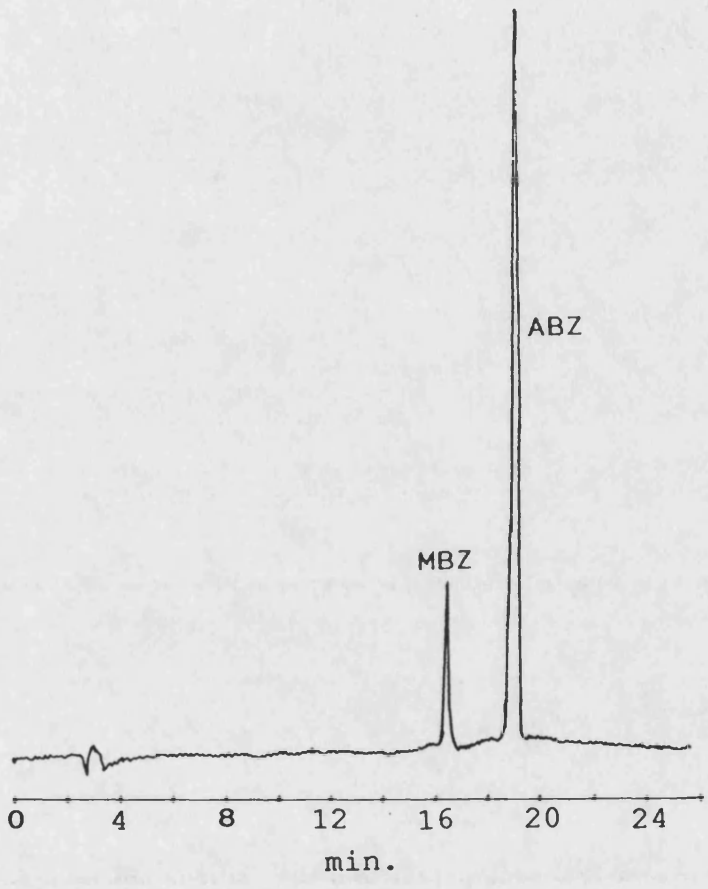


Fig. 4.14 Typical chromatogram of ABZ Solution (10 µg/ml) in 0.1M HCl, sample heated at 55°C for 48 hours.

4.3.2 Solubility Studies

4.3.2.1 Results from Solubility of ABZ in Solvents

(a) DMSO

Table 4.10 shows the solubility of ABZ in DMSO (Method 1, 4.2.2). It is apparent that solubility of ABZ increases with temperature. The corresponding Van't Hoff plot is shown in Fig. 4.15a. The standard enthalpy of the dissolution was estimated from the slope as 44±6 kJ/mole; it is recognised that the data set is limited and further data over this temperature range would needed to be generated to confirm this figure.

Even though the study was carried out in anoxic conditions, some degradation of ABZ still took place. The peaks of the degradation products were first seen in samples at 108hr when the studies were carried out at 23.30°C and in samples at 72hr for 25.20°C and 30.00°C. The degradation products included ABZSO (peak 1), $ABZSO_2$ (peak 2) and ABZ 2-amino compound (peak 6) (Fig. 4.16) which were identified by their HPLC retention times.

(b) 95% EtOH

Solubility of ABZ in 95% EtOH is given in Table 4.10. The plot of $\log_e$ solubility against 1/T for ABZ in 95% EtOH was linear (Fig 4.15b) although the data set is limited. Further data over this temperature range would needed to be generated to confirm this plot. The estimated standard enthalpy for dissolution was 36±2 kJ/mole. ABZ was stable in 95% EtOH at different temperatures (Table 4.11) and HPLC analysis did not show any degradation products present (Fig. 4.17).

Table 4.10 Solubilities of ABZ in DMSO, 95% EtOH and Miglyol.

Temperature (°C)	Solubility (mg/ml)	(moles/l)
DMSO		
23.30	39.05	0.147
25.20	41.03	0.155
30.00	55.60	0.210
95%EtOH		
23.30	0.814	3.072×10^{-3}
25.20	0.900	3.396×10^{-3}
29.10	1.060	4.000×10^{-3}
Miglyol		
20.05	0.460	1.738×10^{-3}
24.75	0.480	1.811×10^{-3}
30.40	0.517	1.951×10^{-3}
37.10	0.580	2.189×10^{-3}

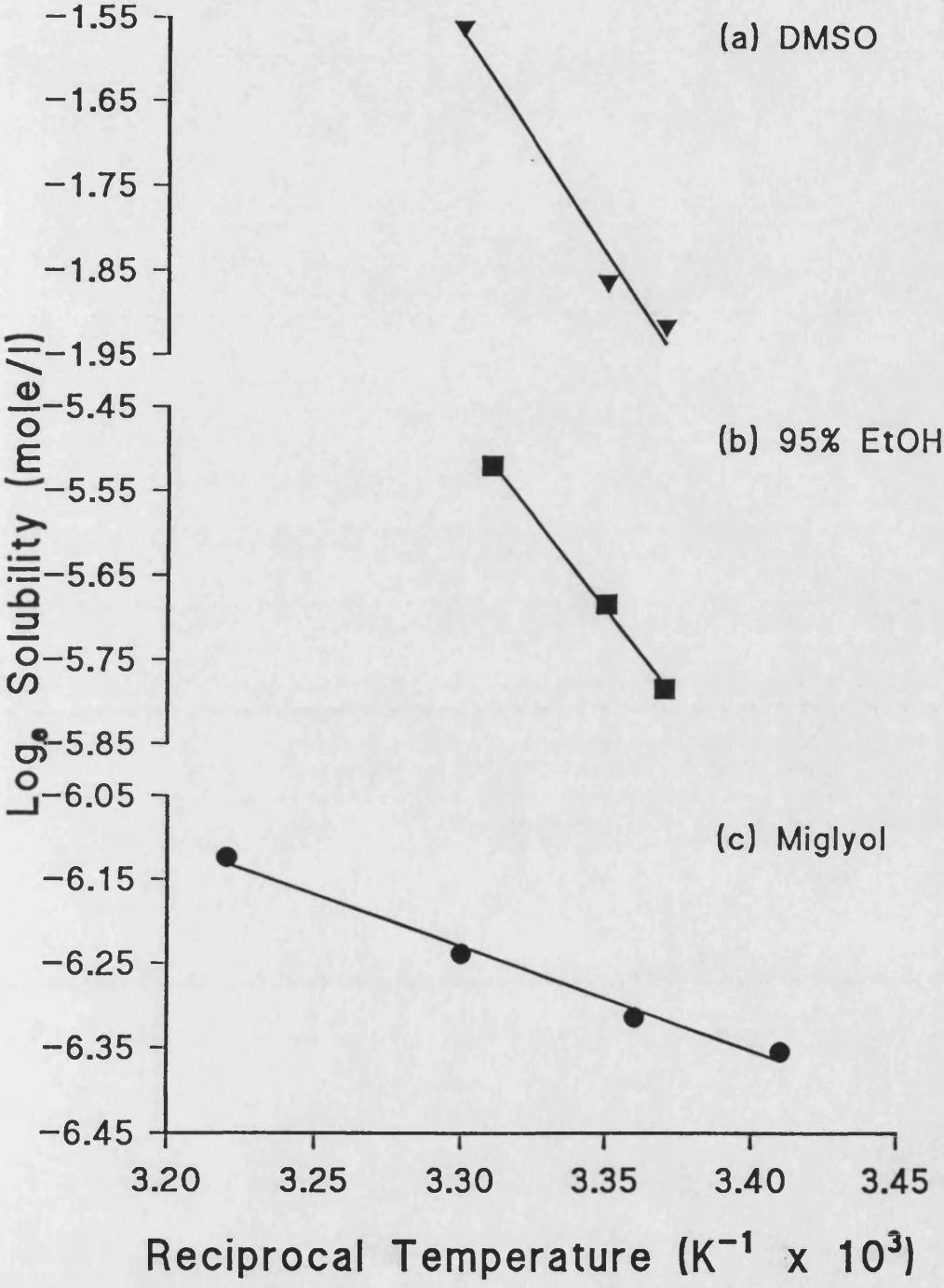


Fig. 4.15 Van't Hoff plot for solubility of ABZ in (a) DMSO, (b) 95% EtOH and (c) Miglyol.

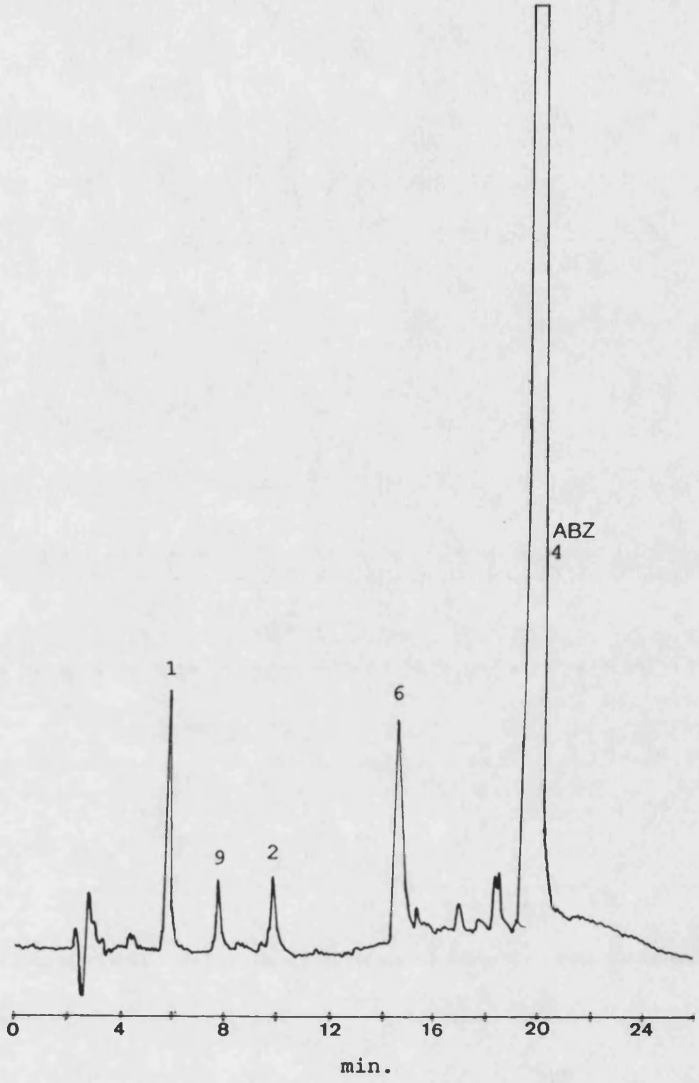
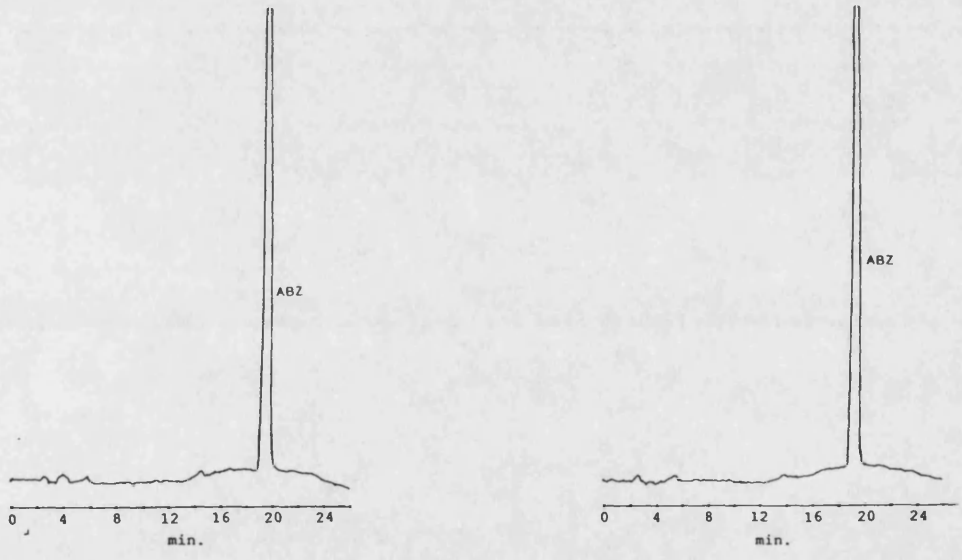


Fig. 4.16 A typical chromatogram of ABZ (4) and the degradation products in DMSO in the solubility study of ABZ. (144hr sample when study was carried out at 23.30°C).

Table 4.11 Concentrations of ABZ in 95% EtOH at different temperatures.

Sampling time (hr)	ABZ concentration (mg/ml) 23.30°C	25.20°C	29.10°C
12.0	0.86	0.96	1.19
24.0	0.80	0.82	1.06
36.0	0.89	0.85	1.09
60.0	0.80	0.95	1.05
96.0	0.85	0.89	0.95
144.0	0.85	0.90	0.93



(a) (b)

Fig. 4.17 Representative chromatograms from solubility study of ABZ in 95% EtOH. (a) 144 hr sample when study was carried out at 23.30°C and (b) 96 hr sample at 29.10°C.

(c) Miglyol

Table 4.10 gives the solubility of ABZ in Miglyol at various temperatures and the Van't Hoff plot (Fig. 4.15c) shows a linear relationship; the calculated standard enthalpy for ABZ dissolution was 10±1 kJ/mole. ABZ was stable in Miglyol at these temperatures (Table 4.12) and degradation products were not observed (Fig. 4.18).

(d) Water

The solubility of ABZ in water was impossible to determine by Method 1 (4.2.2) because the assayed concentrations of ABZ increased until 8 and 36 hours for 23.10°C and 29.10°C, respectively and then gradually decreased as shown in Fig. 4.19. ABZSO was present up to 4% of ABZ analysed.

(e) 0.1M and 0.01M HCl

The measured concentrations of ABZ in 0.1M and 0.01M HCl solutions progressively increased over 5-6 days (Fig. 4.20) and therefore, the solubility of ABZ in these solutions was not established by the Method 1 (4.2.2). ABZSO was detected from 24 hours in both solutions and was present up to 7.0% and 8.0% of the ABZ by 48 hours in 0.1M and 96 hours in 0.01M HCl solution, respectively.. The ABZ 2-amino compound was also found in 0.1M HCl solution from 48 hours. The concentrations of ABZ achieved in 0.1M HCl solution appear to be an order of magnitude higher than those in 0.01M HCl solution.

Table 4.12 Concentrations of ABZ in Miglyol at different temperatures.

Sampling time (hr)	ABZ concentration (mg/ml) 20.02°C	24.75°C	30.40°C	37.10°C
24.0	0.46	0.50	0.51	0.56
48.0	0.46	0.48	0.53	0.54
72.0	ND	0.47	0.41	0.55
96.0	0.46	0.49	0.52	0.63
120.0	0.47	0.49	0.48	0.60

ND: not determined

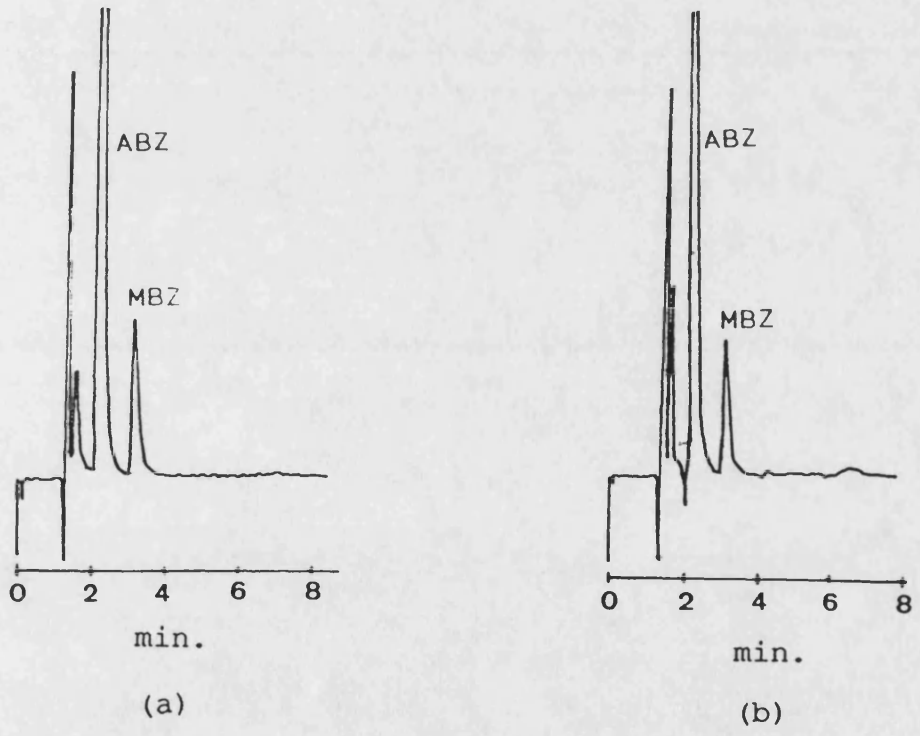


Fig. 4.18 Representative chromatograms from solubility study of ABZ in Miglyol. (a) 96 hr sample when study was carried out at 20.05°C and (b) 144 hr sample at 37.10°C.

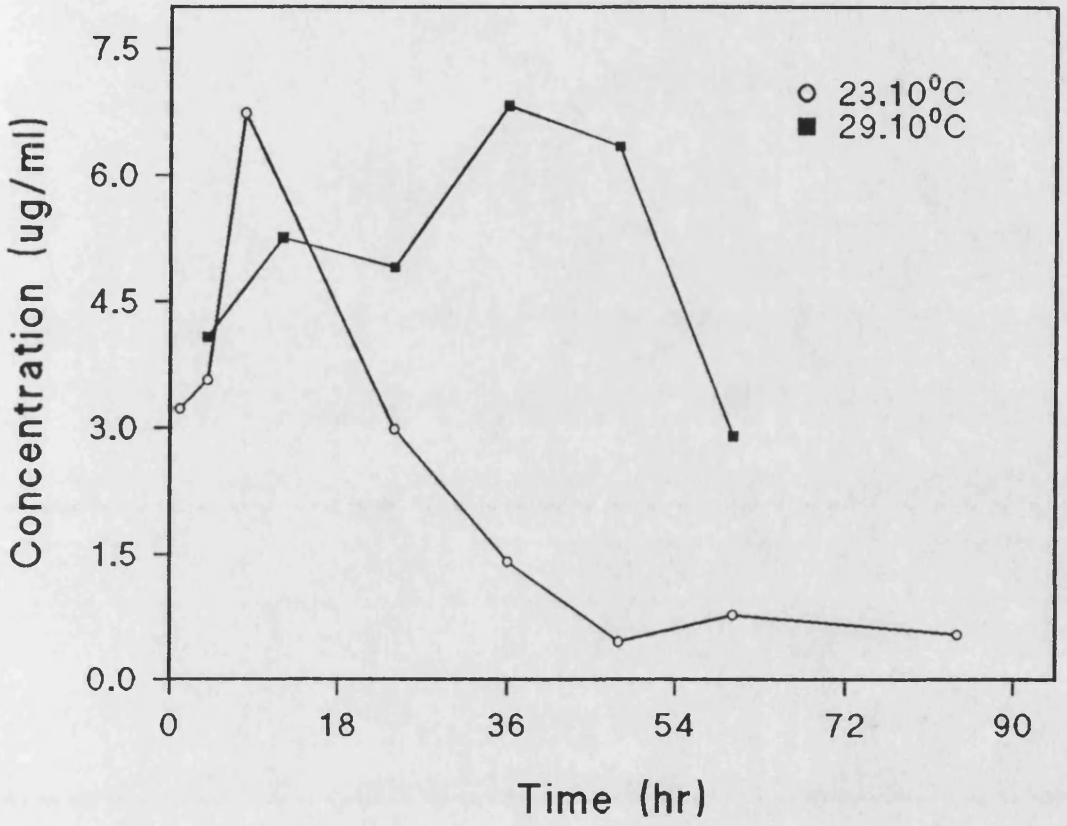
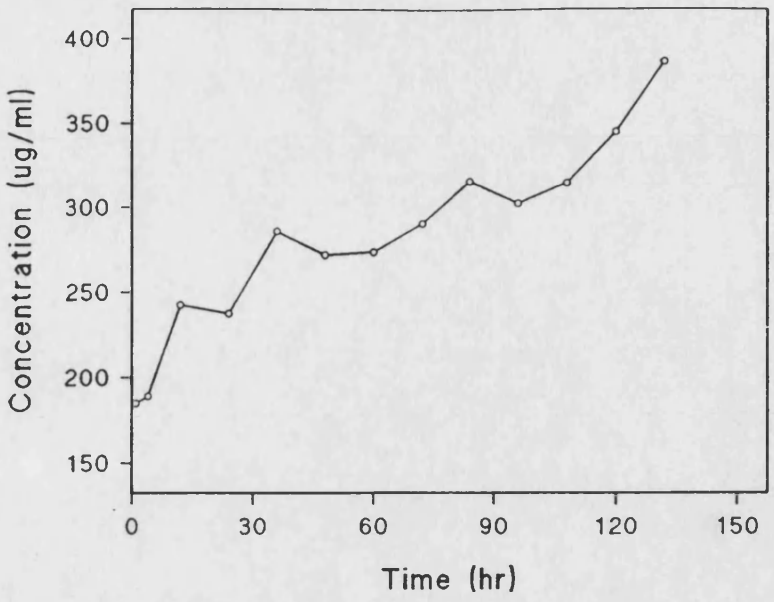
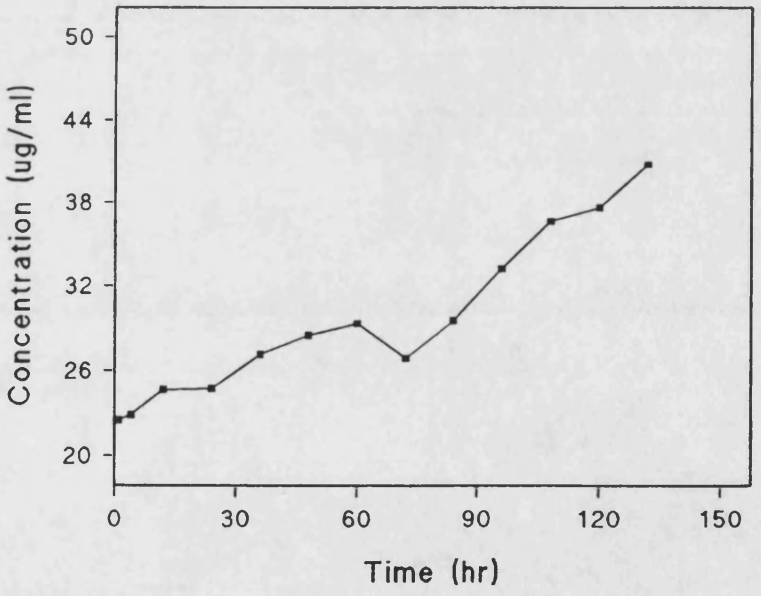


Fig. 4.19 Dissolution profile of ABZ in water at 23.10°C and 29.10°C. Data are shown as concentration against time plot.



(a)



(b)

Fig. 4.20 Dissolution profile of ABZ (a) in 0.1M HCl and (b) in 0.01M HCl, both at 30.30°C. Data are shown as concentration against time plot.

4.3.2.2 Results for Solubility of ABZ in Miglyol, Tween and PEG 400

(a) Method 3

ABZ was soluble up to 1 mg/g in PEG 400 at 37°C. The dissolution of ABZ in any of the drug-vehicle mixtures (Mig:Twn 80; 60:40%, 58:42%, 61:39%, w/w) was very slow. The concentration of ABZ dissolved in these mixtures was 0.5 mg/g after 2 weeks at room temperature.

(b) Method 2

When the solubility of ABZ was determined by using Method 2 (4.2.2) in PEG 400 and mixtures of Tween 80 or 85 and Miglyol (40:60%, w/w), ABZ was soluble up to 2 mg/g in the mixtures and up to 5 mg/g in PEG 400. Results are presented in Table 4.13.

Table 4.13 Results from the solubility study of ABZ in Tween 80/85 and Miglyol mixture and PEG 400 from Method 2.

ABZ concentration (mg/g)	Observation: After heating	Observation: After overnight standing at room temperature
Tween 80:Miglyol (40:60%,w/w)		
2.0	clear	clear
3.0	clear	drug precipitation
5.0	clear	drug precipitation
10.0	cloudy	cloudy
Tween 85:Miglyol (40:60%,w/w)		
2.0	clear	clear
5.0	clear	drug precipitation
10.0	cloudy	cloudy
PEG 400		
4.0	clear	clear
5.0	clear	clear
6.0	clear	drug precipitation
7.5	clear	drug precipitation
9.5	clear	drug precipitation

4.3.2.3 Results from Solubility Studies of "ABZ Solutions"

Tables 4.14, 4.16, 4.18 and 4.21 summarise the absorbance data as the mean value for each replicate run at a given ABZ concentration. However the data for each individual run was subjected to linear regression analysis, the results from which are shown in Tables 4.15, 4.17, 4.19, 4.20 and 4.22. Figs. 4.21-4.23 and 4.25 illustrate typical concentration-absorbance plots obtained from the solubility of ABZ-DMSO solution when added to water and 0.1M HCl, ABZ-Mig:Twn 80 solution when added to 0.1M HCl and ABZ-PEG 400 solution when added to 0.1M HCl, respectively. A typical concentration-volume added plot obtained from the solubility of ABZ-Mig:Twn 80 solution when added to 0.1M HCl solution is shown in Fig. 4.24.

(a) ABZ-DMSO Solution

(i) When added to water

At room temperature (~17°C) a few particles were observed in the solution after 100 µl of ABZ-DMSO solution (5 mg/ml) had been added, giving a final ABZ concentration of 5.00 µg/ml (0.1% DMSO) in the volumetric flask (Method 4 (a)). At a concentration of 7.49 µg/ml ABZ (0.15% DMSO), a milky precipitate formed in the solution. A similar situation occurred at ABZ concentration of 12.47 µg/ml (0.25% DMSO) and 17.89 µg/ml (0.36% DMSO) when using the volumetric flask and jacketed-beaker (Method 4 (b)) techniques, respectively at 37°C. Particles (ABZ) were found on the wall of the volumetric flask or beaker above the solution level.

The absorbances measured at 450 nm to assess turbidity when ABZ-DMSO solution was added to water in volumetric flask (17°C and 37°C) and jacketed-beaker (37°C) are presented in Table 4.14 and the linear

regression data are shown in Table 4.15. Fig. 4.21 shows a typical plot of concentration against absorbance for the solubility of "ABZ-DMSO solution" added to water. The plot axes are reversed from normal to allow solubility determination by linear regression analysis of the data; solubility = intercept. The replicate intercepts for a given method at 17°C (volumetric flask: 4.3±0.5 µg/ml, χ^2 = 8.09, χ^2_{tab} = 11.07) and at 37°C (volumetric flask: 7.5±0.4 µg/ml, χ^2 = 6.09; jacketed-beaker: 11.7± 0.4 µg/ml, χ^2 = 0.64, χ^2_{tab} = 11.07) were not significantly different. However comparison using Student's t-test the solubilities in the flask and jacketed-beaker at 37°C were different at P = 0.05 (t = 8.42, t_{tab} = 2.25).

(ii) When added to 0.1M HCl

When ABZ-DMSO solution was added to 0.1M HCl at 37°C, particles were observed in the solution at ABZ concentration of 0.98 and 0.87 mg/ml in the jacketed-beaker (Method 4(b)) and flask (method 4(a)), respectively. The mean results are shown in Table 4.16 and the regression analysis data in Table 4.17. Fig. 4.22 shows a typical plot of absorbance versus concentration for the solubility of ABZ in 0.1M HCl from DMSO solution. The replicate intercepts were different between a given method; jacketed-beaker: 0.3±0.05 mg/ml, χ^2 = 11.20 and flask: 0.32±0.09 mg/ml, χ^2= 24.48, χ^2_{tab} = 11.07 at P = 0.05. The enhanced solubility of ABZ in 0.1M HCl from DMSO solution at 37°C however were not significantly different between the two techniques (jacketed-beaker and flask, t = 0.99, t_{tab} = 2.25 at P = 0.05).

Table 4.14 Absorbance (mean±SD) of ABZ solution in the solubility study of ABZ-DMSO solution added to water. (n = 6)

ABZ concentration (μg/ml)	DMSO concentration (%)	Absorbance in volumetric flask 17°C	37°C
5.00	0.10	0.007±0.004	
7.49	0.15	0.018±0.004	0.004±0.002
9.98	0.20	0.036±0.006	0.015±0.004
12.47	0.25	0.056±0.007	0.033±0.004
14.96	0.30	0.074±0.007	0.056±0.005
17.44	0.35	0.087±0.008	0.088±0.006
19.92	0.40	0.101±0.008	0.097±0.007
		in jacketed-beaker 37°C	
12.77	0.26	0.006±0.001	
15.30	0.31	0.011±0.002	
17.89	0.36	0.021±0.003	
20.42	0.41	0.034±0.007	
22.94	0.46	0.043±0.007	
25.47	0.51	0.048±0.006	

Table 4.15 Linear regression data for the plots of concentration-absorbance for ABZ-DMSO solution added to water.

Replicate	Slope	SD	Intercept (mg/ml)	SD
Volumetric flask at 17°C				
1	142.2	3.8	3.4	0.3
2	165.0	6.3	4.7	0.5
3	153.3	6.6	4.0	0.4
4	157.6	6.6	4.3	0.4
5	157.5	8.3	4.5	0.6
6	134.0	5.8	4.9	0.4
Mean	154.6	6.2	4.3	0.1
Volumetric fask at 37°C				
1	144.8	6.5	7.3	0.2
2	131.8	4.6	7.0	0.3
3	143.3	7.0	7.2	0.5
4	135.8	8.7	8.1	0.6
5	135.9	5.4	7.3	0.3
6	138.2	6.7	8.0	0.4
Mean	138.3	6.7	7.5	0.4
Jacketed-beaker at 37°C				
1	235.1	44.9	11.5	1.7
2	214.6	43.6	12.3	1.6
3	298.3	30.7	11.5	0.9
4	350.9	47.2	11.2	1.2
5	298.7	29.2	12.2	0.8
6	290.1	26.8	1 1.7	1.2

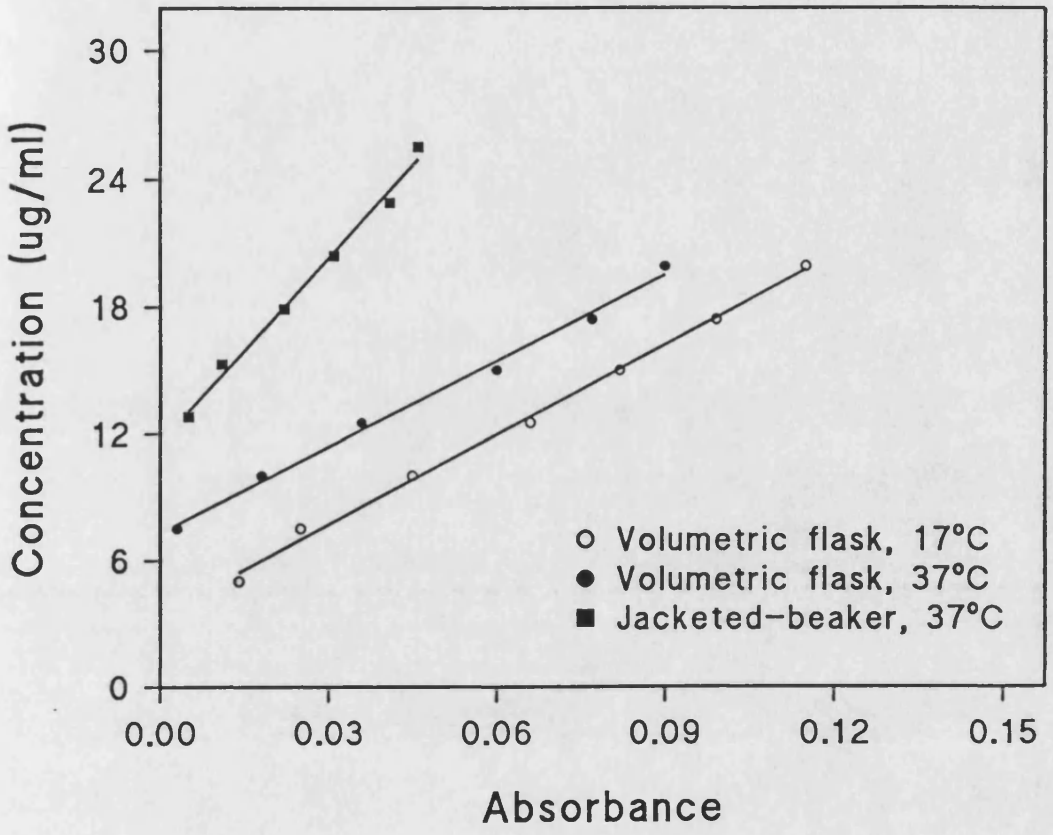


Fig. 4.21 A typical plot of concentration against absorbance obtained from the solubility of " ABZ-DMSO solution" (5 mg/ml) when added to water at 17°C in flask, 37°C in flask and 37°C in jacketed-beaker.

Table 4.16 Absorbance (Mean±SD) of ABZ solution in the solubility study of ABZ-DMSO solution added to 0.1M HCl at 37°C. (n = 6)

ABZ concn (mg/ml)	DMSO concn (%)	Absorbance jacketed-beaker	ABZ concn (mg/ml)	DMSO concn (%)	Absorbance volumetric flask
0.66	2.2	0.003 ±0.001	0.59	2.0	0.004 ±0.001
0.82	2.8	0.005 ±0.001	0.73	2.5	0.005 ±0.001
0.98	3.4	0.007 ±0.002	0.87	3.0	0.007 ±0.001
1.13	3.9	0.009 ±0.002	1.01	3.5	0.008 ±0.002
1.29	4.5	0.010 ±0.002	1.15	4.0	0.010 ±0.001
1.44	5.0	0.011 ±0.002	1.29	4.5	0.012 ±0.001
1.59	5.6	0.013 ±0.002	1.43	5.0	0.014 ±0.001
1.74	6.2	0.014 ±0.002	1.56	5.5	0.016 ±0.002
1.89	6.7	0.016 ±0.002	1.70	6.0	0.017 ±0.001
2.04	7.3	0.018 ±0.002	1.83	6.5	0.018 ±0.002
2.18	7.8	0.019 ±0.003	1.96	7.0	0.020 ±0.001
			2.09	7.5	0.022 ±0.001
			2.22	8.0	0.023 ±0.001

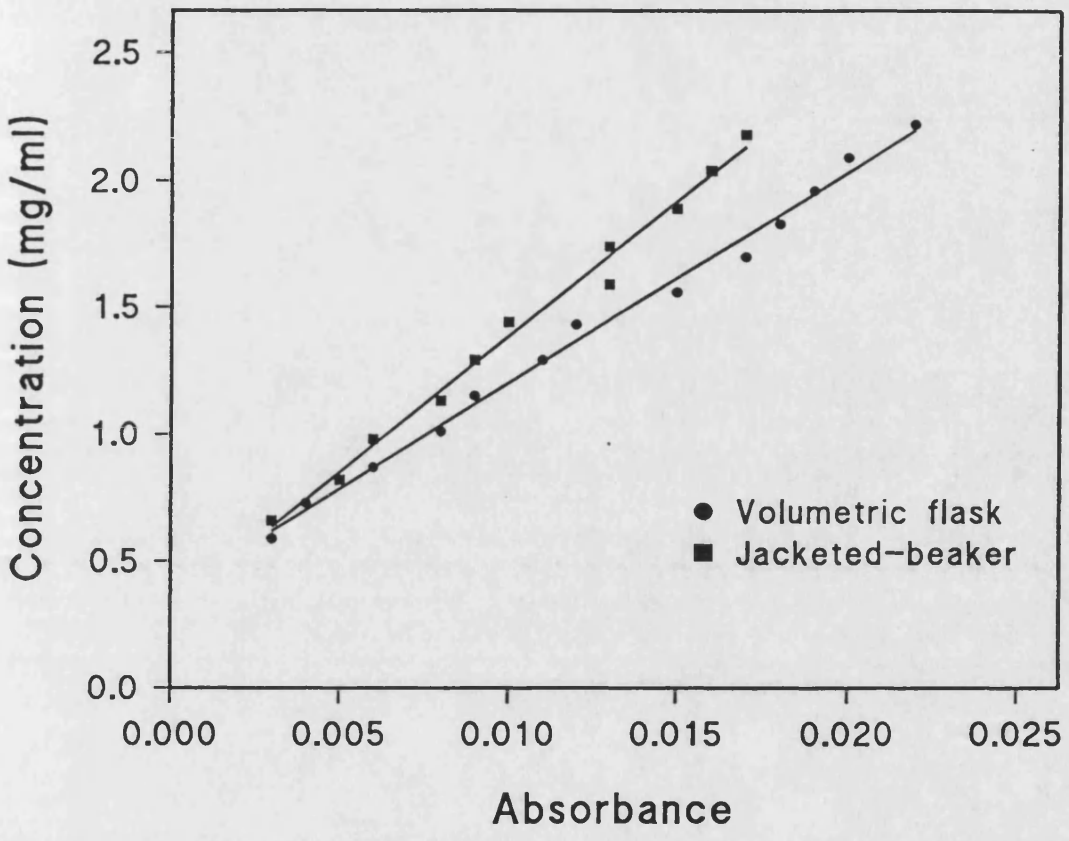


Fig. 4.22 A typical plot of concentration against absorbance obtained from the solubility of " ABZ-DMSO solution" (30 mg/ml) when added to 0.1M HCl at 37°C in the flask and jacketed-beaker.

(b) ABZ-Mig:Twn 80 Solution (Method 4(b))

On addition of ABZ Mig:Twn solution to 0.1M HCl (37°C) the solution became turbid. The mean measured absorbances are presented in Table 4.18. Linear plots of concentration against absorbance were obtained and regression analysis data are presented in Table 4.19. The replicate intercepts were significant difference (χ^2 = 18.38, χ^2_{tab} = 11.07 at P = 0.05) with a mean value of -0.02±0.05, i.e. passing through origin. Fig. 4.23 shows a typical concentration against absorbance plot for the solubility of ABZ in 0.1M HCl from Mig:Twn solution.

Similar behaviour was observed when blank Mig:Twn solution was added to 100 ml 0.1M HCl at the same temperature. The mean absorbance are given in Table 4.18. For comparison with the blank, the volume of Mig:Twn solution added was plotted against the absorbance. The regression analysis data are shown in Table 4.20 and Fig. 4.24 shows a typical plot. The mean intercepts for both systems have negative values; with ABZ: -1.2±2.2 and with no ABZ (blank): -4.5±5.1 and they were significantly different (t = 4.39, t_{tab} = 2.25 at P = 0.05). The negative intercepts suggest that the intercepts for these systems were masked by the presence of Mig:Twn and therefore the solubility of ABZ-Mig:Twn solution added to 0.1M HCl was not established.

Microscopic examination of the emulsion formed by adding ABZ Mig:Twn solution to 0.1M HCl showed that some ABZ crystals were found in the aqueous phase. The scale-drawing of the blank Mig:Twn solution in HCl, ABZ in an aqueous suspension and the emulsion are shown in Fig. 4.25. Although photomicrographs were developed, these gave poor images and have therefore not been included.

Table 4.17 Linear regression data for plots of absorbance-concentration for ABZ-DMSO solution added to 0.1M HCl at 37°C.

Replicate	Slope	SD	Intercept (mg/ml)	SD
Volumetric flask				
1	83.1	2.0	0.37	0.03
2	83.6	2.7	0.29	0.04
3	84.4	1.9	0.27	0.03
4	82.4	1.9	0.36	0.03
5	81.7	1.4	0.27	0.01
6	79.0	2.3	0.27	0.04
Mean	82.4	2.0	0.30	0.03
Jacketed-beaker				
1	109.9	3.4	0.28	0.04
2	106.9	3.5	0.32	0.04
3	97.7	2.8	0.50	0.03
4	99.3	4.0	0.30	0.05
5	105.2	2.7	0.24	0.03
6	106.7	4.0	0.31	0.05
Mean	103.3	3.4	0.32	0.04

Table 4.18 Absorbance (mean±SD) of ABZ solution in the solubility study of ABZ-Mig:Twn solution added to 0.1M HCl at 37°C. (n = 6)

Concentration (µg/ml)	Absorbance ABZ	Volume added (µl)	Absorbance blank
0.17	0.129±0.031	8.50	0.175±0.062
0.34	0.262±0.052	17.10	0.275±0.047
0.51	0.372±0.038	25.60	0.405±0.048
0.69	0.496±0.077	34.20	0.508±0.049
0.86	0.627±0.049	42.70	0.626±0.044
1.03	0.749±0.050	51.20	0.739±0.040
1.20	0.870±0.071	59.80	0.858±0.028
1.37	0.978±0.070	68.30	0.952±0.024
1.55	1.078±0.090		
1.72	1.171±0.087		

Table 4.19 Linear regression data for plots of absorbance-concentration for ABZ-Mig:Twn solution added to 0.1M HCl at 37°C.

Replicate	Slope	SD	Intercept (µg/ml)	SD
1	1.39	0.04	0.02	0.03
2	1.43	0.02	0.07	0.02
3	1.53	0.02	-0.05	0.01
4	1.40	0.04	-0.06	0.03
5	1.41	0.04	-0.07	0.03
6	1.65	0.06	-0.01	0.04
Mean	1.47	0.04	-0.02	0.03

Table 4.20 Linear regression data for plots of absorbance-added volume for ABZ-Mig:Twn solution added to 0.1M HCl at 37°C.

Replicate	Slope	SD	Intercept (µl)	SD
With ABZ				
1	64.3	1.0	3.0	2.5
2	70.3	1.8	-3.2	1.2
3	74.7	1.1	-1.9	0.7
4	65.7	1.4	-0.9	0.9
5	68.0	2.4	-2.7	1.6
6	77.6	3.4	-1.4	1.8
Mean	70.1	1.8	-1.2	1.4
Without ABZ (blank)				
1	78.6	1.8	-6.3	1.1
2	70.0	2.3	0.6	1.4
3	76.6	1.4	-5.8	0.9
4	81.1	1.9	-12.2	1.3
5	74.5	1.1	-5.1	1.1
6	72.6	1.5	1.7	0.9
Mean	75.6	1.7	-4.5	0.2

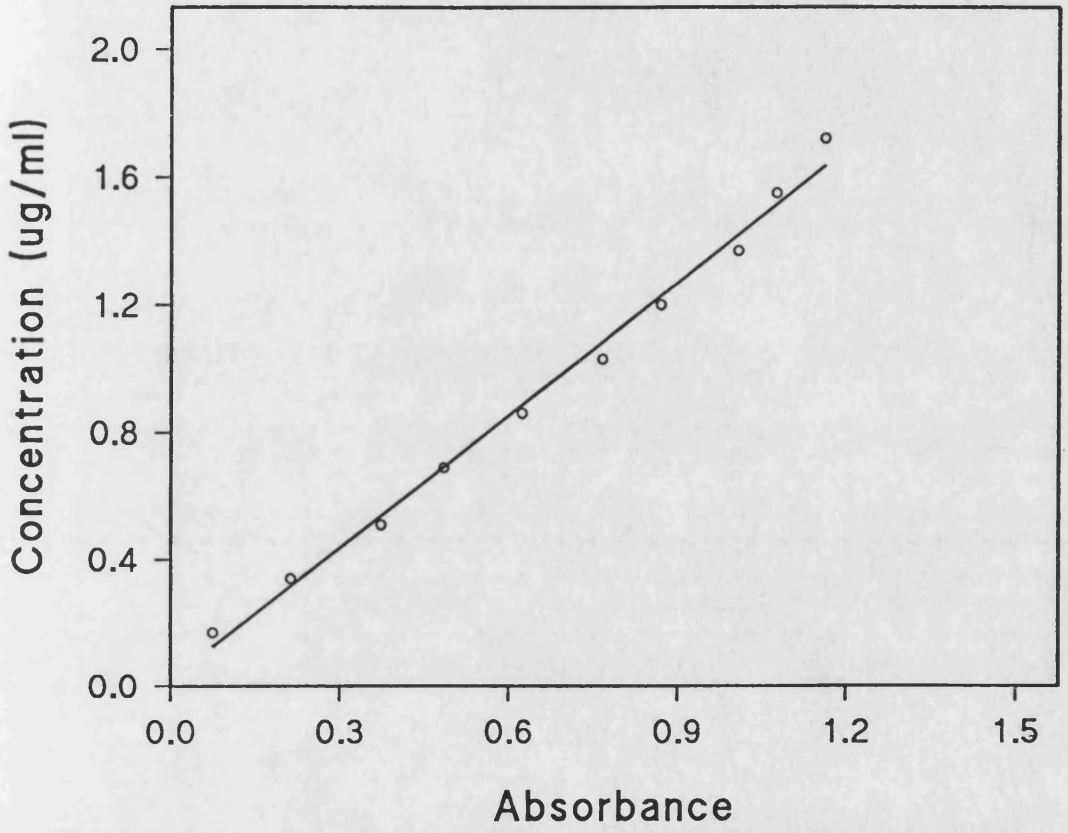


Fig. 4.23 A typical plot of concentration against absorbance obtained from the solubility of "ABZ-Mig:Twn solution" (2 mg/g) when added to 0.1M HCl at 37°C.

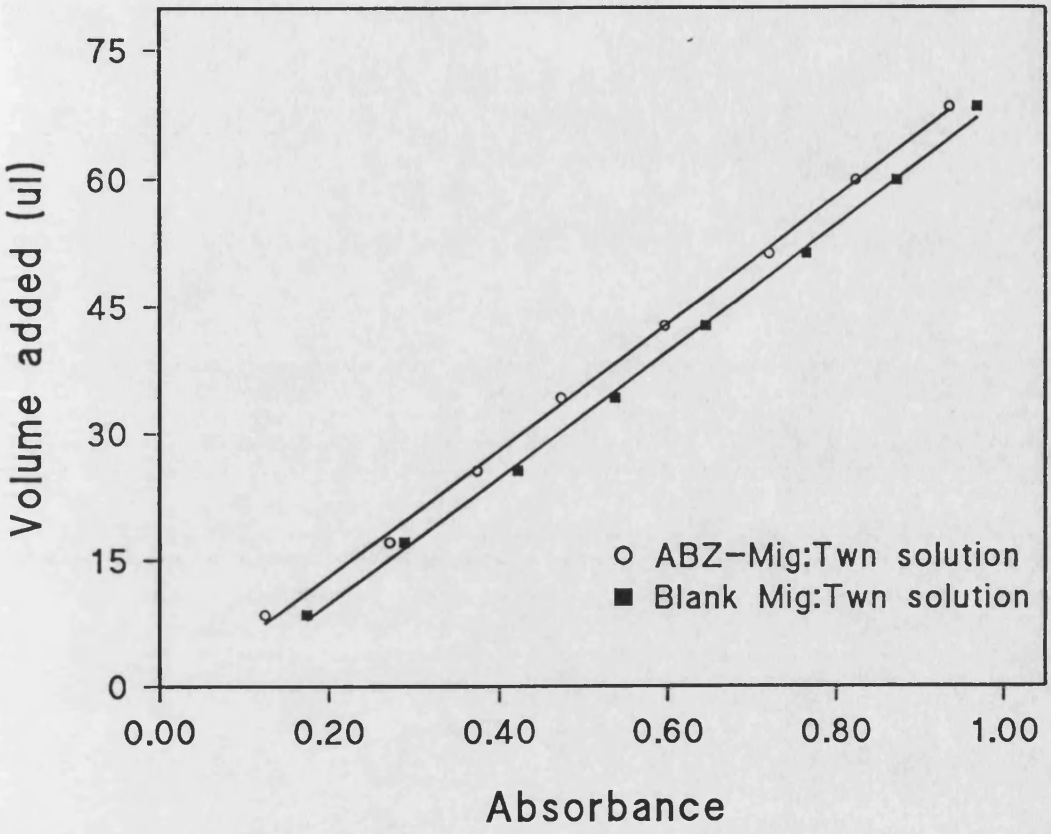
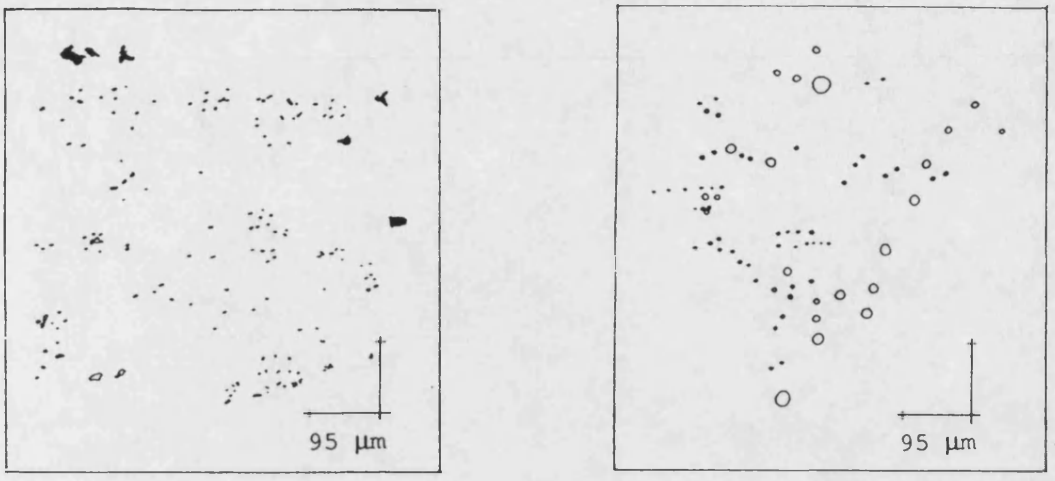
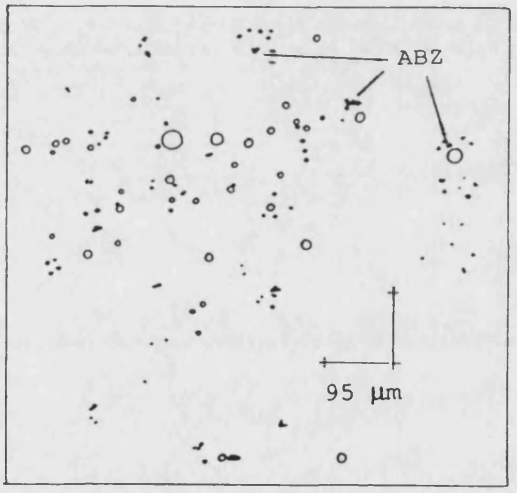


Fig. 4.24 A typical plot of concentration against volume added obtained from the solubility of "Mig:Twn solution" with and without ABZ when added to 0.1M HCl at 37°C.



(a) (b)



(c)

Fig. 4.25 Diagram from the scale-drawing of (a) ABZ in aqueous suspension, (b) blank Mig:Twn in 0.1M HCl and (c) "ABZ-Mig:Twn solution" in 0.1M HCl (the dark spots are ABZ).

(c) ABZ-PEG 400 Solution (Method 4(c))

When 0.1M HCl was added to ABZ-PEG solution, some particles were seen in the solution but then decreased as the volume 0.1M HCl solution added was increased. The absorbances measured were all very low (<0.02) leading to high standard error for slopes. The mean absorbance are shown in Table 4.21 and a typical plot of concentration versus absorbance is presented in Fig.4.26. Table 4.22 gives the linear regression data with mean intercept of 0.2±0.7 and there was no significant difference between the replicates (χ^2 = 1.58, χ^2_{tab} = 9.49 at P = 0.05). However these intercepts passed through zero within ±2 SD, no definitive solubility value could be established.

Table 4.21 Absorbance (mean±SD) of ABZ solution in the solubility study of ABZ-PEG 400 solution added to 0.1M HCl at 37°C. (n = 5)

Concentration (mg/ml)	Absorbance
2.82	0.019±0.003
2.26	0.014±0.007
1.88	0.010±0.005
1.61	0.010±0.005
1.41	0.008±0.003
1.25	0.007±0.003
1.13	0.008±0.003
1.03	0.007±0.002
0.94	0.005±0.002
0.81	0.006±0.002

Table 4.22 Linear regression data for plots of absorbance-concentration for ABZ-PEG 400 solution added to 0.1M HCl at 37°C.

Replicate	Slope	SD	Intercept (mg/ml)	SD
1	229.1	151.7	-0.30	1.2
2	129.8	33.3	-0.33	0.50
3	88.1	76.0	0.68	0.82
4	116.0	25.1	0.47	0.26
5	121.5	92.4	0.60	0.78

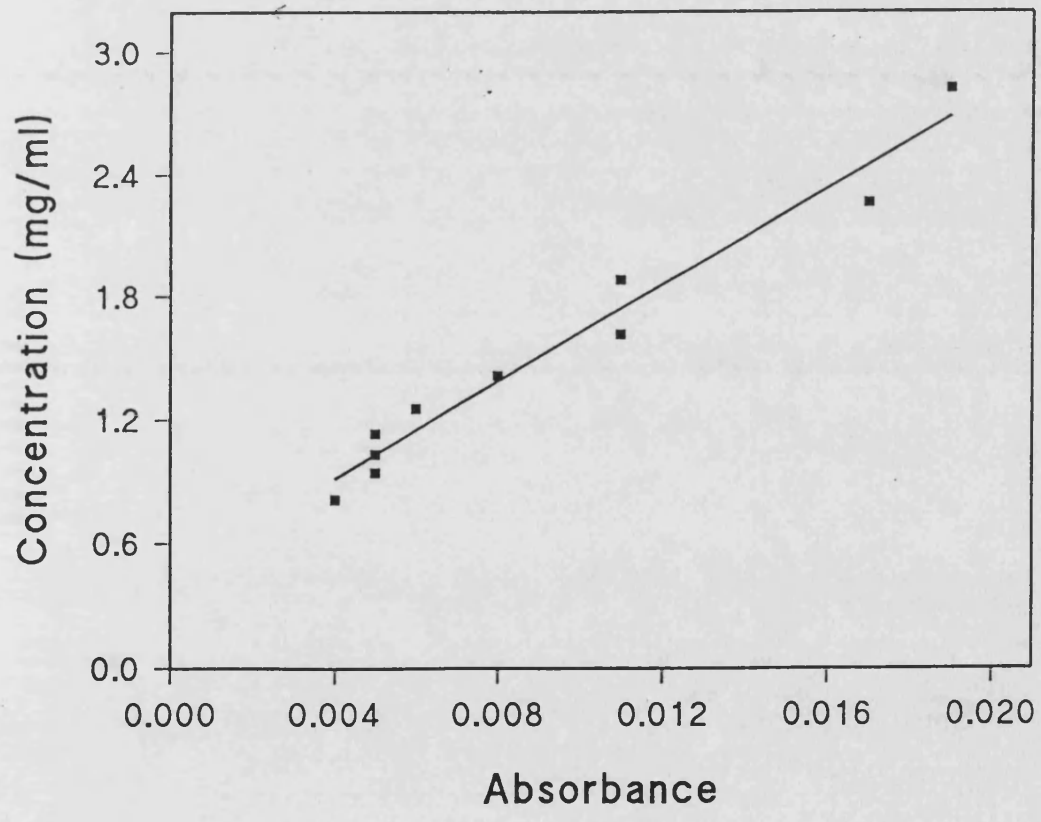


Fig. 4.26 A typical plot of concentration against absorbance obtained from the solubility study of "ABZ-PEG 400 solution" (5 mg/g) when added to 0.1M HCl at 37°C.

4.3.3 Triangular-Phase Diagram for Miglyol, Tween 80 and PEG 400

Fig. 4.27 shows the miscibility phase diagram for the three potential vehicle components; Miglyol, PEG 400 and Tween 80. Miscible regions were found in the lower left end of the triangle and when 10% Tween 80 was mixed with 90% PEG 400. The mixture of 60:40% of Miglyol:Tween 80 was selected for ABZ formulation as it formed one-phase system with the highest volume of the oil.

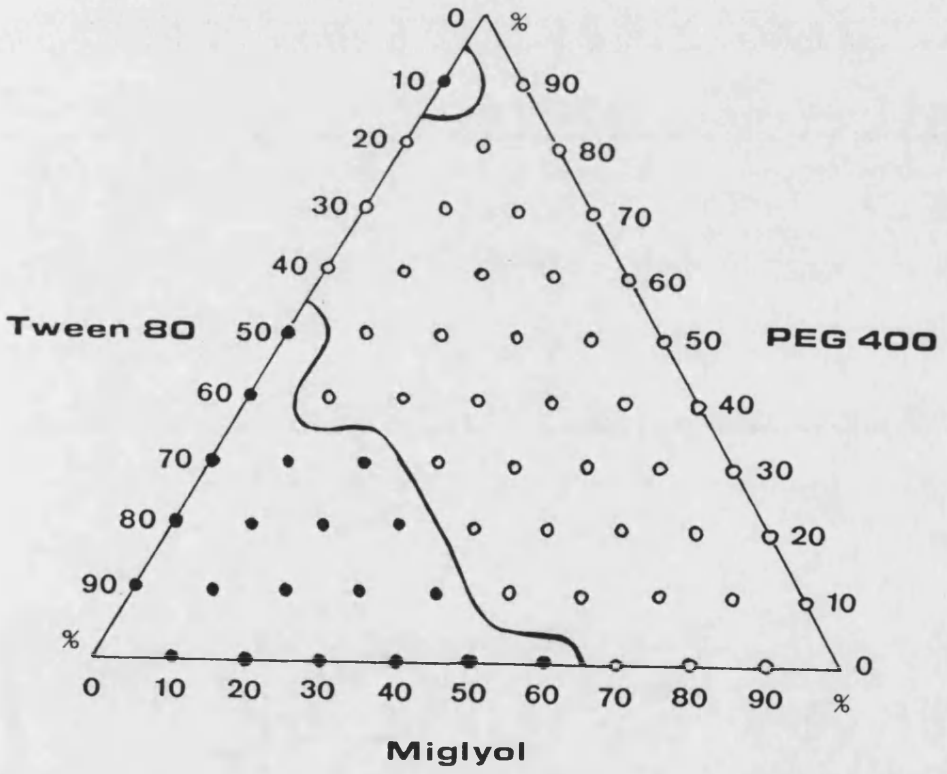


Fig. 4.27 Triangular phase diagram showing the boundaries of micsible region and the 2-layer system of Miglyol, Tween 80 and PEG 400 at room temperature. (● represents miscible region and o represents the 2-layer system).

4.3.4 Solubility of Gluconate Salt

ABZ gluconate salt was formed as sticky solid. It was soluble in PEG 400 (up to 0.5 g/g), slightly soluble in water (0.1 g/ml) and completely insoluble in Miglyol (Table 4.23). Since drug solubility was not improved, further work using this approach was discontinued.

Table 4.23 Results from the solubility study of ABZ gluconate solid product (0.5g) in water, PEG 400 and Miglyol.

Solvent added	Observation: After heating	Observation: After overnight standing at room temperature
Water		
1 ml	clear	drug precipitation
2 ml	clear	drug precipitation
3 ml	clear	clear
PEG 400		
1 g	clear	clear
2 g	clear	clear
3 g	clear	clear
5 g	clear	clear
Miglyol		
5 g	not soluble	
10 g	not soluble	

4.4 DISCUSSION

In the stability studies of ABZ suspensions in the glycine buffer with and without SDS at pH 9.00 and 10.00, the overall reaction can be represented as:

$$A_{suspension} \xrightarrow[\text{Dissolution}]{\text{(I)}} A_{solution} \xrightarrow[\text{Degradation}]{\text{(II)}} B(\text{products})$$

At the start of the process, the solid drug in $A_{suspension}$ started to dissolve slowly in the solution with as the temperature increased to become $A_{solution}$. The degradation of ABZ occurred simultaneously with dissolution process as shown by the detection of the degradation compounds (Figs. 4.6 and 4.10)

Three degradation products were detected by HPLC; ABZSO, Unknown 1 and Unknown 2, suggesting that the degradation of ABZ may involve more than one pathway. Oxidation of ABZ must have taken place in order to produce ABZSO. ABZ also undergoes hydrolysis as evidenced by the formation of Unknown 2, confirmed to be the ABZ 2-amino compound which is consistent with the findings of the alkaline hydrolysis of other carbamate esters (Dittert and Higuchi, 1963).

At pH 9.00, ABZSO was found in both systems. It was found in higher concentration in the system without SDS, although the initial ABZ concentration was 4-fold lower than in the presence of SDS. This observation is similar to the situation with Unknowns 1 and 2, where their rates of formation are faster in the absence of SDS than those in the system with SDS (Table 4.3). However, at pH 10.00 the rate of formation of Unknown 2 was higher in the system with SDS than in that

without SDS. The rate of formation of Unknown 2 was faster at pH 10.00 than at pH 9.00 in both systems, as was the degradation of ABZ (Figs. 4.5 and 4.9). The absence of ABZSO in both systems at pH 10.00 may thus be explained. Unknown 1 was not found in the system with no SDS at pH 10.00 probably due to rapid degradation of ABZ as well as owing to the low initial ABZ concentration in the suspension.

The possibility that SDS micelles solubilize ABZ and therefore protect it from degradation was considered. However, the critical micelle concentration (cmc) of SDS at the experimental conditions is much higher than the concentration of SDS used (0.011% solution). From the plot of cmc of SDS in water against temperature (Flockhart, 1957), the cmc of SDS at 25°C is 0.23%. By extrapolating the curve, the cmc of SDS at 100°C is 0.39%; 1.7 times higher than the value at 25°C. The cmc of SDS is also affected by the presence of electrolytes, the cmc of SDS at total cation concentration of 0.058M (at pH 9.00) was 0.06% at 26°C (Corrin and Harkins, 1947). Therefore, the cmc of SDS can be estimated as 0.10% at 100°C in glycine buffer at pH 9.00. The cmc of SDS at 100°C in glycine buffer at pH 10.00 (total cation concentrations = 0.082M, 0.05% at 26°C) was estimated in the same manner and found to be 0.09%.

Since the rate of formation of the hydrolytic degradation compound (Unknown 2) was higher than the formation of ABZSO, it may be assumed that the hydrolysis of ABZ dominates the oxidative degradation and the overall reaction is more likely to follow first-order kinetics. Therefore this assumption was tested by plotting log concentration against time for ABZ in aqueous suspension with and without SDS at pH 9.00 and 10.00. These plots are shown in Figs. 4.28 and 4.29. For pH

10.00 the fit is reasonable, although the data are limited. this is not the case for pH 9.00 where the points are scattered badly around the line of the best fit (Fig. 4.28). This indicates that at pH 10.00, hydrolysis dominates the overall degradation pattern of ABZ whilst at pH 9.00 other reactions (e.g. oxidation) occurring simultaneously with hydrolysis are probably a greater contribution to the degradation rate. This was also evidenced from the $t_{1/2}$ values of ABZ (Table 4.24). The $t_{1/2}$s calculated from the log concentration-time plot (Fig. 4.29) for the suspensions (with and without SDS) at pH 10.00 were the same as that measured from the simple concentration-time plot (Fig. 4.31) whereas for suspensions at pH 9.00 the calculated and measured $t_{1/2}$s were very different (Figs. 4.28 and 4.30).

The hydrolysis of ABZ at pH 10.00 was 2-times faster in the system with SDS compared to the system without SDS. The rates of degradation of ABZ at pH 9.00 in the presence and absence of SDS however were not significantly different (χ^2 = 0.89, χ^2_{tab} = 3.84 at P = 0.05). An increase from pH 9.00 to 10.00 would gave a theoretical increase in degradation rates of ~10-fold if specific OH^- catalysis is operating. However the rates of ABZ degradation at pH 10.00 were increased by 7.5-fold for system with SDS and 5.6-fold for system with no SDS which suggests the degradation of ABZ involved other pathways.

The stability studies of ABZ in aqueous suspensions showed that the degradation of ABZ was fastest at pH 10.00 at 100°C and the process was dominated by the hydrolysis as the rate of hydrolysis depends on the pH.

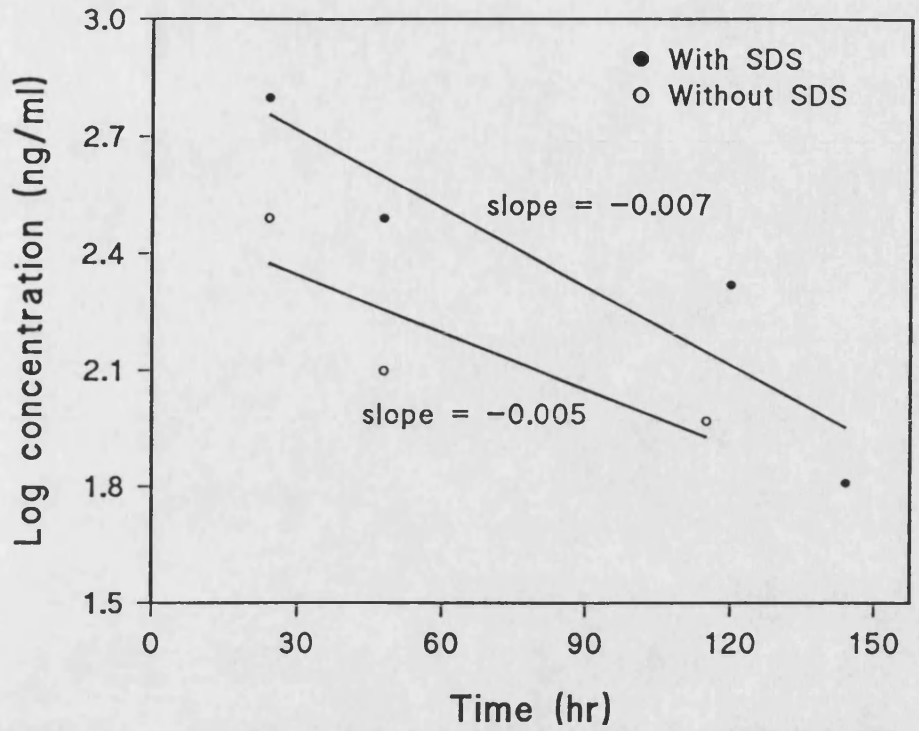


Fig. 4.28 Plot of log concentration against time showing the degradation of ABZ after heating 0.1% ABZ suspension at 100°C with and without SDS at pH 9.00. (The line of best fit is shown).

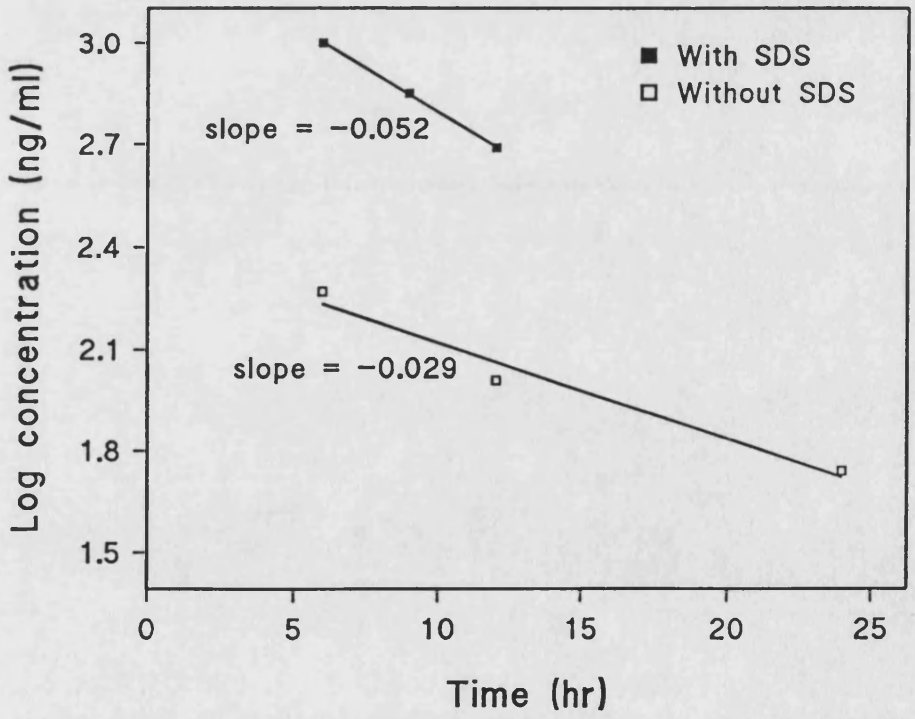


Fig. 4.29 Plot of log concentration against time showing the degradation of ABZ after heating 0.1% ABZ suspension at 100°C with and without SDS at pH 10.00. (The best fit line is shown).

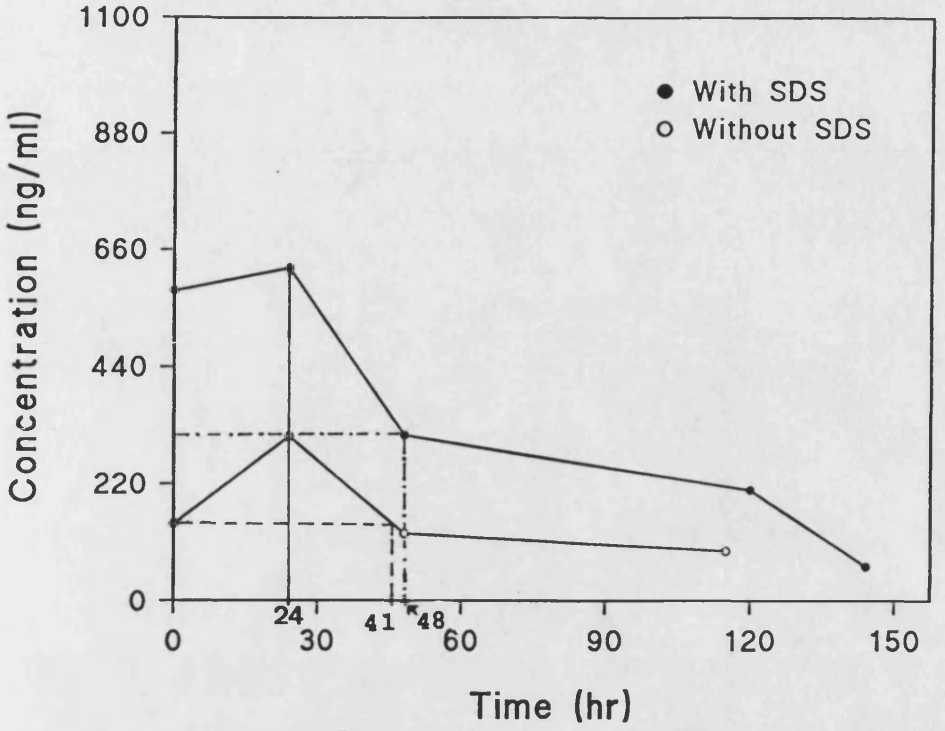


Fig. 4.30 Plot of concentration against time showing the degradation of ABZ after heating 0.1% ABZ suspension at 100°C with and without SDS at pH 9.00

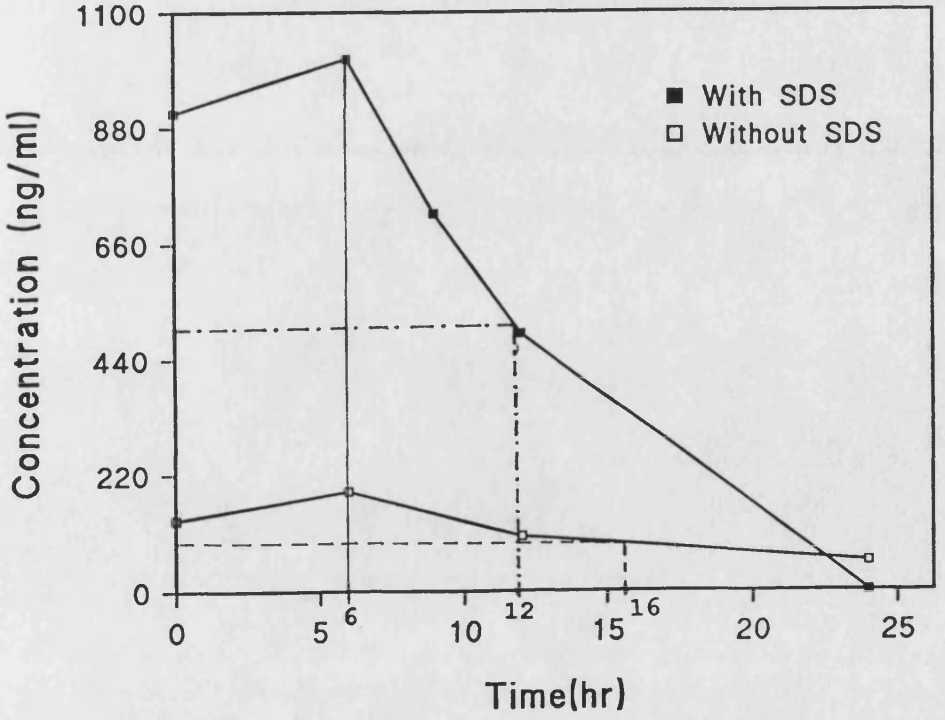


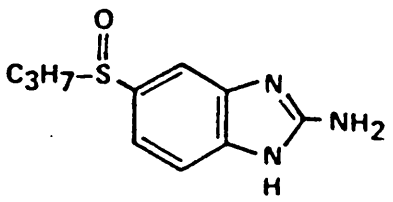
Fig. 4.31 Plot of concentration against time showing the degradation of ABZ after heating 0.1% ABZ suspension at 100°C with and without SDS at pH 10.00

Table 4.24 The rates of reaction (k) and half-lifes ($t_{1/2}$) of ABZ when heated at 100°C in aqueous suspension with and without SDS at pH 9.00 and 10.00.

Suspension	k (hr^{-1})	Calculated $t_{1/2}$ (hr)	Measured* $t_{1/2}$ (hr)
At pH 9.00			
With SDS	0.016	43	24
Without SDS	0.012	58	17
At pH 10.00			
With SDS	0.120	6	6
Without SDS	0.067	10	10

* the $t_{1/2}$s were measured from the plots of concentration against time of ABZ suspension heated at 100°C with and without SDS at pH 9.00 and 10.00 shown in Figs. 4.29 and 4.30.

Only one unidentified peak (Unknown 3) was found when ABZSO aqueous suspension with SDS at pH 10.00 was heated at 100°C. The rate of formation of the Unknown 3 was much faster than any of the other degradation compounds found in ABZ suspension under the same conditions. There was no evidence of any $ABZSO_2$ formed. There are 2 possible explanations; either oxidation of ABZSO did not take place or $ABZSO_2$ was formed by oxidation of ABZSO but was being degraded as it was formed and therefore would not be seen. If ABZSO behaves similarly to ABZ, then ABZSO will undergo hydrolysis in alkaline solution in the same manner as ABZ. Thus, Unknown 3 could probably be 5-(propylsulfinyl)-1*H*-benzimidazol-2-amine.



This characterization is supported by HPLC analysis. The peak of Unknown 3 having earlier retention time than ABZSO indicating that Unknown 3 is more polar than ABZSO.

Assuming that a hydrolytic reaction dominates in the degradation of ABZSO, the degradation data were fitted to first-order kinetic relationship (Fig. 4.32). From the curve, the calculated rate of reaction and $t_{1/2}$ were 0.85±0.11 hr^{-1} and 0.82 hr, respectively. The measured $t_{1/2}$ from the plot of ABZSO concentration against time (Fig. 4.33) was 0.9hr. The difference between the measured and the calculated t1/2 was 0.1 hr suggest that the data reasonably fit the first-order kinetics and therefore the overall degradation of ABZSO was also dominated by hydrolytic reaction at pH 10.00. The rate of reaction was rapid which explains the absence of ABZSO in the ABZ suspensions under the same conditions.

ABZSO, $ABZSO_2$, ABZ 2-amine compound (Unknown 2) and the other 4 unidentified peaks were found after samples of ABZ in DMSO were kept in the freezer (control samples) and when the samples were heated at 22°C, 37°C and 55°C for 48 hours. A possible explanation for ABZ degradation observed in the control samples may be due to DMSO reaction with ABZ while the samples were being thawed before the HPLC analysis. However degradation of samples at 55°C may be due to thermal effects as the ABZ 2-amine compound formed in these samples was higher compared to the control samples and samples at 22°C and 37°C. ABZ was also found to be degrading in DMSO during the solubility study, after 108 hour at 23.30°C and 72 hour at 25.20°C.

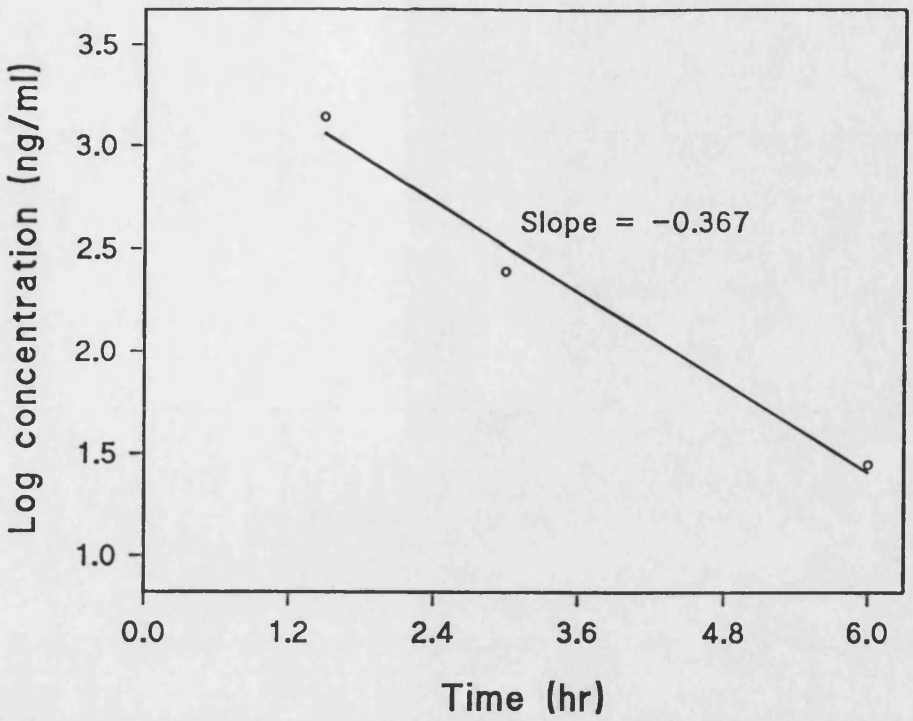


Fig. 4.32 Plot of log concentration against time showing the degradation of ABZSO after heating 0.1% ABZSO suspension at 100°C with SDS at pH 10.00. (The best fit line is shown).

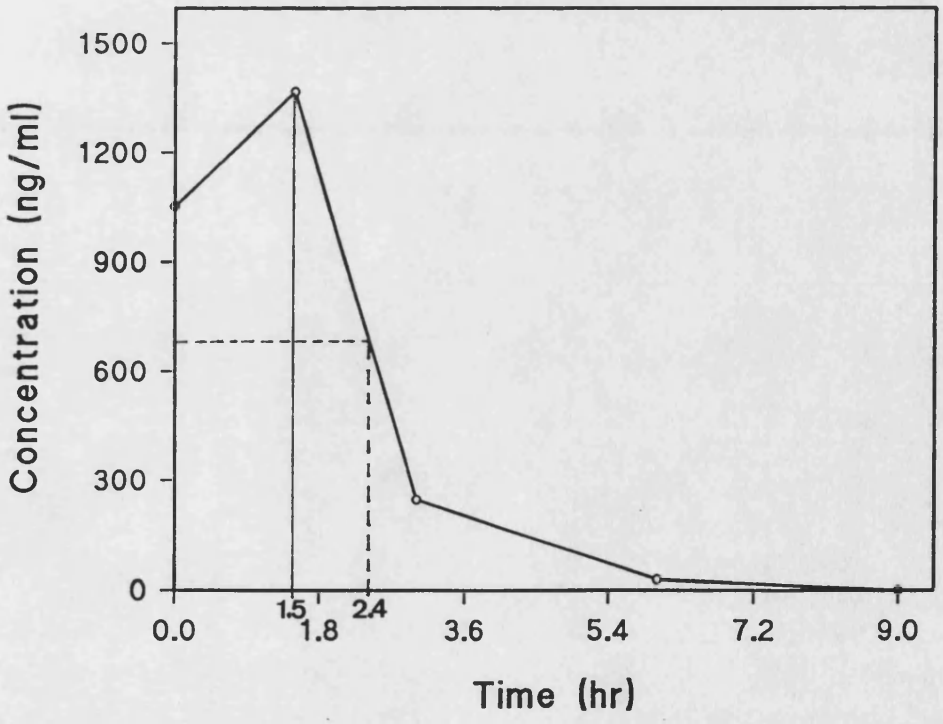


Fig. 4.33 Plot of concentration against time showing the degradation of ABZSO after heating 0.1% ABZSO suspension at 100°C with SDS at pH 10.00.

The mechanisms of reactions of DMSO are not certain. DMSO is known to serve as an oxidising agent in several organic and inorganic reactions (Ranky and Nelson, 1961). Therefore DMSO could oxidise ABZ to ABZSO as shown in Fig. 4.34. As DMSO does not oxidise sulphoxide to sulphone, the formation of $ABZSO_2$ may be due to oxidation of ABZSO with molecular oxygen. DMSO is very hygroscopic (absorbing over 70% of its own weight of water at 20°C and 65% relative humidity (Ranky and Nelson, 1961)) and ABZ may therefore react with water in the DMSO sample to form other products including ABZ 2-amino compounds. Once ABZSO and $ABZSO_2$ are formed, they may also form corresponding 2-amine compounds in the same manner as ABZ.

ABZ when heated in 0.1M HCl solution at 22°C, 37°C and 55°C for 48 hours was relatively stable. However during the solubility study in 0.01M and 0.1M HCl, ABZ was found to be degrading (7-8% ABZSO was found by 48-96 hours). There were several differences in these studies; (1) the HCl solutions in the solubility studies were prepared with constant ionic strength (0.154M). The degradation rate was faster in HCl solution with ionic strength of 0.154M because the rate of reaction increases as ionic strength increases (Florence and Attwood, 1988). (2) ABZ concentration in the stability studies was 10 µg/ml whereas ABZ was at the saturation concentration in the solubility studies (>22 µg/ml). Since rate of oxidation is often dependent on the concentration of the oxidising molecule, oxidation could well proceed faster in the HCl solutions in the solubility studies. (3) As the initial concentration of ABZ was much lower (in the thermal stress study), the products formed would also be in low concentration and therefore may not be detected during HPLC analysis (see section 3.3.1 for limit of detection).

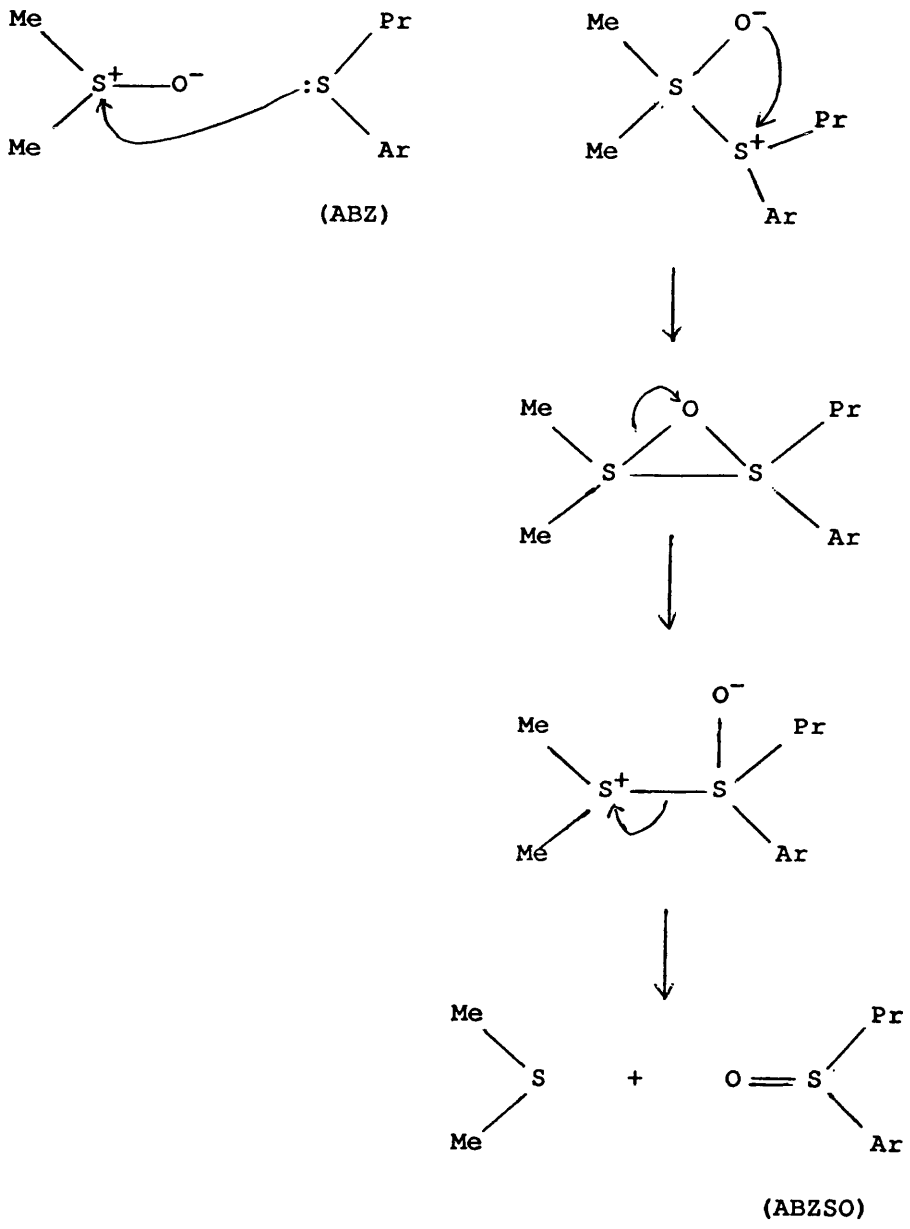


Fig 4.34 The possible mechanism for ABZ oxidation to ABZSO by DMSO.

It is apparent that the solubilities of ABZ in DMSO, 95%EtOH and Miglyol increase with temperature. ABZ appears to have higher solubility in DMSO than 95%EtOH and Miglyol. This can be explained on the basis of the higher dielectric constant of DMSO (Table 4.0) and DMSO also has a high hydrogen bonding capacity compared to EtOH (Craver, 1970). As expected from its high partition coefficient ($P_{octanol/water}$ = 40, section 1.5.1), solubility of ABZ in Miglyol is higher than in water. However, the solubility of ABZ in 95%EtOH and Miglyol was still <1 mg/ml.

A mixture of Mig:Twn 80, 60:40%, w/w was selected from the triangular phase diagram study of Miglyol, Tween 80 and PEG 400 (section 4.3.3, Fig. 4.27) for solubility study. The solubility of ABZ in this mixture was higher when ABZ was ground with the mixture followed by heating at high temperature (Method 2) giving a final concentration of 2 mg/g whereas only 0.5 mg/g was obtained when ABZ was shaken in the mixture at 25°C and 37°C and left at room temperature for 2 weeks (Method 3). This is because the dissolution of ABZ in Mig:Twn 80 was very slow. However the solubility of ABZ was higher in the mixture than in Miglyol alone, presumably due to the micellisation/liquid crystal formation.

The solubility of ABZ in water was not determined using Method 1 as concentration of ABZ was found to decrease with time at the two temperatures studied. ABZ could probably be undergoing degradation and/or some other processes were occurring simultaneously. No other peaks were detected by HPLC except for ABZSO which was present in a small amount (4% of ABZ) and it appears that the loss of ABZ was much faster than the formation of the sulphoxide. It was considered that

it could be due to the possibility of polymorphic changes of ABZ which would result in different solubilities in water. However the DSC thermogram (Fig. 4.35) indicates this was unlikely.

The solubility of ABZ in water was determined by titrating ABZ solution prepared in DMSO into water. However the solubility values found at 37°C were different ($P<0.05$) using different techniques. This could be due to several reasons including the different in method of introducing ABZ-DMSO solution, method of mixing and the type of vessel used.

The solubility of ABZ in 0.1M and 0.01M HCl was not established using Method 1 due to increasing ABZ concentration and degradation of ABZ. More ABZ dissolved in the HCl solution due to the low pH and the formation of the protonated drug form. However the solubility of ABZ in 0.1M HCl at 37°C was determined using Methods 4(a) and (b) by titrating ABZ solution prepared in DMSO into 0.1M HCl solution. The solubility values found using the two different techniques were not statistically different ($P<0.05$); 0.30±0.05 mg/ml when carried out in volumetric flask and 0.32±0.09 mg/ml in the jacketed-beaker. These values were obtained after addition of 1.0-1.1% DMSO solution to 100 ml of 0.1M HCl, thus the co-solvent influence of DMSO was negligible.

When "ABZ-Mig:Twn 80 solution" was added to 0.1M HCl solution, the system became turbid almost immediately. The solubility of ABZ in 0.1M HCl from Mig:Twn solution was not established because the intercepts of the plot of concentration against absorbance passed through zero within ±2 SD. Therefore no definitive value could be determined. A similar situation was observed when blank Mig:Twn

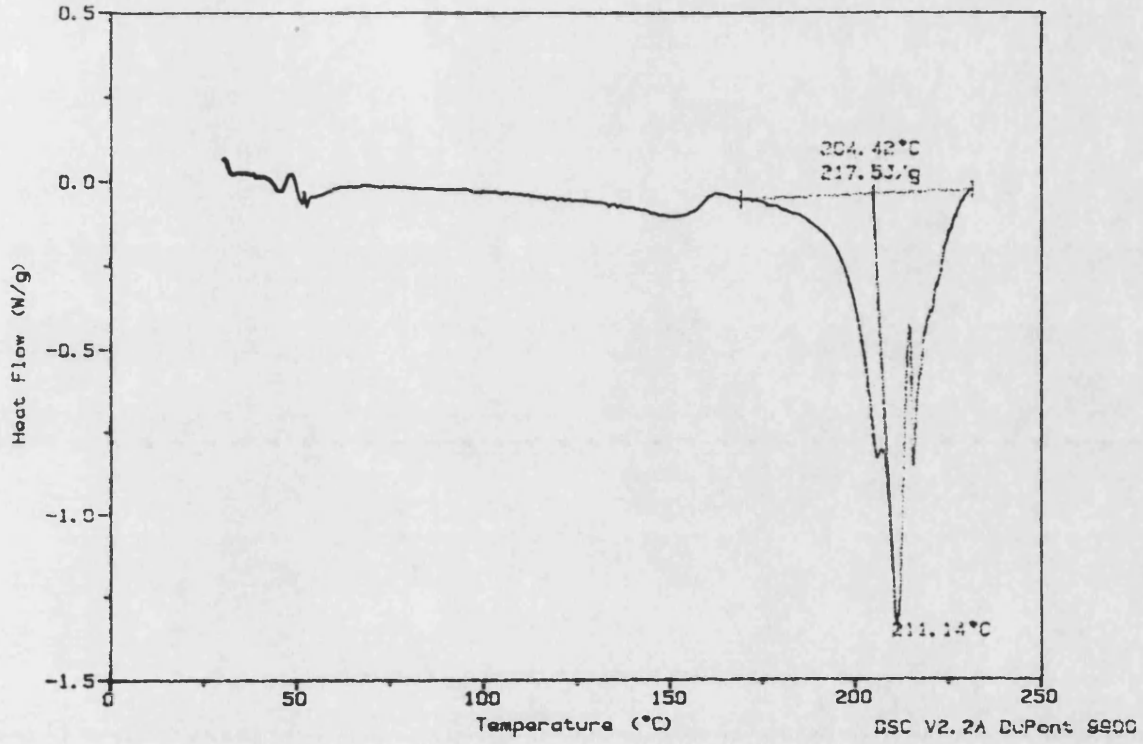


Fig. 4.35 DSC analysis of ABZ.

solution was added to 0.1M HCl. The turbidity of the solution probably was caused by Miglyol self-emulsification.

Solubility determination of PEG 400 in 0.1M HCl was carried out in the reverse method from the ABZ-DMSO and ABZ-Mig:Twn solution (i.e. 0.1M HCl solution was added to ABZ-PEG 400 solution). The solubility of ABZ in 0.1M HCl when added to PEG 400 solution (at 37°C) was not established due to low absorbance values and due to the intercepts of the plots of concentration against absorbance being not definitive (passing through zero within ±2 SD).

When 0.1M HCl was added to the ABZ-PEG solution, the HCl effect is to increase the solubility of ABZ. However addition of the "water" to the PEG solution would decrease the solubility of ABZ. At 50:50 PEG solution and 0.1M HCl, the overall concentration is supposed to be "0.05M HCl" but H^+ concentration in the solution would not be 0.05M as 50% PEG solution interferes with HCl ionisation. The general effect therefore was likely to decrease ABZ solubility as PEG solution is mixed with 0.1M HCl.

The solubility of "ABZ solutions" in 0.1M HCl at 37°C was determined to simulate the condition present in the stomach when these solutions are introduced. Volumes of 0.18-0.19 ml (1.8-1.9 mg ABZ, dose of 5 mg ABZ/kg) and 0.35-0.39 ml (3.5-3.9 mg ABZ, dose of 10 mg ABZ/kg) ABZ-DMSO solutions were given to rats. Considering the fasting volume of aqueous fluid in rat gastric lumen is 0.15±0.01 ml (Yamahira *et al.*, 1979), ABZ concentration in the rat stomach was 5.45-5.59 mg/min and 7.00-7.22 mg/ml. As these values are higher than the solubility value of ABZ in 0.1M HCl obtained from DMSO solution (concentration of 30

mg/ml) at 37°C, it is expected that ABZ would precipitate in the rat stomach. However when 50:50 ABZ-DMSO solution (10 mg/ml - concentration used to dose the rat) and 0.1M HCl were mixed in a test-tube, no precipitation occurred. Therefore it is likely that ABZ remains in solution in the rat stomach.

At a given dose of 6 mg ABZ/kg, 0.20-0.35 ml of ABZ-PEG 400 solution (1.0-1.7 mg ABZ) was introduced to rat stomach. Although the solubility of ABZ from PEG 400 in 0.1M HCl was not fully determined, it is likely that 0.59-0.92 mg/ml ABZ in solution in the stomach.

The results from the microscopic examination of ABZ-Mig:Twn in 0.1M HCl shows that solid ABZ was present in the aqueous phase, indicating that ABZ is at the saturation in both the oil droplets and aqueous phase. Therefore, there is a dual reservoir for albendazole present in the stomach environment.

In conclusion, ABZ undergoes oxidative and hydrolytic degradation *in vitro* when exposed to high temperature. The rate of ABZ degradation was faster at pH 10.00 than at pH 9.00 under the same conditions. ABZ was found to degrade in DMSO solution and it is believed that DMSO reacted with ABZ. ABZ was relatively stable in 0.1M HCl, 95%EtOH and Miglyol.

The solubility of ABZ was higher in DMSO compared to the other solvents studied. Although the solubility of ABZ in Mig:Twn was higher than in Miglyol alone, the solubility of ABZ is <10 mg/ml and thereby indicates the need for a suspension preparation. The solubilities of ABZ in water and 0.1M HCl (at 37°C) were determined

from DMSO solution whereas solubilities of ABZ in 0.1M HCl from Mig:Twn and PEG 400 solutions were not established.

CHAPTER 5

IN VIVO AND *IN VITRO* BIOLOGICAL STUDIES

5.1 INTRODUCTION

ABZ, although poorly soluble, has the advantage of greater absorption following oral administration over the other benzimidazole carbamates (Morris *et al.*, 1983). *In vitro* studies in perfused rat stomachs demonstrated that plasma concentrations of ABZ found were 40% higher than those for MBZ (Prieto et al., 1991). Bioavailability of ABZ was shown to be improved when co-administered with a fatty meal (Lange *et al.*, 1988) or administered as a suspension in oil/milk (Marriner *et al.*, 1986). An increase in plasma concentrations of MBZ has also been observed when MBZ was administered with a fatty meal (Munst *et al.*, 1980), in olive oil (Dawson and Watson, 1985) or as a suspension in sunflower-seed oil (Lurie and Scherbakov, 1987; Lurie *et al*, 1988). Recently, a similar finding was reported when oxfendazole was administered to sheep as an oil-based formulation (Ali and Chick, 1992).

These studies demonstrate that co-administration with lipid enhances the bioavailability of benzimidazole carbamates which may be beneficial in the treatment of a systemic infection such as filariasis. In this chapter, the effect of an "oily" formulation on the systemic bioavailability of ABZ is investigated. Plasma concentrations after a single dose in a healthy volunteer and multiple dose of ABZ in loa loa patients are reported.

5.2 METHODS

5.2.1 Formulations

ABZ and ABZSO used for the following preparations were micronised standards. ABZ and ABZSO solutions were prepared in DMSO (10 mg/ml) for the rat studies. No impurities were detected in these solutions when analysed by HPLC (3.2.1.1.(a)). The chromatograms are shown in Appendix 2 (Fig. 2A.1).

ABZ in Miglyol:Tween 80 (Mig:Twn), 60:40% w/w was prepared by grinding up ABZ, using a pestle and mortar, and adding Mig:Twn (60:40%, w/w) solution in small amounts whilst mixing. The mixture was transferred to a conical flask, heated (~70^{o}C) until the solution turned clear and cooled. The final concentration of ABZ was 2 mg/g. ABZ in PEG 400 was prepared in a similar manner to give a final concentration of 5 mg/g which was used as a "control" formulation.

ABZ tablet (Zentel, SmithKline & French, France) was formulated in Mig:Twn and PEG 400 using the above procedure to give final concentrations of 2 mg/g and 5 mg/g, respectively.

5.2.2 Animals

The experimental animals used were male Wistar albino rats (Bath University strain) weighing between 313 and 385 g, and female Wistar rats weighing between 168 to 270 g, housed in ventilated rooms at a temperature of 24^{o}C. They were maintained on commercial diet pellets and tap water, *ad libitum*.

Female Wistar albino rats from the School of Pharmacy, USM breeding colony, weighing between 168-250g were employed. They were housed in a ventilated rooms at ambient temperature and maintained on a commercial diet pellet and tap water, *ad libitum*.

8 healthy *Macaca Fascicularis* monkeys (2 males and 6 females) weighing between 2.5 and 6.4 kg from a breeding colony at the Institute of Medical Research, Kuala Lumpur, Malaysia were used. They were housed in individual cages and maintained on vegetables, fruits and tap water, *ad libitum*.

5.2.3 Human Volunteer and Patients

A single male human volunteer (age 26 years and body weight, 65 kg) clinically healthy and not receiving any other drugs was recruited.

The "albendazole treatment for human loiasis" study (study number: 90-1-101) was conducted by the National Institute of Health of the United of States of America and the Centre National Universitaire at Hospitalier du Benin, Africa. The study was carried out in Benin with Dr. Thomas Nutman as the principal investigator. Loa loa patients: apparently healthy men and non-pregnant women (18-70 years) with levels of microfilariae in the blood 100-10,000 microfilariae/ml as determined by blood filtration who gave their informed consents were enrolled for ABZ treatment.

5.2.4 Administration of Drugs to Animals and Humans

(a) Animal Studies

ABZ or ABZSO were given orally in various doses and formulations using an oral dosing cannula for rats and a stomach tube for monkeys. ABZ and ABZSO administered to rats in the initial studies were prepared in DMSO or as otherwise stated. The animals were starved overnight prior to dosing.

In the initial studies, 10 male rats (352-385 g) were randomly divided into 2 groups. One group was treated with 5 mg/kg body weight of ABZ and the other group received 10 mg/kg ABZ in DMSO. The study was repeated with 2 other groups of 10 male rats (364-385 g and 350-385 g) on separate days. An inter-rat variability study was carried out by dosing 8 male rats (265-280 g) with 10 mg/kg ABZ in DMSO. A dose of 10 mg/kg of ABZSO was also given to a group of 10 male rats (313-335 g).

ABZ standard in Mig:Twn and tablet in PEG 400 were given to 20 female rats (168-250g) which were divided into 2 groups. The dose of ABZ in both instances was 5 mg/kg. This study was carried out at USM.

72 female rats (185-270 g) were treated with ABZ standard prepared either in Mig:Twn or PEG 400, both at 6 mg/kg. Additionally, 0.6 mg/kg ABZ in Mig:Twn and PEG 400 were given to 32 female rats (190-220 g).

A four "solutions" study was carried out in 6 monkeys. 3 monkeys (Grp 1) received ABZ tablet in PEG 400 (Prep.A) and the other 3 monkeys (Grp 2) received ABZ standard in Mig:Twn (Prep.B) on the first arm of

the study. After an interval of 2 weeks, the second leg was carried out where the Grp 1 monkeys received ABZ tablet in Mig:Twn (Prep.C) and the Grp 2 received ABZ standard in PEG 400 (Prep.D). 2 monkeys (one from each group) died before the second part of the study commenced and they were replaced (for the final dose only). The details of the monkeys are shown in Table 5.1.

Table 5.1 The details of the monkeys used in the study.

Monkeys	Body weight (kg)	Sex	Stomach pH before treatment	Rectal temp. (°C)	Formulation
First part of the study					
513	3.7	F	8.0	38.7	A
533	5.5	M	8.0	38.6	A
535	2.5	F	6.0	38.2	A
434	4.5	F	8.0	37.5	B
486	5.1	M	9.0	38.3	B
435	3.1	F	6.0	38.1	B
Second part of the study					
252	6.4	F	8.0	38.4	C
533	5.5	M	7.0	38.3	C
535	2.5	F	8.0	38.2	C
434	4.5	F	7.0	37.8	D
486	5.1	M	8.0	38.8	D
316	5.5	F	4.0	37.2	D

F = female, M = male
A = ABZ tablet in PEG 400
B = ABZ standard in Mig:Twn
C = ABZ tablet in Mig:Twn
D = ABZ standard in PEG 400

(b) Human Studies

The single volunteer received 400 mg (approximately 6.2 mg/kg) ABZ tablet after fasting overnight. A dwelling cannula was inserted into a forearm vein until 12 hours after drug administration for blood sampling. Food and drink were allowed as normal 2 hours after dosing.

The loa loa patients were given orally 200 mg ABZ or placebo twice a day for 21 days in a double-blind study. Both ABZ and placebo were packaged identically by SmithKline Beecham (SB,USA).

5.2.5 Blood Sampling

(a) Animal Studies

Rats were anaesthetized using diethyl ether or carbon dioxide, and blood samples (1-5 ml) were drawn by cardiac puncture. Samples were collected at 0.5, 1.0 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 and 7.0 hours after drug administration. Rats were bled at most 2 times. In the study where rats were given ABZ in Mig:Twn and PEG 400, samples were collected for up to 24 hours. In the variability study, samples were collected at 2, 4 and 6 hours after dosing. Blood was placed into plain eppendorf or heparinised glass tubes, and centrifuged at 10,000 g or 2500 g for 10 minutes to obtain serum or plasma, respectively. The separated serum or plasma were stored in plastic vials at $-20^{o}C$ until required for analysis.

Monkeys were anaesthetized using Ketalar (ketamine hydrochloride) prior to blood collection. Blood samples (3 ml) were drawn predose and at 1.0, 2.0, 3.0, 4.0, 5.0, 8.0, 12.0, 24.0 and 48.0 hours after dosing by venepuncture of the femoral vein. Blood was drawn into heparinised vacutainers, thoroughly mixed with the anticoagulant, and

centrifuged at 2500 g for 10 minutes. The plasma was separated and stored at -50°C until time of analysis.

(b) Human Studies

Serial blood samples (5 ml) were collected in the vacutainer prior to dosing and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 12.0, 24.0, 32.0 and 48.0 hours after drug administration for the single subject study. The plasma samples were maintained at -50°C until analysis.

Blood samples from loa loa patients were drawn 4-6 hours after the patients received their dose (generally around 7.00 a.m.). Serum samples were collected for drug measurement on days 0, 1, 3, 6, 9, 14 and 20 post-treatment. Samples were transferred to USM packed in solid carbon dioxide and examination on arrival showed the samples were still frozen.

5.2.6 Intestinal and Stomach Preparations

Highly variable ABZ, ABZSO and $ABZSO_2$ concentrations were observed in the initial studies in rats. *In vitro* experiments were carried out to investigate whether this variability was caused by the metabolism of ABZ by the GI tract, degradation of ABZ by the gastric pH, damage of GI tract membrane by DMSO, or indeed was the results of variable absorption.

ABZ in DMSO was administered orally to 3 rats at a dose of 10 mg/kg. 2 or 4 hours after dosing blood was collected by cardiac puncture, the rat killed and the stomach and intestine removed. Stomachs and intestines were flushed with 50 ml of distilled water using a syringe

which was then filtered under gravity. The clear filtrate was collected and 1 ml was used for HPLC analysis.

A rat (400 g) was anaesthetized with hypnorm/medazolam and the abdomen was opened. The stomach was identified and both ends of the stomach were occluded to form a "bag" like which 0.4 ml of 10 mg/ml ABZ in DMSO was introduced via a cannula. The stomach was then placed into a small beaker of saline and placed in a water-bath at 37^{o}C. After 6 hours incubation, the stomach was removed from the water-bath. The following washings were collected:

(1) The fluid in the stomach was flushed out with 30 ml distilled water using a syringe.

(2) The stomach was then cut open and the contents were transferred into a beaker and washed with 20 ml distilled water.

(3) The mucosal layer of the stomach was scraped off and washed with 20 ml distilled water.

All the washings from (1), (2) and (3) were filtered separately and 1 ml of the clear filtrate was assayed by HPLC.

Stomachs and intestines were removed from untreated rats. The mucosal layers were scraped off and put into saline (20 ml) in test-tubes which were placed in a water-bath at 37^{o}C. 0.4 ml of 10 mg/ml ABZ in DMSO was introduced to the solutions and mixed thoroughly. Saline solution without mucosa was set-up as a control. Samples (1 ml) were taken at 1, 2, 4, 5, and 6 hours after addition of ABZ. Each sample drawn from the test-tubes was replaced with saline solution (1 ml).

5.2.7 Analysis of Samples

Serum and plasma samples (0.2-1.0 ml) and the washings from stomach and intestinal preparations were extracted using the SPE procedure described in section 3.2.1.3. The samples from rat studies were assayed by the HPLC method described in section 3.2.1.1(a) and samples from the rat study carried out at USM, monkey studies and human studies were analysed by the HPLC method described in section 3.2.1.1(b). Spiked serum, plasma and saline solutions were extracted and assayed together with the samples as quality control.

ABZSO and $ABZSO_2$ concentrations were also presented as ABZ equivalents in the intestinal and stomach preparation studies.

5.2.8 Pharmacokinetic Analysis

The time to reach the maximum concentration (T_{max}) and the value for maximum concentrations (C_{max}) of ABZ, ABZSO and $ABZSO_2$ were determined by inspection. The area under the concentration-time curve (AUC) was calculated using the trapezoidal method (Gibaldi and Perrier, 1982).

5.2.9 Statistical Analysis

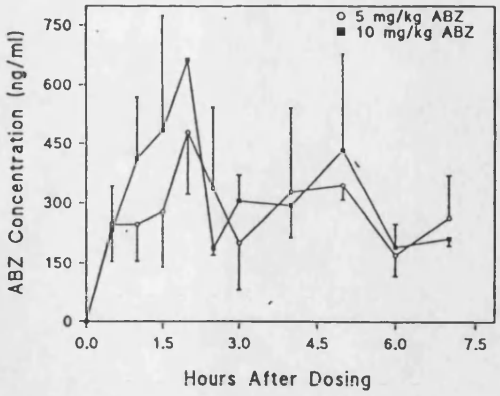
Statistical comparison was carried out using the Student's t-test. t values of $P>0.05$ were rejected.

5.3 RESULTS

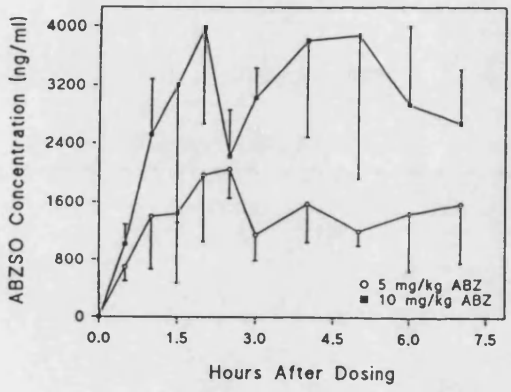
5.3.1 Results from Preliminary Studies in Rats

Single oral dose studies of 5 and 10 mg/kg ABZ in rats were carried out on three separate days collecting one sample at each time point per day. Fig. 5.1 shows the mean concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ following both dosage. The individuals concentrations are tabulated in Appendix 3 (Tables 3A.1-3A.2). ABZ appeared to have two distinct phases (Fig. 5.1(a)) with the first peak at around 2.0hr and second at 5.0hr for both dosages. The mean maximum concentration of 479±157 ng/ml was achieved when 5 mg/kg ABZ was administered and 664±291 ng/ml following a dose of 10 mg/kg at 2.0hr after dosing. ABZSO (Fig.5.1(b)) behaved similarly to ABZ with mean maximum concentrations at 2.5hr (2046±392 ng/ml) and 2.0hr (3978±132 ng/ml) after administration of 5 mg/kg and 10 mg/kg ABZ, respectively. The mean maximum concentration for $ABZSO_2$ was achieved at 7.0hr (408±187 ng/ml) and 5.0hr (613±91 ng/ml) following 5 and 10 mg/kg ABZ, respectively.

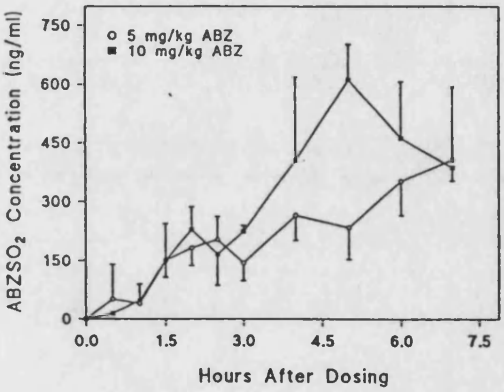
The pharmacokinetic parameters of ABZ, ABZSO and $ABZSO_2$ were calculated individually for each set of results obtained from the three days and the parameters are summarised in Table 5.2. The ratio of the AUC (5/10 mg/kg) for ABZSO (0.47) and $ABZSO_2$ (0.68) were comparable with the dose ratio (0.5). In contrast, the AUC for ABZ were similar for both dosage (ratio of 0.89). The ratios of AUC ABZSO/ABZ were 4.68 and 8.75, $ABZSO_2$/ABZ were 0.72 and 0.93 and $ABZSO_2$/ABZSO were 0.15 and 0.11 for 5 and 10 mg/kg, respectively indicating dose-proportionality for the ABZSO only.



(a)



(b)



(c)

Fig. 5.1 The concentration-time profiles of (a) ABZ, (b) AZBSO and (c) $ABZSO_2$ following a single oral dose of 5 mg/kg and 10 mg/kg ABZ in DMSO in rats. (n = 3)

Table 5.2 Summary of the pharmacokinetic parameters (mean±SD) after a single oral dose of 5 mg/kg and 10 mg/kg ABZ in DMSO in rats. (n =3)

Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
5 mg/kg			
ABZ	2.7±1.2	614± 82	2052± 136
ABZSO	2.0±0.5	2364±699	9609±2759
$ABZSO_2$	6.3±0.6	463±154	1484± 357
10 mg/kg			
ABZ	1.7±0.6	731± 343	2313± 780
ABZSO	4.3±2.1	3886±5003	20303±3011
$ABZSO_2$	5.0±0.0	613± 91	2161± 275

Concentrations of ABZ, ABZSO and $ABZSO_2$ were found to be highly variable. A variability study in rats at three time points after receiving 10 mg/kg ABZ orally showed that ABZ concentration ranged from 152 to 955 ng/ml at 2.0hr, 259 to 1327 ng/ml at 4.0hr and 126 to 506 ng/ml at 6.0hr. ABZSO concentrations were 1649-4038 ng/ml at 2.0hr, 2220-4969 ng/ml at 4.0hr and 2085-5169 ng/ml at 6.0hr. Concentrations of $ABZSO_2$ found at 2.0hr were 144-281 ng/ml, 320-650 ng/ml at 4.0hr and 344-795 ng/ml at 6.0hr. The $AUC_{(0-6hr)}$ for ABZ varies from 1115 to 3877 ng/ml.hr, 9809-19413 ng/ml.hr for ABZSO and 1063-2649 ng/ml.hr for $ABZSO_2$. The mean, standard deviation (SD) and relative standard deviation (RSD) of ABZ, ABZSO and $ABZSO_2$ are shown in Table 5.3. These results indicate a significant inter-rat variation in absorption rates or in bioavailability.

Table 5.3 The variation of ABZ, ABZSO and $ABZSO_2$ concentrations following 10 mg/kg ABZ in DMSO in rats.

Sampling time (hr)	Serum concentration (ng/ml) mean	SD	RSD(%)	AUC(0-6hr) mean[b]	SD	RSD(%)
ABZ						
2.0[a]	545	336	62			
4.0[a]	664	494	82	2138	1429	67
6.0[b]	344	114	33			
ABZSO						
2.0[a]	2476	1306	53			
4.0[a]	3830	1259	33	14792	3726	25
6.0[b]	1214	1214	34			
$ABZSO_2$						
2.0[a]	211	59	28			
4.0[a]	450	142	32	1710	516	30
6.0[b]	574	162	28			

[a] : n = 4
[b] : n = 7

Fig. 5.2 shows the concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ in serum of rats treated with 10 mg/kg ABZSO. Each point represents a mean of two samples obtained from two different rats. The individual concentrations are given in Table 3A.3 (Appendix 3). The trend of the profiles for ABZ, ABZSO and $ABZSO_2$ in rats receiving 10 mg/kg ABZSO were found to be similar to those in rats that received 5 or 10 mg/kg ABZ, with ABZ and ABZSO having 2 peaks. The pharmacokinetic parameters were obtained from the mean concentration-time curves (Fig.5.2) and are summarised in Table 5.4. The ratio of AUC ABZ/ABZSO was 0.08 and for $ABZSO_2$/ABZSO 0.14.

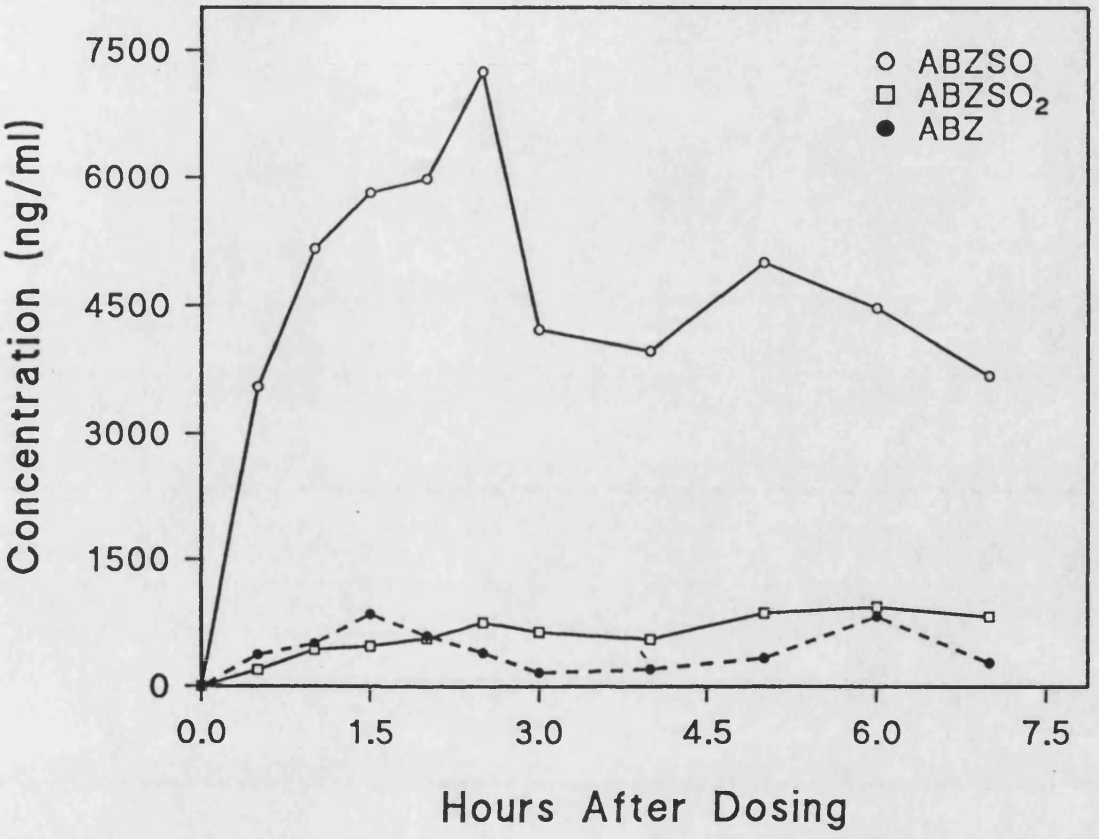


Fig. 5.2 The mean concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ following a single oral dose of 10 mg/kg ABZSO in DMSO in rats.

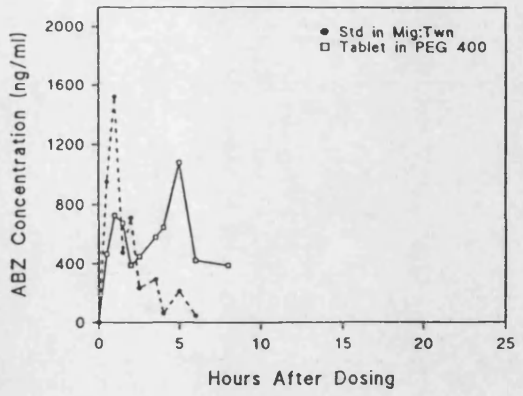
Table 5.4 Summary of the pharmacokinetic parameter after a single oral dose of 10 mg/kg ABZSO in DMSO in rats.

Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
ABZ	1.5	853	2487
ABZSO	2.5	7252	32344
$ABZSO_2$	6.0	941	4478

5.3.2 The Influence of a Mig:Twn Formulation on the Systemic Availability of Albendazole

The mean concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ in rats which received 5 mg/kg standard in Mig:Twn or tablet in PEG 400 are shown in Fig. 5.3. Each point represents a mean of two samples and the individual concentrations are given in Appendix 3 (Tables 3A.4-3A.5). Peak concentration of ABZ when standard ABZ was given was attained within 1.0hr after dosing. When ABZ tablet formulation was given, ABZ was absorbed to a lesser extent(~48%) with an initial peak at 1.0hr although the maximum concentration was reached within 5.0hr after drug administration. The ratio of $AUC_{(0-6hr)}$ (standard/tablet) of ABZ was 0.68.

The concentrations of ABZSO were extremely variable for both formulations. Maximum concentrations of ABZSO were achieved at 2.0hr for standard formulation and 4.0hr for tablet formulation with the latter having a 9% higher concentration. ABZSO was still measured at 24.0hr whereas ABZ was undetectable after 6.0hr when ABZ was given in standard form. The ratio of $AUC_{(0-12hr)}$ (standard/tablet) of ABZSO was 0.95.



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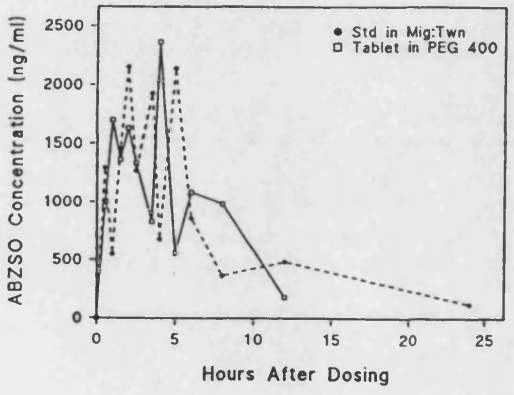

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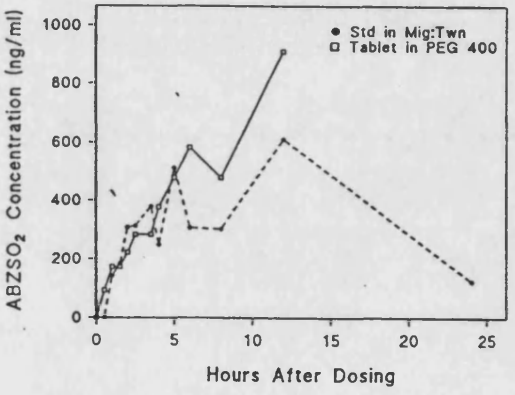


(c)

Fig. 5.3 The mean concentration-time profiles of (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ following a single oral dose of 5 mg/kg ABZ standard in Mig:Twn and tablet in PEG 400 in rats.

$ABZSO_2$ showed a similar pattern with peak concentrations at 12.0hr for both formulations. The peak concentration was higher when ABZ given as tablet and the ratio of $AUC_{(0-12hr)}$ (standard/tablet) of $ABZSO_2$ was 0.73. The T_{max}, C_{max} and AUC for ABZ, ABZSO and $ABZSO_2$ are given in Table 5.5.

ABZ standard in Mig:Twn or PEG 400 was administered to rats at a dose of 6 mg/kg. The mean plasma concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ are shown in Fig.5.4 . Each point represents the mean of three samples and the individual concentrations are tabulated in Appendix 3 (Tables 3A.6-3A.7). The pharmacokinetic parameters were obtained from the mean concentration-time curves (Fig.5.4). The time to reach the peak concentration of ABZ for both formulations was 2.0hr, although ABZ achieved a concentration approximately 1.4 times higher after the PEG formulation than the oily formulation. The AUC's of ABZ were comparable for both formulations as shown in Table 5.6.

The time to maximum plasma concentration for ABZSO were 4.0hr and 5.0hr in rats receiving ABZ in Mig:Twn or PEG 400, respectively. The maximum concentrations were similar (Table 5.6) and the AUC ratio for Mig:Twn/PEG 400 ratio was 0.82.

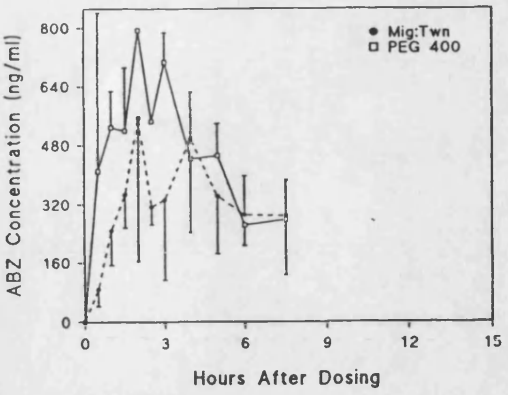
The maximum concentrations of $ABZSO_2$ were approximately 20% of those of ABZSO for both formulations. The T_{max}'s were 7.5hr and 6.0hr when ABZ was given in Mig:Twn and PEG 400, respectively. The ratio of AUC's (Mig:Twn/PEG 400) for $ABZSO_2$ was 0.91.

Table 5.5 Summary of the pharmacokinetic parameters after a single oral dose of 5 mg/kg ABZ standard in Mig:Twn and ABZ tablet in PEG 400 in rats.

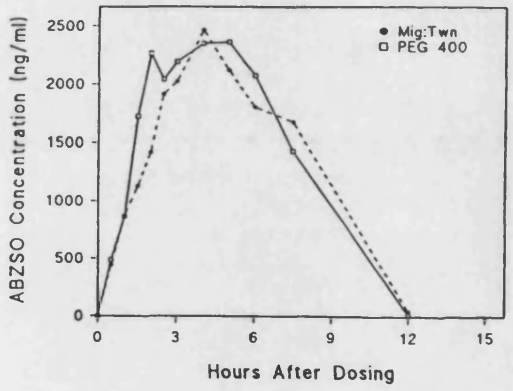
Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
Standard in Mig:Twn			
ABZ	1.0	1521	2510
ABZSO	2.0	2156	14762
$ABZSO_2$	12.0	612	8538
Tablet in PEG 400			
ABZ	5.0	1080	4477
ABZSO	4.0	2366	11721
$ABZSO_2$	12.0	912	5654

Table 5.6 Summary of the pharmacokinetic parameters after a single oral dose of 6 mg/kg ABZ standard in Mig:Twn and PEG 400 in rats.

Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
Mig:Twn			
ABZ	2.0	544	2446
ABZSO	4.0	2467	13905
$ABZSO_2$	7.5	485	3297
PEG 400			
ABZ	2.0	794	3362
ABZSO	5.0	2363	16956
$ABZSO_2$	6.0	535	3626



(a)



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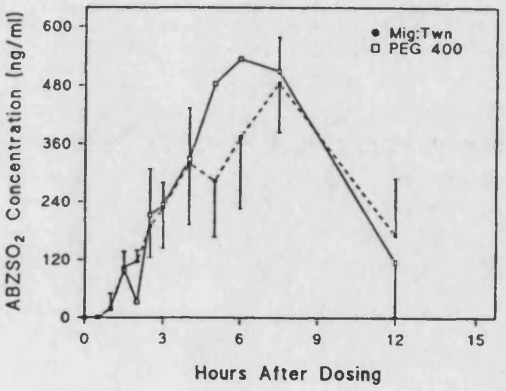


(c)

Fig. 5.4 The mean concentration-time profiles of (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ following a single oral dose of 6 mg/kg ABZ standard in Mig:Twn and PEG 400 in rats.

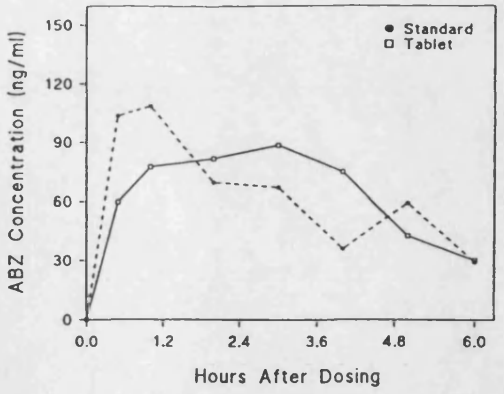
Fig.5.5 shows the mean concentration-time curves of ABZ, ABZSO and $ABZSO_2$ following a single oral dose of 0.6 mg/kg ABZ standard and tablet in Mig:Twn. Each point represents the mean of two samples. The individual concentrations are given in Tables 3A.8-3A.9 (Appendix 3). When ABZ standard was administered, absorption of ABZ was rapid with the maximum concentration being attained at 1.0hr after dosing. On the other hand when powdered ABZ tablet was administered, absorption was slower (T_{max} = 3.0hr) but the maximum concentration achieved was higher. The ratio of the AUC was 1.11 (Table 5.7).

The T_{max} for ABZSO was 2.0hr for both formulations, although the maximum concentration was approximately 20% lower in rats receiving ABZ standard compared to the rats that received ABZ tablet (Table 5.7). The ratio of $AUC_{(0-6hr)}$ (standard/tablet) of ABZSO was 0.85.

A delayed absorption of $ABZSO_2$ was observed after administration of ABZ standard or tablet in Mig:Twn and the T_{max} was achieved at 5.0hr for both formulations.

Table 5.7 Summary of the pharmacokinetic parameters after a single oral dose of 0.6 mg/kg ABZ standard and tablet in Mig:Twn in rats.

Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
Standard			
ABZ	1.0	109	435
ABZSO	3.0	425	1689
$ABZSO_2$	5.0	178	599
Tablet			
ABZ	3.0	89	392
ABZSO	3.0	565	2048
$ABZSO_2$	5.0	202	568



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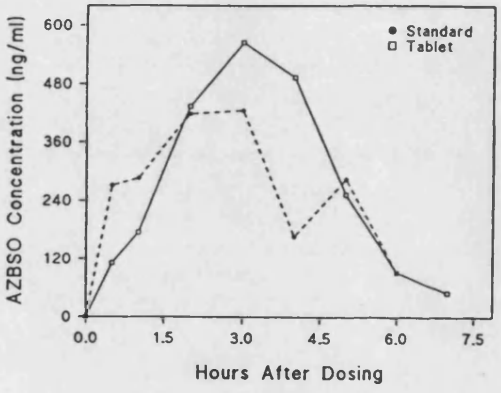

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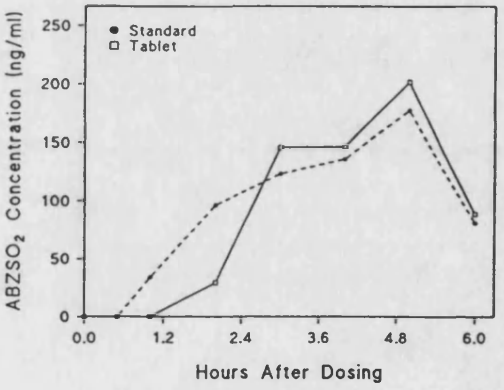

(c)

Fig. 5.5 The mean concentration-time profiles of (a) ABZ, (b) ABZSO and (c) $ABZSO_2$ following a single oral dose of 0.6 mg/kg ABZ standard and tablet in Mig:Twn in rats.

The plasma concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ following oral doses of 6 mg/kg ABZ in four different formulations in monkeys are shown in Fig. 5.6, Fig. 5.7 and Fig. 5.8, respectively. The individual plasma levels are tabulated in Appendix 3 (Tables 3A.10-3A.13). Monkey 513 was omitted since there were no samples after 5.0hr due to difficulty in blood sampling. The absorption of ABZ in monkeys receiving Prep.A was faster compared to that from the other three preparations when maximum concentration of ABZ was reached within 2.0-5.0hr (Fig. 5.6). Interestingly, the maximum concentration of ABZ was achieved at 24.0hr in monkeys receiving Prep.B except for one monkey (434) which displayed the C_{max} at 4.0 hr. The absorption rate of ABZ was variable for all the formulations demonstrated by T_{max} shown in Table 5.8. ABZ appeared to have a second absorption phase in 6 out of the 12 monkeys. C_{max}'s for ABZ achieved were higher in monkeys receiving preparations made in PEG 400 compared to those receiving Mig:Twn preparations. There were no significant differences in $AUC_{(0-24hr)}$ of ABZ for any of the formulations ($F = 0.47$, $F_{tab} = 5.89$ at $P = 0.05$) as shown in Table 5.9.

The plasma concentration-time profiles of ABZSO in monkeys (Fig. 5.7) shows a similar pattern to ABZ. The time to reach peak concentration of ABZSO in monkeys receiving Prep.A was 4.0-5.0hr which was faster than that in monkeys which received Prep.B (24.0hr), Prep.C (4.0-12.0hr) or Prep.D (8.0hr). The plasma concentrations of ABZSO were 2.5 to 20 fold the plasma concentrations of the parent ABZ. Consequently, the metabolite $AUC_{(0-24hr)}$ were substantially higher than the $AUC_{(0-24hr)}$ found for ABZ. The mean T_{max}, C_{max} and $AUC_{(0-24hr)}$ for ABZSO are tabulated in Table 5.8. There were no

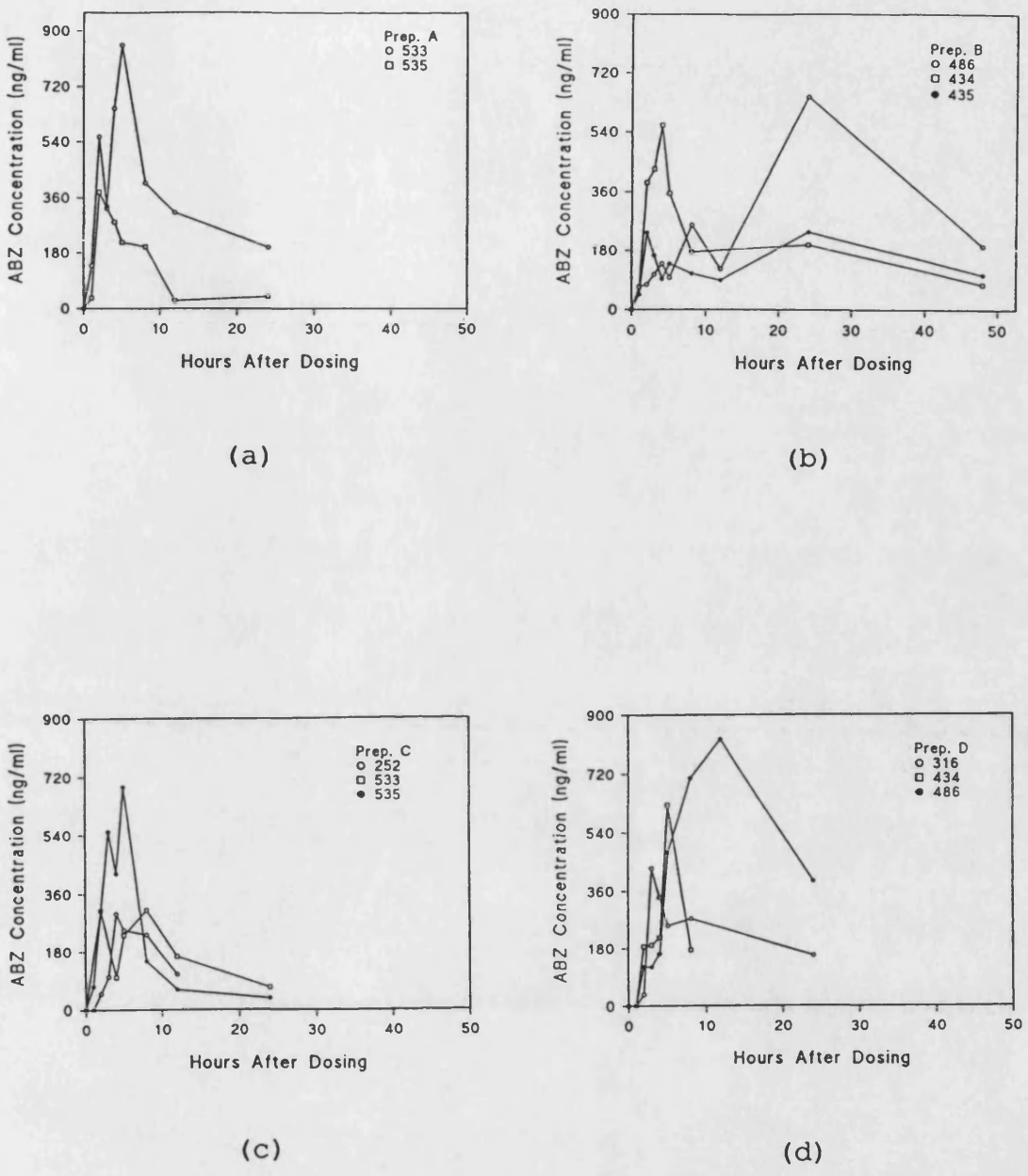

Fig. 5.6 The plasma concentration-time profiles of ABZ following a single oral dose of 6 mg/kg ABZ as (a) Prep.A-tablet in PEG 400, (b) Prep.B-standard in Mig:Twn, (c) Prep.C-tablet in Mig:Twn and (d) Prep.D-standard in PEG 400 in monkeys.

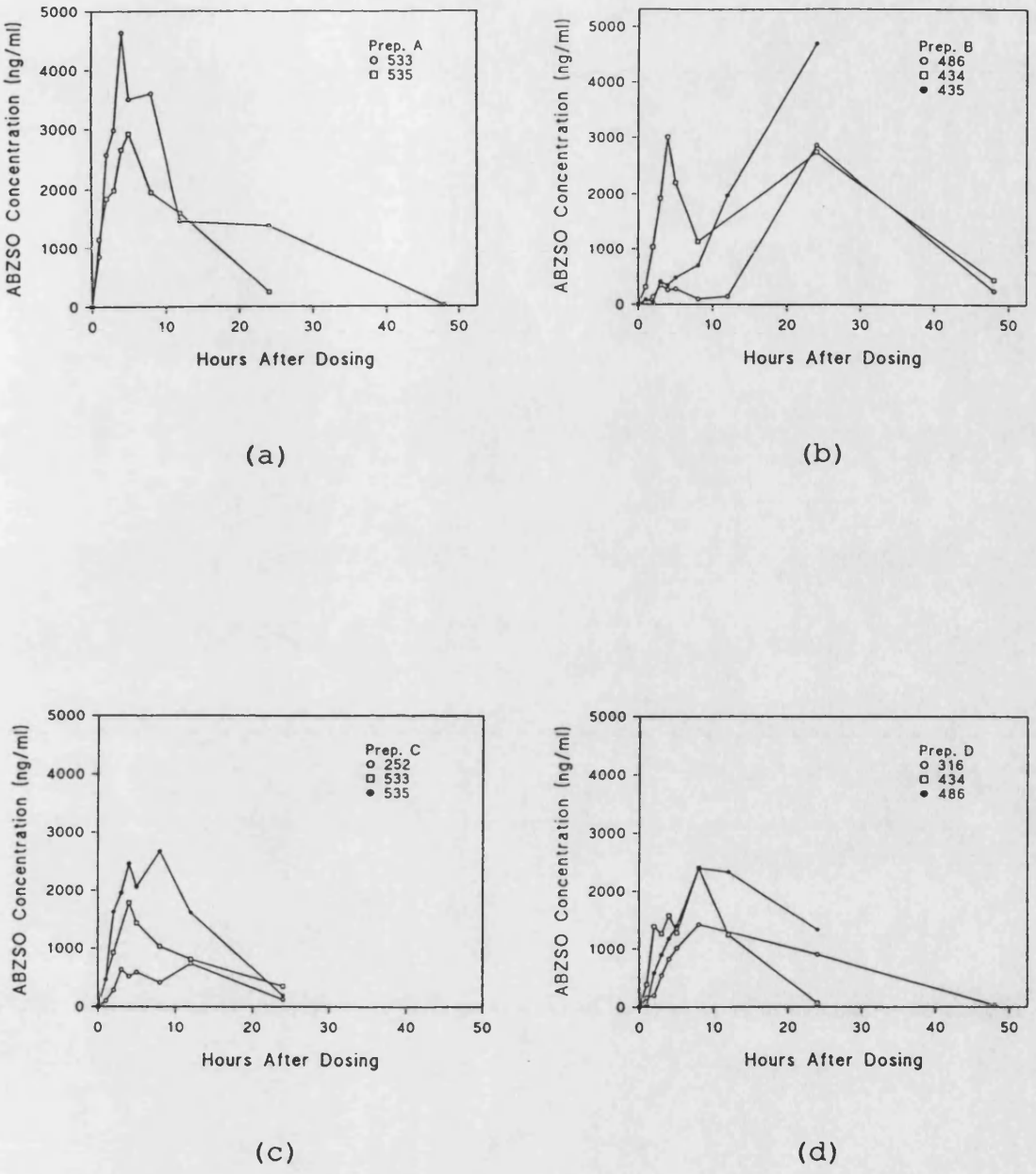

Fig. 5.7 The plasma concentration-time profiles of ABZSO following a single oral dose of 6 mg/kg ABZ as (a) Prep.A-tablet in PEG 400, (b) Prep.B-standard in Mig:Twn, (c) Prep.C-tablet in Mig:Twn and (d) Prep.D-standard in PEG 400 in monkeys.

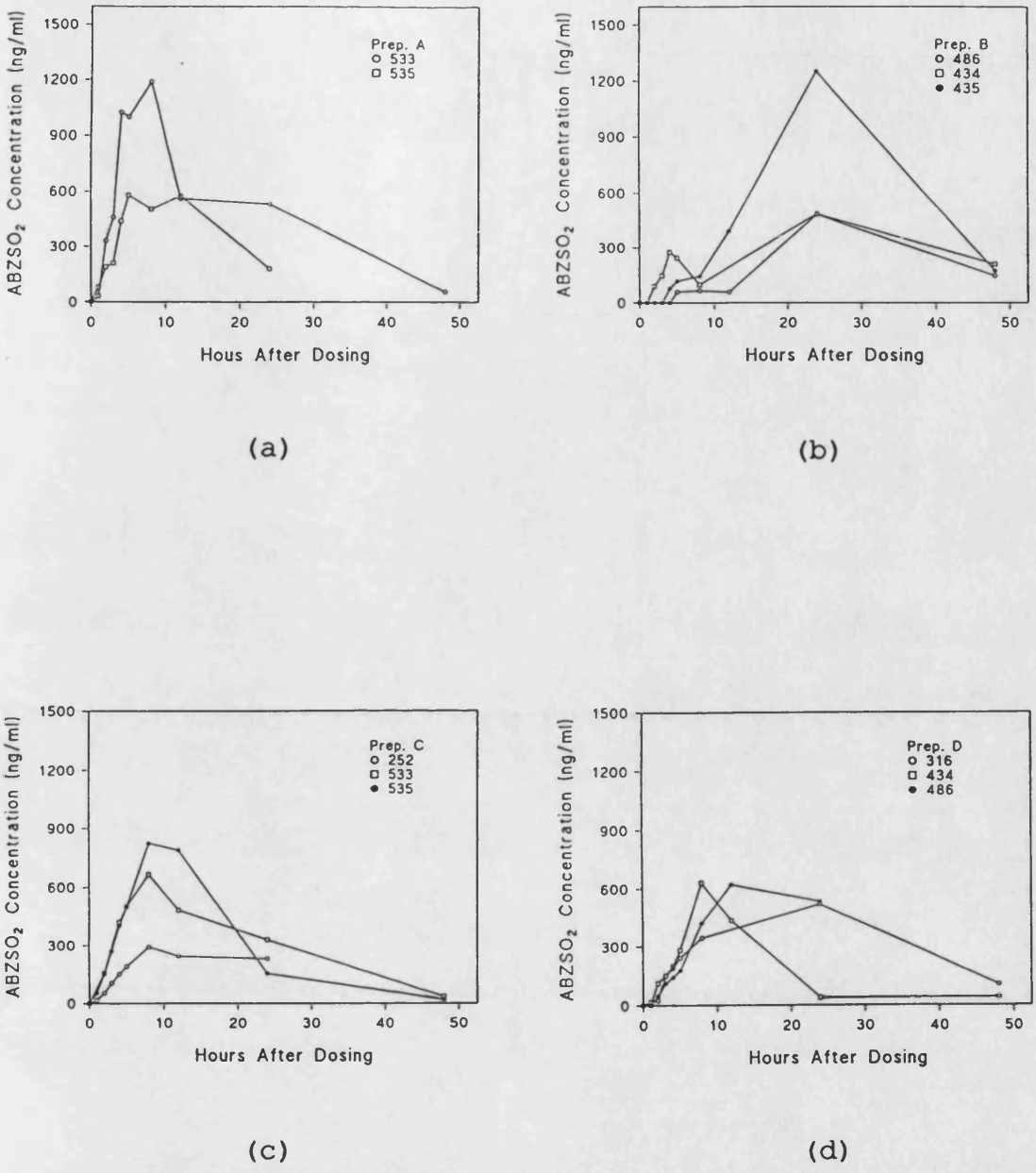

Fig. 5.8 The plasma concentration-time profiles of $ABZSO_2$ following a single oral dose of 6 mg/kg ABZ as (a) Prep.A-tablet in PEG 400, (b) Prep.B-standard in Mig:Twn, (c) Prep.C-tablet in Mig:Twn and (d) Prep.D-standard in PEG 400 in monkeys.

Table 5.8 Summary of the pharmacokinetics parameters (mean+SD) after oral dose of 6 mg/kg ABZ as Prep.A (tablet in PEG 400), Prep.B (std. in Mig:Twn), Prep.C (tablet in Mig:Twn) and Prep.D (std. in PEG 400) in Monkeys. (n=3)

Compound	T_{max} (hr)	C_{max} (μg/ml)	$AUC_{(0-24hr)}$ (μg/ml.hr)
Prep.A*			
ABZ	3.5±2.1	0.62±0.34	5.55±4.19
ABZSO	4.5±0.7	3.78±1.21	42.6±11.3
$ABZSO_2$	6.5±2.1	0.89±0.43	12.6±4.36
Prep.B			
ABZ	17.3±1.5	0.48± 0.22	5.03±1.55
ABZSO	17.3±11.5	3.53±1.02	56.7±43.4
$ABZSO_2$	24.0±0.0	0.74±0.44	7.01±4.00
Prep.C			
ABZ	5.7±2.1	0.43±0.22	3.32±1.17
ABZSO	8.0±4.0	1.66±0.88	21.4±11.7
$ABZSO_2$	8.0±0.0	0.59±0.27	9.04±3.58
Prep.D			
ABZ	6.7±4.7	0.63± 0.20	6.68±5.50
ABZSO	8.0±0.0	2.07±0.56	30.2±8.87
$ABZSO_2$	14.7±8.3	0.59±0.06	8.48±1.70

* n=2

Table 5.9 The ratios of $AUC_{(0-24hr)}$ from the monkey studies. (A - tablet in PEG 400, B - standard in Mig:Twn, C - tablet in Mig:Twn and D - standard in PEG 400).

	ABZ	ABZSO	$ABZSO_2$
$\frac{AUC_D}{AUC_A}$	1.20	0.71	0.67
$\frac{AUC_B}{AUC_C}$	1.51	2.65	0.78
$\frac{AUC_B}{AUC_D}$	0.75	1.88	0.83
$\frac{AUC_C}{AUC_A}$	0.60	0.50	0.72

5.3.3 Results from Human Studies

The plasma concentration profiles of ABZ, ABZSO and $ABZSO_2$ after a single oral dose of 400 mg (approximately 6.2 mg/kg) ABZ tablet in a single volunteer are illustrated in Fig.5.9 and the concentrations are quoted in Table 3A.14 (Appendix 3). Some ABZ was absorbed at 1.0hr but the maximum concentration was achieved at 3.0hr. The peak concentration for ABZSO was 2-fold greater than the peak concentration for ABZ, whereas the peak concentration for $ABZSO_2$ was 80% that of ABZ. The T_{max}, C_{max} and AUC for ABZ and its metabolites are shown in Table 5.10.

Samples of 5 patients from loa loa study were analysed. These samples were picked at random and it was not known whether they had received placebo or ABZ. ABZ and metabolites were not detected in any of the serum samples of 2 out of the 5 patients which indicated that they had probably received the placebo. The concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ for the 3 patients are shown in Fig. 5.10 and the concentration given in Table 3A.15 (Appendix 3). $ABZSO_2$ and ABZ concentrations were low and ABZSO were variable in all 3 patients.

Table 5.10 Summary of the pharmacokinetic parameters following a single dose of 400 mg ABZ tablet in one healthy male volunteer.

Compound	T_{max} (hr)	C_{max} (ng/ml)	$AUC_{(0-t)}$ (ng/ml.hr)
ABZ	3.0	74	354
ABZSO	2.5	151	2729
ABZSO2	3.0	59	1070

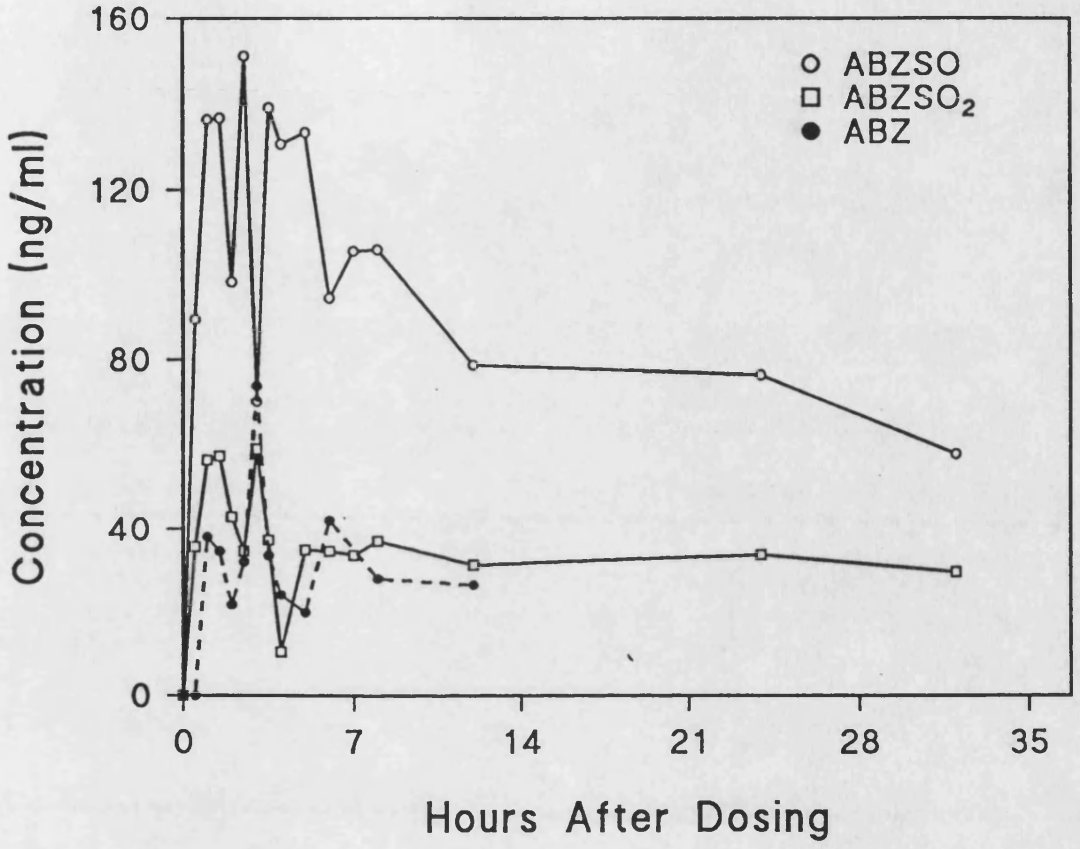

Fig. 5.9 The plasma concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ following a single oral dose of 400 mg ABZ tablet in a single healthy volunteer.

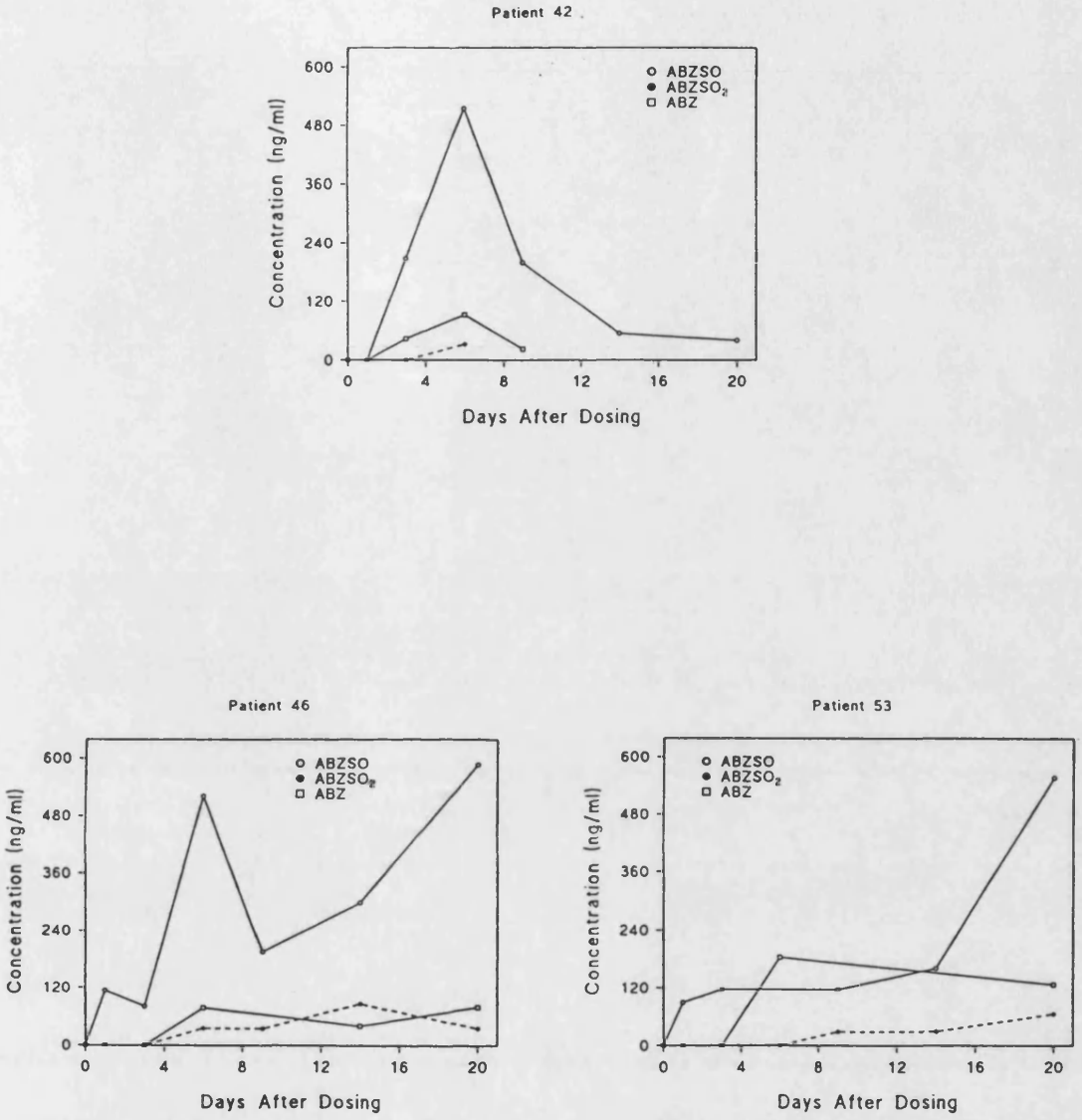

Fig. 5.10 The plasma concentration-time profiles of ABZ, ABZSO and $ABZSO_2$ in loa loa patients after receiving 200 mg ABZ twice a day over 21 days.

5.3.4 Results from the Stomach and Intestinal Preparations

ABZ (10 mg/kg in DMSO directly into the stomach) was metabolised into ABZSO and $ABZSO_2$ in the stomach and/or intestine of rats (Table 5.11). The concentration of ABZSO found in the stomach at 2.0hr was 1.24 times and 0.09-1.22 times at 4.0hr the concentration of remaining ABZ. ABZSO found in the intestine was higher, 1.51 times that of ABZ at 2.0hr and 1.71-2.13 times at 4.0hr. The concentrations of $ABZSO_2$ found in the stomach were 3-6% of the ABZ concentration and in the intestine were 14-34%. Total drug concentrations in plasma, stomach and intestine wash were 7.02 µg/ml at 2.0hr, 4.72 and 4.82 µg/ml at 4.0hr (ABZ equivalent).

After 6.0hr incubation of ABZ in the rat stomach, ABZ and ABZSO were found in the stomach wash and contents, and the mucosal layer. The conversion of ABZ to ABZSO was highest in the mucosal layer with a ratio of ABZ to ABZSO of 1.28 compared to 2.67 in the stomach wash and 8.30 in the stomach contents. The concentrations of ABZ and ABZSO are presented in Table 5.12.

Fig. 5.11 shows the concentration-time profiles for ABZ and ABZSO after ABZ in DMSO (0.4 ml of 10 mg/ml) was incubated with mucosal scrapings taken from the stomach and intestine. The decrease in ABZ concentration was observed in samples with mucosa. After 6.0 hr incubation, 4.15% and 4.58% ABZSO (ABZ equivalent) were present in the control samples, 21.70% (at 5.0hr) and 16.18% in the stomach samples and, 82.69% and 24.41% in the intestine samples. Concentration levels are presented in Table 3A.16.

Table 5.11 ABZ, ABZSO and $ABZSO_2$ levels in stomach and intestinal wash following oral dose of 10 mg/kg ABZ in rats.

Sampling time (hr)	Amount in 50 ml sample (µg) ABZSO a	ABZSO b	$ABZSO_2$ a	$ABZSO_2$ b	ABZ
Stomach					
2.0(1)	58.2	54.7	1.61	1.44	44.1
4.0(3)	46.8	44.1	2.25	2.01	36.3
4.0(4)	46.4	43.8	3.24	2.89	46.7
Intestine					
2.0(1)	9.98	9.41	0.99	0.88	6.22
4.0(3)	10.6	9.96	1.63	1.45	4.67
4.0(4)	6.78	6.39	1.43	1.28	3.74

a: actual concentrations
b: ABZ equivalent

Table 5.12 ABZ and ABZSO levels following incubation of ABZ in stomach for 6.0hr at 37°C.

Samples	Amount of drug (ng) ABZSO a	ABZSO b	ABZ
Stomach washing	840	792	2118
Stomach contents	2752	2595	21536
Mucosal layer	4023	3794	4856

Q
a: actual concentrations
b: ABZ equivalent

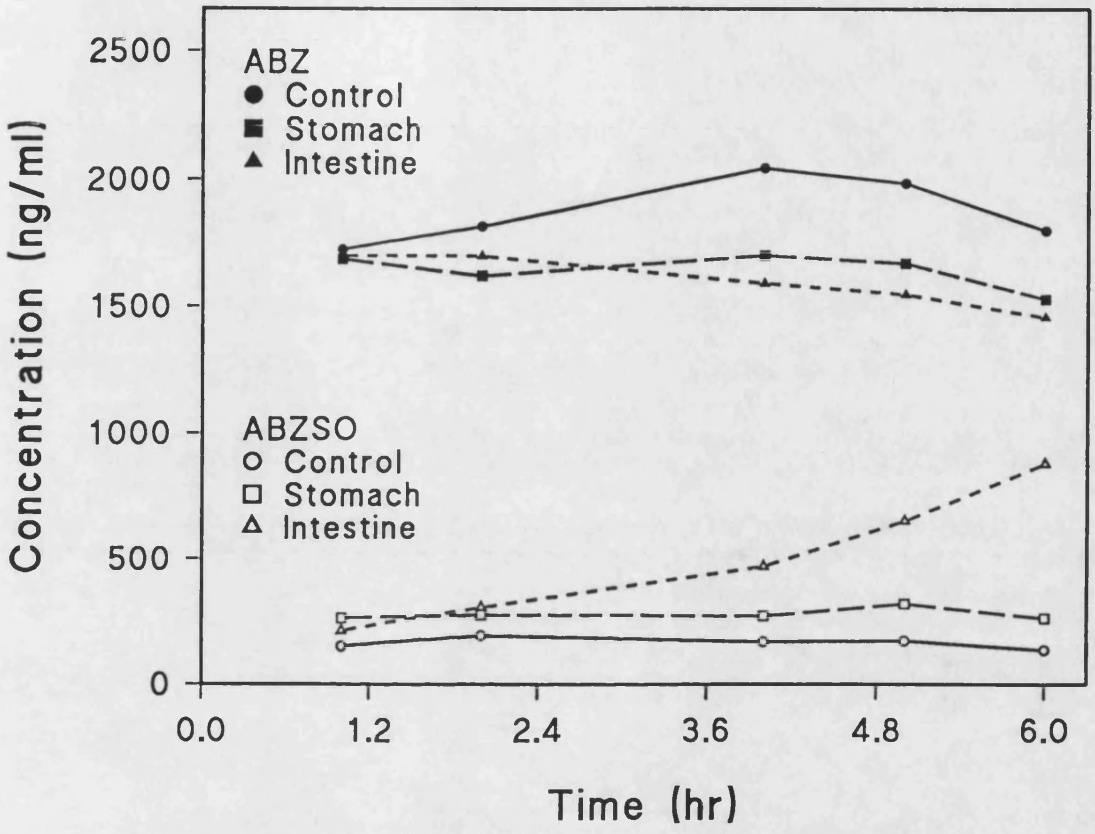

Fig. 5.11 Mean concentration-time profiles of ABZ and ABZSO following incubation of ABZ with mucosal layer of the stomach and intestine at 37°C.

5.4 DISCUSSION

ABZ kinetics have been described in this chapter.The absorption of ABZ and metabolites were highly variable and the variation in concentrations between rats were large. This variability may be caused by variation in the individual rates of absorption as well as differences in the rates of metabolism. The concentration profiles of ABZ and its metabolites show secondary absorption suggesting a possibility of enterohepatic cycling. Prieto *et al.* (1988, 1991) suggested the main absorption route of ABZ is by gastric absorption with a secondary step of intestinal absorption for ABZ and/or metabolites excreted in the bile.

The ratios of C_{max} and AUC for ABZSO and $ABZSO_2$ after 5 and 10 mg/kg ABZ were comparable with the dose ratio of 0.5, indicating that concentrations of ABZSO and $ABZSO_2$ increase proportionally to dose. In contrast, the ratios for ABZ were close to unity suggesting that there may be a saturating effect on the absorption process. This finding is consistent with previously observed data in the perfused rat gut following 0.5 and 1.0 mg/ml ABZ dissolved in DMSO (Prieto *et al.*, 1988).

The metabolism of ABZSO to $ABZSO_2$ has been exclusively attributed to cytochrome P-450c in perfused rat liver (Souhaili-El Amri *et al.*, 1988) and this conversion is dependent upon the ABZSO concentrations in the medium. The rat studies demonstrated that the conversion of ABZSO to $ABZSO_2$ is consistent with the AUC ratios which were almost identical irrespect of ABZ (5 and 10 mg/kg) or ABZSO (10 mg/kg) administered.

It has been previously suggested that conversion of ABZ to ABZSO is reversible in the liver (Gyurik *et al.*, 1981). Studies in rats receiving 10 mg/kg ABZSO demonstrated that rats were able to reduce ABZSO to ABZ. AUC for ABZ was 8% of the AUC for ABZSO. There was no evidence of reduction of ABZSO *in vitro* in sheep and cattle microsomes (Lanusse *et al.*, 1992). ABZ was formed however when ABZSO was incubated in sheep and cattle ruminal and ileal fluids (Lanusse *et al.*, 1992). Studies on sulphoxide-containing drugs have shown that the gastrointestinal tract is the principal site for reduction of these drugs (Renwick *et al.*, 1986). It has been suggested that this metabolic reduction may act as a source of ABZ (Lanusse *et al.*, 1992) and therefore be of importance for the efficacy of ABZ against parasites as ABZ has greater affinity for parasite tubulin than ABZSO (Lubega and Prichard, 1991).

Oxidation of ABZ may also take place in the gastrointestinal tract as ABZSO was found in higher concentrations than ABZ in the stomach and intestine after oral doses of ABZ in rats. $ABZSO_2$ was also present in small amounts. $ABZSO_2$ was not detectable in any of the samples after incubation of ABZ in the stomach. ABZ and ABZSO were found in the stomach wash and content. Galtier *et al.* (1991) have postulated the occurence of extrahepatic sulphoxidation of ABZ in other body tissues or fluids may explain the 2 to 3 times greater total body clearance of ABZ than that previously determined in hepatic blood flow in sheep. Consistently, there was evidence that ABZ was converted to ABZSO and $ABZSO_2$ in sheep and cattle ruminal and ileal fluids following anaerobic incubation (Lanusse *et al.*, 1992).

ABZ and ABZSO were found in the mucosal layers of the stomach after ABZ was incubated in the stomach for 6 hours with an ABZ/ABZSO ratio of 1.28. Upon incubation of the mucosal layer of the stomach and intestine, ABZ was progressingly oxidised to ABZSO in the samples with mucosal layers. In stability study of ABZ in DMSO in Chapter 4, it was found that ABZ degraded in DMSO. Therefore conversion of ABZ to ABZSO in the stomach and intestine preparations may be due to degradation of ABZ by DMSO and/or enzymatic degradation.

ABZ has low bioavailability which may be due to its limited dissolution in the gastrointestinal fluids. The enhanced absorption of ABZ when taken after fatty food/oil (Lange *et al.*, 1988; Marriner *et al.*, 1986) may be rationalised in term of delayed gastric emptying and prolonged gastrointestinal transit which allows more complete dissolution of the drug and longer residence at the sites favouring the passage of the drug into the systemic circulation.

Rat studies looking at the kinetics of ABZ standard in Mig:Twn and PEG 400 showed there were no significant differences in the AUC between the formulations. As the rat models were established, a study in higher animal (monkey) was initiated to assess the plasma levels of ABZ and metabolites before going onto human studies. Four formulations of ABZ (standard or tablet in Mig:Twn; standard or tablet in PEG 400) were administered to monkeys at a dose of 6 mg/kg. There were no significant differences between the formulations. Plasma levels of ABZ, ABZSO and $ABZSO_2$ were comparable to those found in rats. Interestingly, plasma levels of ABZ on a comparable mg/kg dosing are approximately one order of magnitude higher in rats and monkeys than man. The C_{max} is approximately 7 times higher in rats

and 6 times in monkeys than in man. The AUC are also higher in rats and monkeys by 10- and 14-fold, respectively. The enhanced absorption observed in animals may be due to (i) a consequence of the formulations used or the powdered form of ABZ used or (ii) a difference in absorption due to differences in animal and human gastrointestinal tract. In monkeys it may be due to the high pH of the stomach (pH 4-9). As ABZ is a basic drug, in accordance to the pH-partition hypothesis, its absorption will be favoured in the high pH stomach since a larger fraction of ABZ in solution will be in the unionised form.

For potential human studies we needed to err on the safety side and therefore assumed that the new formulation would improve absorption, requiring a decrease in dose. 0.6 mg/kg ABZ was given to rats as ground-up tablet or standard, both prepared in Mig:Twn. The ground-up tablet was given to animals to compare the absorption with the standard since standard ABZ for human use was not available. The results showed no significant difference in the kinetic profiles between the 2 sources of ABZ and dose proportionality was maintained. To resolve the question as to whether powdering up the ABZ tablet or the new formulation was responsible for improved absorption, a study in man to compare ABZ tablet and/or 10% ground-up tablet in capsule, and 0.6 mg/kg new formulation in capsule would be necessary.

Plasma levels of ABZ and its two major metabolites were measured in a single volunteer who received a 400 mg ABZ tablet. The AUC for ABZSO found was comparable to previous findings (Penicaut *et al.*, 1983; Marriner *et al.*, 1986; Lange *et al.*, 1988). Concentrations of ABZ and $ABZSO_2$ found were <100 ng/ml. ABZ levels were measured for the first

time following an oral dose of ABZ in a healthy man. This may due to the sensitive and selective analytical method used as discussed in Chapter 3.

ABZ was also found in loa loa patients. Steady-state concentrations of ABZ and both metabolites were not achieved after administration of 200 mg ABZ twice a day over 21 days in the three patients since the concentrations were highly variable. The variation of ABZSO concentrations were difficult to explain. It may be attributed to extensive hepatic metabolism (Marriner *et al.*, 1986) and rapid gastrointestinal metabolism of ABZ to ABZSO (Lanusse *et al.*, 1992). A recent study in onchocerciasis patients following multiple doses of 800 or 1200 mg ABZ for 3 days, a steady-state level of ABZSO was not observed as the concentration continued to decrease over the 72 hours sampling time period (Hoaksey, 1990). It was suggested that it may be due to ABZ autoinduction, since in rats ABZ is known to induce cytochrome P-450c, an isoenzyme which is responsible for the conversion of ABZSO to $ABZSO_2$ (Souhaili-El Amri *et al.*, 1988) and therefore, it is possible that ABZ to undergo autoinduction in man. The measured concentrations of $ABZSO_2$ however did not indicate this but it may be undergoing some other reactions. According to Gyurik *et al.* (1981), $ABZSO_2$ is not reduced to ABZSO, but in turn may metabolised to three metabolites as identified in the urine of rats (Gyurik *et al.*, 1981) and man (Penicaut *et al.*, 1983) which were not looked for in this study as they were only present in trace amount (Gyurik *et al.*, 1981; Penicaut *et al.*, 1983).

In conclusion, there was no significant difference in the bioavailability between the "oily" (Mig:Twn) and PEG 400 ABZ formulations in animals (rats and monkeys). However, the ABZ concentrations were an order of magnitude higher in rats and monkeys than in man receiving 400 mg ABZ tablet. The absorption of ABZ was improved (C_{max} = 6-7 times higher) and AUC was also higher (10-14 times) in animals which necessitate a study in man to evaluate the bioavailability following administration of the "oily" formulation.

Concentrations of ABZ, ABZSO and $ABZSO_2$ were highly variable in both animals and human, and there is evidence of reversible conversion of ABZ to ABZSO in *in-vitro* studies. ABZ was also detected for the first time in a healthy volunteer receiving 400 mg ABZ tablet. No tendency of attainment of the steady-state concentrations of ABZ, ABZSO and $ABZSO_2$ were observed in loa loa patients after receiving 200 mg ABZ twice daily over 21 days.

CHAPTER 6

CONCLUDING DISCUSSION

Benzimidazole carbamates have been shown to be active against both larvae and adult filarial nematodes (Van den Bossche *et al.*, 1982; Cook, 1990). However due to low bioavailability and side-effects, mebendazole and flubendazole were not recommended for the treatment of filarial infections (Tropical Disease Research, 1984). Although albendazole has low bioavailability, it has better absorption in the gastrointestinal tract following oral administration (Morris *et al.*, 1983) and greater potency (Awadzi *et al.*, 1990) compared to mebendazole. Currently, albendazole is being used in the treatment of systemic infections such as hydatid disease and cysticercosis (Todorov *et al.*, 1992a, b; Jung *et al.*, 1992). However due to its low bioavailability, large doses of albendazole are required. An improved bioavailability requiring a smaller dose is desirable since this would decrease any side-effects associated with the drug as well as the cost.

The primary aim of this study was to produce a formulation of albendazole which would improve the bioavailability when administered orally. The literature suggests that when albendazole is given with a fatty meal or co-administered with "oil", its bioavailability is improved (Marriner *et al.*, 1986; Lange *et al.*, 1988). The analysis was however based on the appearance of the sulphoxide metabolite in the blood, as albendazole was apparently undetected in human plasma.

The initial aim of the study was consequently to improved the methodology so that bioavailability could be assessed on albendazole and not a metabolite. This was achieved by development a sensitive and selective gradient elution reversed-phase HPLC method for quantitation of albendazole and its two major metabolites, the

sulphoxide and sulphone in plasma. The method was sufficiently sensitive to measure 20 ng/ml albendazole and the sulphone, and 10 ng/ml sulphoxide metabolite in 1 ml plasma sample with acceptable precision (<10% RSD). Using this method for the first time, albendazole was detected in volunteer/patients plasma who received a single clinical dose (400 mg) of albendazole. This demonstrates the capability of this methodology as the working tool for the determination of albendazole and therefore the investigation on albendazole could proceed further.

There were no significant differences in bioavailability of albendazole in both rat and monkey when "oily" formulation and when PEG 400 formulation were administered. However the C_{max} and AUC of albendazole were 6-7 times and 10-14 times higher in animals, respectively when compared to that of a healthy volunteer who received an albendazole tablet (on comparing mg/kg dosing, 6 mg/kg). The AUC of albendazole sulphoxide were 5 times higher in rats and, 8-20 times in monkeys, which are higher than that reported by Lange *et al.* (1988) in man after given albendazole with a fatty meal (4.5 times).

The improved absorption of albendazole may be due to the consequence of the its formulation. It has been established that the mechanism of drug absorption *in vivo* from lipid-based dosage form involves the drug partitioning into the aqueous phase before absorption through the gastrointestinal wall (Armstrong and James, 1980). Since dissolution process is not involved, albendazole is readily available and absorption is more rapid. However if the transfer across the mucosa is the rate limiting step, the absorption rate will dependent

on the concentration gradient across the mucosa. In this situation, as the aqueous and oily phase are in equilibrium, the partition coefficient of albendazole would determine the rate of absorption (Kakemi *et al.*, 1972). On the other hand if the rate of drug release is the rate determining step, the equilibrium concentration in the aqueous phase would not be achieved as a lipophilic drug has a greater affinity for oil. However this is not the case in this situation since the microscopic examination of albendazole in Mig:Twn solution when added to simulated gastric fluid showed some of albendazole present in aqueous phase which indicates that albendazole was present in excess.

The large oil/water interfacial area of the Mig:Twn system would promote the diffusion of albendazole from the oil phase into the luminal contents. Digestion of the oil would be expected to modify the interfacial area. Miglyol in self-emulsifying system has been shown to inhibit lipolysis rate (Challis, 1991). The presence of surfactant layer around the oil droplet will also hindered the action of the lipase. However Tween 80 was found to be rapidly hydrolysed (Challis, 1991). Therefore lipolysis of Miglyol in the system probably be slow. The composition of oil and aqueous phases would also change as lipolysis of the Miglyol would decrease the volume of the oil phase (Playoust and Isselbacher, 1964; Greenberger *et al.*, 1966). The fatty acid and monoglyceride produced by hydrolysis of triglyceride of Miglyol are more polar and will associate with the bile salt before being absorbed by the intestinal mucosa. It has been suggested that in the presence of the fatty acids in the intestinal epithelium cell membrane, they may cause an increase in membrane fluidity and thereby increase the diffusion rate of the drug across

the membrane (Grisafe and Hayton, 1978).

The excess albendazole has to be dissolved before absorption. In this situation the solid albendazole may dissolve in the aqueous phase as the drug is being absorbed and the presence of Tween 80 may increase the dissolution rate of albendazole into the aqueous phase. It is also possible that the drug in the aqueous phase partitions into the oil droplets, although it is generally accepted that only non-ionised species partitions from the aqueous phase into the non-aqueous phase (Florence and Attwood, 1988b).

In the presence of the bile salt, the dissolution rate of albendazole may also be increased and therefore may increase the absorption across the membrane. Del Estal *et al.* (1991) demonstrated that the intestinal absorption of albendazole was increased when it was administered in sodium taurocholate solution. The enhanced absorption of albendazole observed in rats may be due to the effect of bile salt. Since the rat has no gall bladder, the bile is continuously flowing into the duodenum and immediately available whereas in humans, a neural or hormonal stimuli induced by feeding is necessary to empty the gall bladder into the duodenum (Poelma *et al.*, 1990). However the composition of bile and the concentration of bile salts in human and rat bile are similar, taurocholate being the major bile salt. The effect of bile salts in the dissolution rates of a drug is by micellar solubilization and/or by wetting effect (Miyazaki *et al.*, 1979; Poelma *et al.*, 1990). This probably caused the large variation in absorption between animals.

The enhanced absorption of albendazole is not due to the gastric

emptying rate as medium-chain triglycerides did not cause any gastric retention (Yamahira *et al.*, 1979). On the other hand, Miglyol when formulated as self-emulsifying system improved the reproducibility of the gastric emptying (Challis, 1991). The results from the solubility study of albendazole in Miglyol (Chapter 4) showed that albendazole was stable at least for 5 days and therefore it is possible that Miglyol promotes albendazole absorption by protecting the molecule from the degradation in the stomach environment because at high concentration in acidic solution albendazole may degraded as observed in the stability and solubility studies of albendazole in 0.1M HCl (Chapter 4).

Charman *et al.* (1992) observed an improvement of plasma levels of a lipophilic drug following oral administration of lipid-based preparation (self-emulsifying system: Neobee M5 a medium-chaim triglyceride oil, Tagat TO a nonionic surfactant and a lipophilic drug, 40:25:35%, w/w) in two separate studies. However the levels were inconsistent and erratic, and proposed that the formulation is most likely to be a function of solubilisation and dispersion of the drug and not the function of lipid component in the formulation. The concentrations of albendazole following administration of this "oily" formulation were highly variable. It may be that Mig:Twn behaves similar to the Neobee M5:Tagat TO system (Charman *et al.*, 1992).

The powdered form of albendazole used as opposed to a compressed tablet/standard albendazole may also affect the absorption. According to the Noyes-Whitney equation (Eqn. 4.4 in section 4.1.2) an increase the surface area of the drug will increase the dissolution rate of the drug. This was demonstrated by the results from the comparison

study of the standard albendazole and ground-up tablet, both in Mig:Twn in rats. There was no difference statistically in the kinetic profiles from the two sources.

A study in man is necessary to evaluate the bioavailability of albendazole following the administration of the "oily" formulation since improved absorption was observed in animals. This study would also address the question raised as to whether the absorption was improved due to the formulation, or to the powdered form of albendazole used or simply due to the difference in absorption. However there is a solubility problem in the new formulation. The concentration of albendazole in Mig:Twn is 2 mg/g. In order to dose a healthy volunteer of 65 kg with 0.6 mg/kg albendazole, approximately 19 ml of the Mig:Twn solution (2 mg/g) is required which is approximately two tablespoon of the formulation. However due to the palatability and patient compliance, this may not be possible. Therefore for oral dosage form in man the formulation may have to be filled in soft gelatine capsules. Unfortunately, a gelatine capsule can only be filled with a dose volume of 0.10 ml to 0.80 ml (range from the round to oblong shape capsule). Therefore it is not possible to dose a man with this formulation.

The work resulting from this investigation that should be considered for further study is to reformulate albendazole as a suspension in Mig:Twn for assessment of bioavailability of albendazole in humans. Various investigations have been undertaken to increase drug aqueous solubility by using cyclodextrin. Therefore another possibility is to increase aqueous solubility of albendazole and therefore its bioavailability by formulating albendazole as cyclodextrin complexes.

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APPENDIX 1

The Bartlett Test is calculated using the following equations

$$\chi^2 = \frac{(x - \bar{x})^2}{\sigma^2}$$

where σ^2 is given by

$$\sigma^2 = \frac{\Sigma\, n_i s_i^2}{\Sigma\, n_i}$$

where x = slope value

$\bar{x}$ = mean slope

s_i = standard error of slope

n_i = number of points on curve

APPENDIX 2

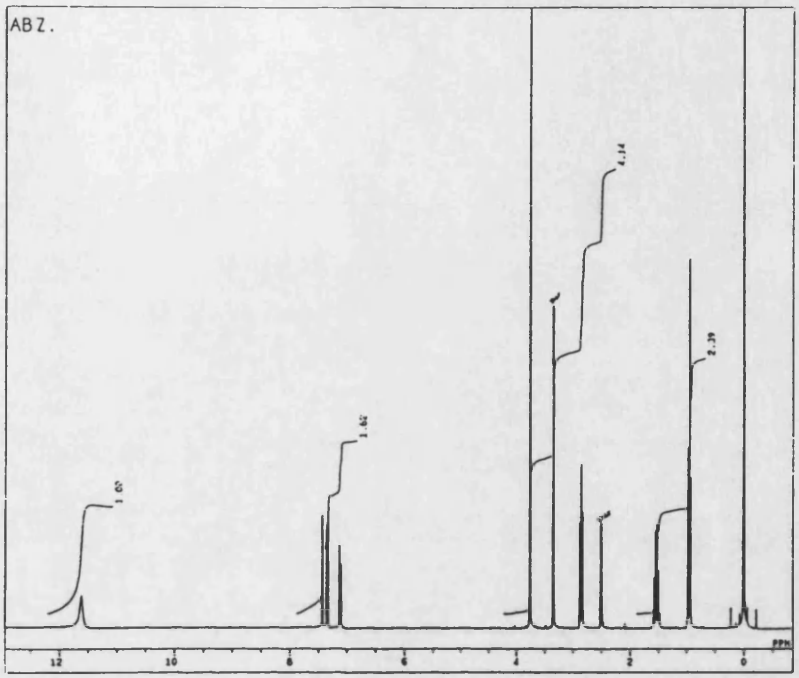

Fig. 2A.1 NMR spectrum of ABZ

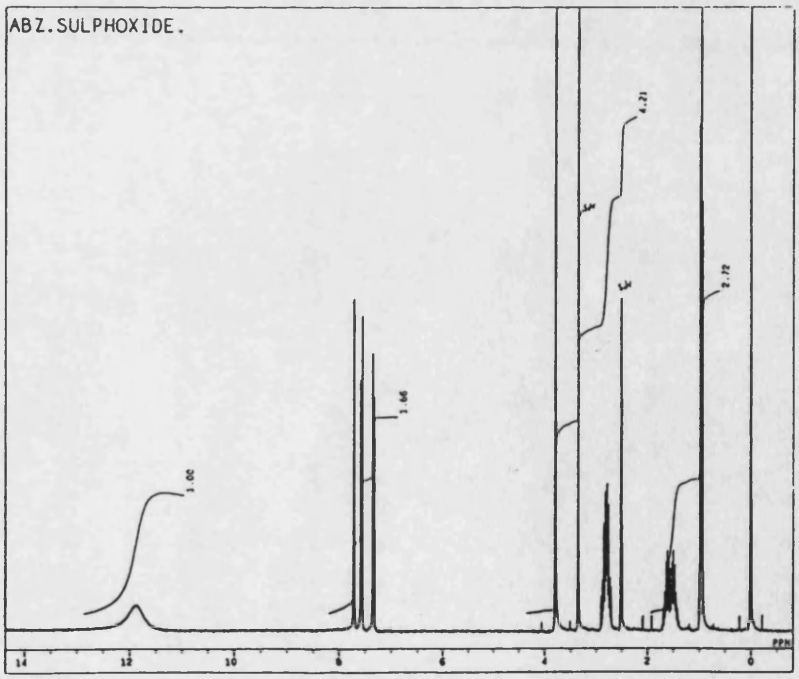

Fig. 2A.2 NMR spectrum of ABZSO

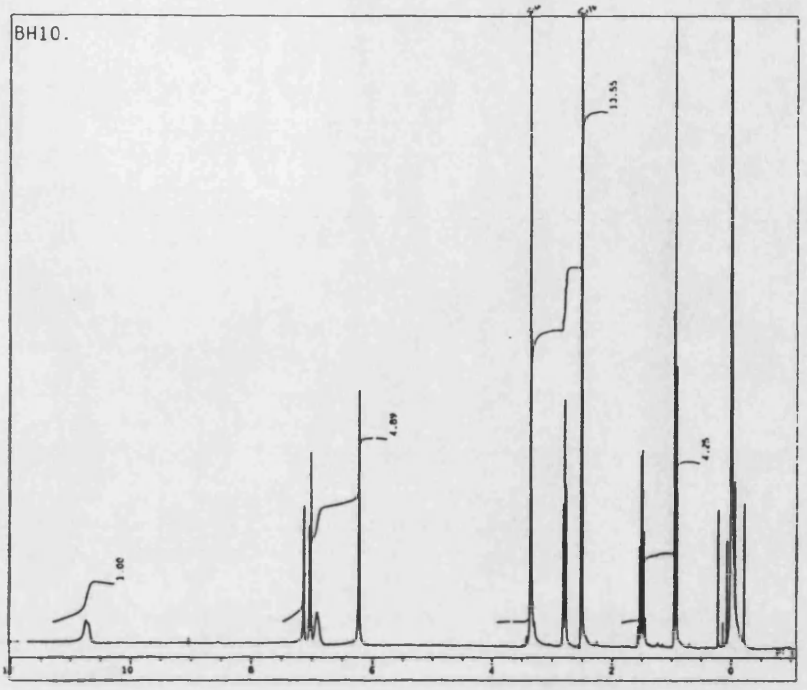

Fig 2A.3 NMR spectrum of Unknown 2 (ABZ 2-amine)

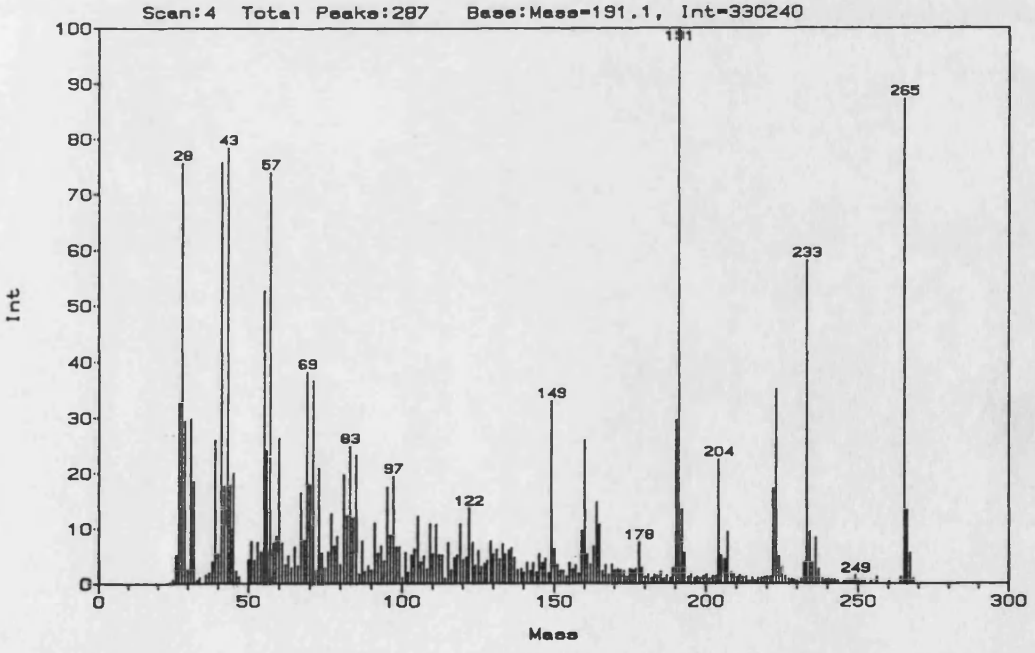

Fig. 2A.4 MS spectrum of ABZ

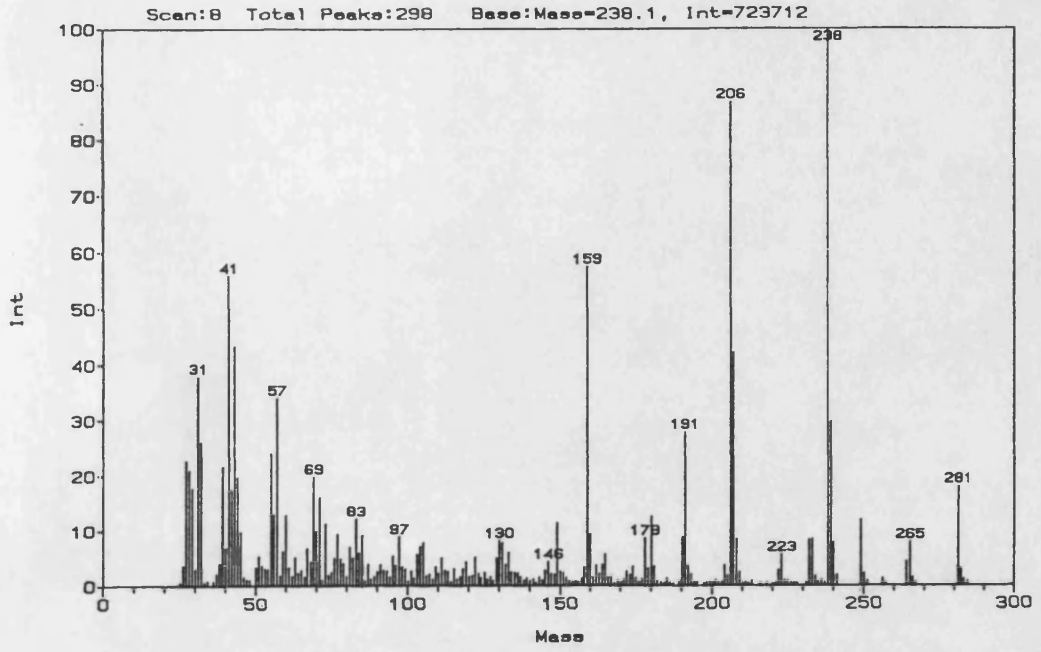

Fig. 2A.5 MS spectrum of ABZSO

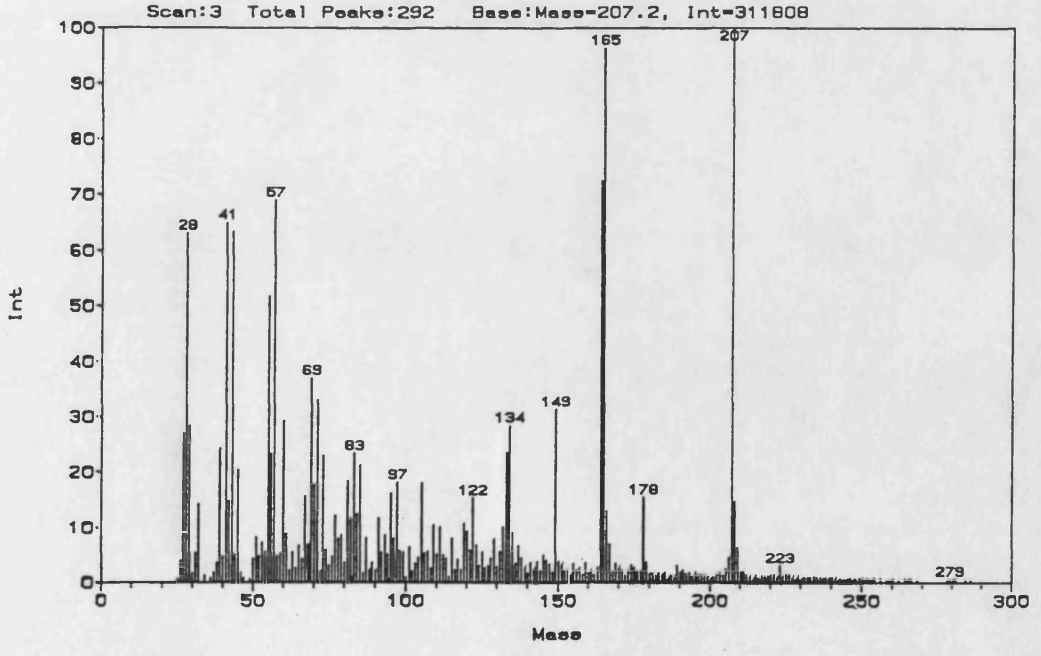

Fig. 2A.6 MS spectrum of Unknown 2 (ABZ 2-amine)

APPENDIX 3
Results from the *in vitro* and *in vivo* studies in animal and human studies.

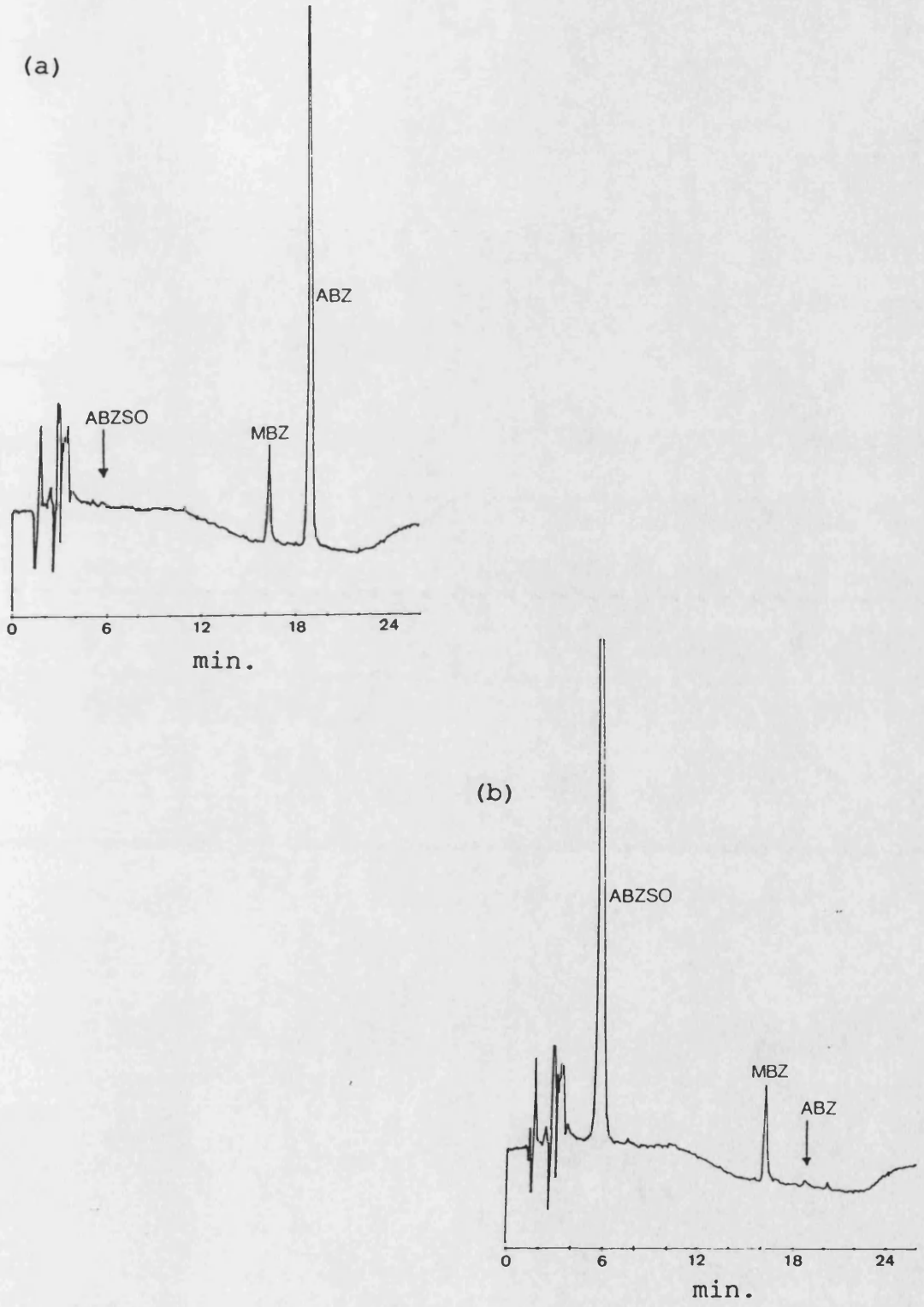

Fig.3A.1 Chromatograms of (a) ABZ and (b) ABZSO standards in DMSO.

Table 3A.1 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 5 mg/kg ABZ in DMSO in rats. (ND:not detectable)

Sampling time (hr)	ABZ concentration (ng/ml) 1	2	3	Mean±SD
0.5	174	357	209	247± 97
1.0	347	233	163	248± 93
1.5	439	183	215	279±140
2.0	594	544	300	479±157
2.5	543	340	130	337±206
3.0	100	332	171	201±119
4.0	263	403	705	329±214
5.0	389	319	331	346± 38
6.0	228	121	166	172± 54
7.0	367	273	153	264±107
	ABZSO concentration (ng/ml) 1	2	3	Mean±SD
0.5	530	641	915	696±198
1.0	857	1104	2228	1397±731
1.5	1053	721	2534	1436±965
2.0	1142	2962	1791	1965±922
2.5	1595	2311	2233	2046±393
3.0	929	1547	944	1140±352
4.0	1420	1136	2179	1578±539
5.0	976	1228	1363	1189±197
6.0	528	1923	1844	1431±784
7.0	649	2219	1842	1570±819
	$ABZSO_2$ concentration (ng/ml) 1	2	3	Mean±SD
0.5	155	ND	ND	52± 89
1.0	120	ND	ND	40± 69
1.5	188	103	163	151± 44
2.0	233	168	148	183± 44
2.5	267	150	195	204± 59
3.0	189	146	98	145± 46
4.0	306	193	302	267± 64
5.0	325	161	224	237± 83
6.0	291	316	451	353± 86
7.0	623	313	289	408±187

Table 3A.2 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 10 mg/kg ABZ in DMSO in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	ABZ concentration (ng/ml) 1	2	3	Mean±SD
0.5	314	155	233	234± 80
1.0	232	481	522	412±157
1.5	474	198	781	484±291
2.0	590	283	1121	665±424
2.5	205	171	187	187± 17
3.0	383	272	263	306± 67
4.0	351	238	NS	295± 80
5.0	351	242	711	435±245
6.0	126	215	234	192± 58
7.0	211	196	231	213± 18
	ABZSO concentration (ng/ml) 1	2	3	Mean±SD
0.5	1167	696	1172	1012± 274
1.0	2413	1801	3332	2516± 771
1.5	4216	1019	4365	3200±1890
2.0	4163	2579	5192	3978±1317
2.5	2466	2696	1505	2222± 631
3.0	2865	3493	2709	3022± 415
4.0	4756	2873	NS	3815±1331
5.0	6057	2223	3372	3884±1967
6.0	1716	3761	3297	2925±1072
7.0	2545	2576	2125	2670± 750
	$ABZSO_2$ concentration (ng/ml) 1	2	3	Mean±SD
0.5	44	ND	ND	15± 25
1.0	77	ND	67	48± 42
1.5	150	57	247	151± 95
2.0	284	170	236	230± 57
2.5	189	233	78	167± 80
3.0	210	224	243	226± 16
4.0	557	255	NS	406±213
5.0	691	513	635	613± 91
6.0	303	500	585	463±145
7.0	370	366	426	387± 34

Table 3A.3 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 10 mg/kg ABZSO in DMSO in rats.

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	383	2255	119
	362	4853	266
1.0	492	4847	282
	520	5485	588
1.5	584	7029	526
	1123	4603	427
2.0	551	6017	548
	647	5938	590
2.5	217	7172	661
	577	7332	850
3.0	100	1985	221
	220	6455	1066
4.0	161	4053	582
	242	3889	534
5.0	414	5777	1074
	257	4244	662
6.0	218	4718	993
	431	4226	888
7.0	245	2201	415
	319	5170	1242

Table 3A.4 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 5 mg/kg ABZ standard in Mig:Twn in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	1128	1256	ND
	771	1333	ND
1.0	238	786	129
	2802	3122	155
1.5	428	1495	166
	518	1416	200
2.0	910	1907	266
	510	2405	350
2.5	234	1268	313
	NS	NS	NS
3.5	359	2244	251
	229	1615	506
4.0	126	865	247
	ND	488	250
5.0	336	1968	413
	95	2314	611
6.0	ND	603	312
	96	1110	304
8.0	ND	731	413
	ND	ND	193
12.0	ND	775	976
	ND	194	248
24.0	ND	244	247
	ND	ND	ND

Table 3A.5 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 5 mg/kg ABZ tablet in PEG 400 in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	303	938	100
	623	983	84
1.0	596	1934	225
	855	1464	119
1.5	711	1722	164
	636	1001	182
2.0	357	1979	292
	419	1283	151
2.5	535	1083	295
	359	1585	272
3.5	339	132	299
	816	1530	269
4.0	832	2420	416
	591	2288	336
5.0	462	1024	397
	1698	89	559
6.0	532	840	634
	311	1326	534
8.0	NS	NS	NS
	388	985	479
12.0	ND	362	1379
	ND	ND	445
24.0	ND	ND	ND
	ND	ND	ND

Table 3A.6 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ standard in Mig:Twn in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	154	417	ND
	1106	464	ND
	NS	NS	NS
1.0	347	1101	ND
	242	843	ND
	157	666	55
1.5	264	1092	140
	445	1003	94
	332	1283	79
2.0	530	1986	104
	956	974	143
	176	1290	102
2.5	371	1586	116
	289	2072	214
	282	2010	236
3.0	579	2222	141
	156	2038	222
	262	1814	315
4.0	644	2605	265
	424	2480	229
	425	2316	467
5.0	443	2162	304
	425	2432	389
	161	1773	158
6.0	367	1279	270
	215	2330	478
	NS	NS	NS
7.5	358	1172	414
	219	2181	556
	NS	NS	NS
12.0	ND	ND	ND
	ND	ND	116
	ND	135	399
24.0	ND	ND	ND
	ND	ND	ND
	ND	ND	ND

Table 3A.7 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ standard in PEG 400 in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	294	454	ND
	51	162	ND
	888	837	ND
1.0	599	967	36
	459	757	ND
	NS	NS	NS
1.5	333	1723	89
	549	1711	99
	675	1732	108
2.0	752	2329	96
	507	1063	ND
	310	2144	79
	328	1445	300
	1120	2332	209
	421	2248	263
3.0	650	1850	238
	670	2606	278
	800	2127	185
4.0	606	1612	213
	218	2404	363
	508	3034	412
5.0	549	1785	272
	379	2501	537
	428	2803	643
6.0	223	1755	336
	303	2396	734
	NS	NS	NS
7.5	452	1393	472
	188	1408	467
	192	1483	590
12.0	ND	ND	ND
	ND	ND	120
	ND	41	225
24.0	ND	ND	ND
	ND	ND	ND
	ND	ND	ND

Table 3A.8 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 0.6 mg/kg ABZ tablet in Mig:Twn in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	90	64	ND
	30	158	ND
1.0	53	128	ND
	102	221	ND
2.0	44	290	58
	120	578	ND
3.0	96	533	130
	81	597	162
4.0	20	122	63
	131	864	230
5.0	38	193	150
	47	310	254
6.0	ND	ND	48
	60	185	130
7.0	ND	32	ND
	ND	68	ND

Table 3A.9 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 0.6 mg/kg ABZ standard in Mig:Twn in rats. (ND:not detectable, NS:no sample)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	109	337	ND
	98	206	ND
1.0	133	354	ND
	85	218	67
2.0	89	540	130
	50	297	63
3.0	47	373	150
	87	478	97
4.0	36	125	132
	36	207	140
5.0	51	172	117
	68	395	239
6.0	ND	135	21
	58	49	141
7.0	ND	ND	ND
	ND	ND	ND

Table 3A.10 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ tablet in PEG 400 (Prep. A) in monkeys. (ND:not detectable)

	Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
513	1.0	334	938	87
	2.0	490	2739	432
	3.0	712	1376	347
	4.0	391	1572	436
	5.0	986	2708	743
533	1.0	139	854	31
	2.0	557	2555	332
	3.0	333	2985	459
	4.0	649	4634	1024
	5.0	855	3510	999
	8.0	408	3606	1191
	12.0	313	1454	562
	24.0	199	1380	534
	48.0	ND	22	55
535	1.0	34	1147	79
	2.0	379	1847	190
	3.0	326	1986	210
	4.0	280	2657	437
	5.0	215	2928	581
	8.0	200	1954	506
	12.0	26	1596	575
	24.0	39	248	181
	48.0	ND	ND	ND

Table 3A.11 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ standard in Mig:Twn (Prep.B) in monkeys. (ND:not detectable, NS:no sample)

	Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
486	1.0	69	36	ND
	2.0	76	140	ND
	3.0	106	350	ND
	4.0	140	260	ND
	5.0	97	280	60
	8.0	258	100	66
	12.0	123	145	59
	24.0	648	2864	483
	48.0	194	250	148
434	1.0	69	314	ND
	2.0	388	1040	91
	3.0	429	1923	148
	4.0	561	3014	277
	5.0	355	2208	246
	8.0	175	1136	100
	12.0	NS	NS	NS
	24.0	198	2843	487
	48.0	76	447	211
435	1.0	46	90	ND
	2.0	235	46	ND
	3.0	164	416	ND
	4.0	92	346	78
	5.0	139	480	119
	8.0	109	701	146
	12.0	89	1975	392
	24.0	237	4698	1251
	48.0	105	ND	175

Table 3A.12 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ tablet in Mig:Twn (Prep.C) in monkeys. (ND:not detectable, NS:no sample)

	Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
533	1.0	NS	NS	NS
	2.0	308	921	155
	3.0	NS	NS	NS
	4.0	99	1789	419
	5.0	231	1429	500
	8.0	310	1032	667
	12.0	165	810	481
	24.0	68	347	329
	48.0	ND	ND	33
535	1.0	70	465	51
	2.0	306	1626	150
	3.0	551	1957	270
	4.0	422	2463	400
	5.0	690	2063	500
	8.0	150	2673	824
	12.0	61	1612	788
	24.0	35	188	153
	48.0	ND	ND	19
252	1.0	ND	100	18
	2.0	48	283	51
	3.0	100	635	103
	4.0	298	519	150
	5.0	246	587	189
	8.0	233	413	292
	12.0	109	736	243
	24.0	ND	119	230
	48.0	ND	ND	ND

Table 3A.13 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 6 mg/kg ABZ standard in PEG 400 (Prep.D) in monkeys. (ND:not detectable, NS:no sample)

	Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
486	1.0	0	31	ND
	2.0	121	586	45
	3.0	121	897	110
	4.0	162	1171	141
	5.0	478	1397	176
	8.0	708	2390	420
	12.0	825	2323	620
	24.0	390	1327	533
	48.0	ND	ND	ND
434	1.0	0	387	ND
	2.0	185	1384	112
	3.0	190	1256	150
	4.0	213	1573	199
	5.0	625	1266	281
	8.0	174	2395	629
	12.0	ND	1231	433
	24.0	ND	64	37
	48.0	ND	ND	39
316	1.0	0	160	18
	2.0	35	196	25
	3.0	429	541	150
	4.0	339	821	188
	5.0	250	1010	241
	8.0	273	1415	343
	12.0	NS	NS	NS
	24.0	157	900	518
	48.0	ND	27	104

Table 3A.14 Concentrations of ABZ, ABZSO and $ABZSO_2$ following oral dose of 400 mg ABZ in healthy volunteer. (ND:not detectable)

Sampling time (hr)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
0.5	ND	89	36
1.0	38	136	56
1.5	35	137	57
2.0	22	98	43
2.5	32	151	35
3.0	74	70	59
3.5	34	139	37
4.0	24	130	40
5.0	20	133	35
6.0	42	95	35
7.0	ND	106	34
8.0	28	106	37
12.0	26	79	31
24.0	ND	76	34
32.0	ND	58	30

Table 3A.15 Concentrations of ABZ, ABZSO and $ABZSO_2$ following 200 mg ABZ twice a day over 21 days in loa loa patients. (ND:not detectable)

	Sampling time (day)	Concentration (ng/ml) ABZ	ABZSO	$ABZSO_2$
42	0	ND	ND	ND
	1	ND	ND	ND
	3	44	207	ND
	6	93	515	33
	9	22	199	ND
	14	ND	55	ND
	20	ND	40	ND
46	0	ND	ND	ND
	1	ND	114	ND
	3	ND	81	ND
	6	78	522	35
	9	ND	195	34
	14	40	300	86
	20	79	587	35
53	0	ND	ND	ND
	1	ND	89	ND
	3	ND	116	ND
	6	182	ND	ND
	9	ND	116	28
	14	ND	159	29
	20	126	557	65

Table 3A.16 Concentrations of ABZ and ABZSO following incubation of ABZ with mucosal layer of stomach and intestine at 37°C.

Sampling time (hr)	Concentration (ng/ml) ABZSO a	ABZSO b	ABZ
Control1			
1.0	124	117	1687
2.0	178	168	1758
4.0	150	142	2099
5.0	149	140	2069
6.0	129	121	1806
Control2			
1.0	175	165	1746
2.0	204	192	1858
4.0	187	176	1988
5.0	196	185	1899
6.0	144	136	1789
Stomach1			
1.0	232	219	1791
2.0	239	225	1429
4.0	251	237	1829
5.0	270	255	1937
6.0	262	247	1528
Stomach2			
1.0	290	273	1576
2.0	308	291	1509
4.0	291	274	1369
5.0	368	347	1600
6.0	NS	NS	NS
Intestine1			
1.0	208	196	1715
2.0	314	296	1587
4.0	700	660	1611
5.0	1009	951	1775
6.0	1420	1340	1620
Intestine2			
1.0	212	200	1681
2.0	293	277	1806
4.0	238	224	1569
5.0	290	274	1318
6.0	334	316	1293

a: actual concentration
b: ABZ equivalent
NS: no sample