MICROEMULSIONS - A NEW PERSPECTIVE IN THE TREATMENT OF PAEDIATRIC AND GERIATRIC TUBERCULOSIS PATIENTS.

THESIS

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Abstract

Tuberculosis(TB)was declared to be a global emergency in 1993, with South Africa declaring it to be the country's top health priority in 1996, but ineffective treatment strategies have led to fewer than half of all treated patients in South Africa being cured. At present, paediatric treatment remains a problem, as the antitubercular preparations of rifampicin, isoniazid and pyrazinamide, that are currently available, were not initially designed for the treatment of paediatric TB patients, providing a motivation for this project. The aim of this project is thus the development of a microemulsion dosage form for the oral delivery of RIF(Rifampicin), INH(Isoniazid) and PZA(Pyrazinamide) in combination. RIF, INH and PZA were adequately characterised with reference to the monograph standards referenced and were found to be sufficiently pure to be used in subsequent work. A chromatographic system and conditions were selected and validated as being optimal for HPLC analysis of RIF, INH and PZA in combination, with a drug partitioning method for miglyol 812 developed and validated. Ternary and pseudo-ternary phase diagrams were constructed and reported, all employing miglyol 812 as the lipid. It was undoubtedly the imwitor 308 and crillet 3 combination o/w microemulsion system that proved most successful, maintaining homogeneity on dilution. The microemulsion used in formulation comprised imwitor 308 (27.63%), crillet 3 (27.63%), miglyol 812 (23.68%) and water (21.06%). The stability of RIF, INH and PZA was investigated in aqueous solution, miglyol 812, corn oil, 10%m/v cremophor RH, 5%m/v imwitor 308, 10%m/v crillet 3 and 70%m/v sorbitol solution. Trends in the stability assessments conducted on RIF, INH and PZA were noted, with slight variation depending on the formulation component being evaluated. RIF invariably demonstrated temperature and oxidation dependent degradation in all vehicles, with a definite distinction possible between samples stored at 25, 40 and 60^0C over a 7 day trial period. A definite advantage of storing RIF solutions under nitrogen was observed, with these solutions showing less degradation over the course of the trial, than those stored under air. INH produced a pronounced increase in the degree of degradation of RIF, whereas PZA had a negligible effect on it's stability. INH proved to be most stable in the 70%m/v sorbitol solution with no significant oxidation or temperature dependent degradation indicated. Temperature dependent degradation was only noticable when INH was in combination with RIF, most significant in crillet 3 solution. PZA was the most stable of the three drugs, remaining relatively unaffected by temperature and the presence of air, independent of the vehicle employed, although the drug remaining did decrease slightly in the presence of RIF.

Due to drug dose specifications and solubility limitations, the final formulation assessed, only contained RIF and INH, despite INH and PZA having no significant effect on the stability of each other. The solubility of PZA in the lipid and aqueous components of the microemulsion was not great enough to achieve the required 500 mg/10ml dose, while RIF and INH could achieve the respective 150mg/10ml and 100mg/10ml dose. RIF stability was improved, as anticipated, with the incorporation of RIF into the internal phase decreasing contact with INH which has been shown to affect it's stability. RIF behaved as predicted, possessing greater stability than shown in the individual formulation components, however, INH did not, being less stable in formulation in the absence of antioxidant, than in it's presence. A novel microemulsion formulation capable of delivering the incompatible RIF and INH in combination, with numerous microemulsion systems mapped, with the ability of being used for the delivery of other lipophilic drugs and drug combinations, was produced. The final formulation provided valuable information into possible future improvements of the microemulsion to improve drug stability.

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List of Abbreviations

AUFS	-	Area Under Full Scale
Co-SA	-	Cosurfactant
COSY	-	Correlated Spectroscopy
CPP	-	Critical Packing Parameter
DDRP	-	DNA dependent RNA polymerase
DOTS	-	Directly Observed Treatment Short-course
DSC	-	Differential Scanning Calorimetry
E	-	Stable coarse emulsion region
E_a	-	Stable coarse emulsion region (a)
FDA	-	Food & Drug Administration
HLB	-	Hydrophile-lipophile balance
HPLC	-	High Performance Liquid Chromatography
INH	-	Isoniazid
ICH	-	International Committee on Harmonisation
IPM	-	Isopropyl Myristate
IR	-	Infrared Spectroscopy
L	-	Stable isotropic region
L_a	-	Stable isotropic region (a)
LC	-	Liquid crystalline region
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
MDR-TB	-	Multi-Drug Resistant Tuberculosis
MS	-	Mass Spectrometry
m/e	-	mass-to-charge ratio
N	-	Column efficiency
NMR	-	Nuclear Magnetic Resonance Spectrometry

o/w	-	oil in water
PIT	-	Phase Inversion Temperature
PZA	-	Pyrazinamide
R	-	Resolution
R^2	-	Correlation Coefficient
R_f	-	Retention factor
RIF	-	Rifampicin
RSD	-	Relative Standard Deviation
Rt	-	Retention time
SA	-	Surfactant mixture
SAA	-	Surfactant/Surface Active Agent
SAMF	-	South African Medicines Formulary
SAR	-	Structure Activity Relationship
TB	-	Tuberculosis
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet Spectroscopy
w/o	-	water in oil
WHO	-	World Health Organisation
WISP	-	Waters Intelligent Sample Processor

Chapter One

Introduction

1.1 Disease State

Tuberculosis(TB) is a chronic, recurrent bacterial infection, caused by the organism *Mycobacterium tuberculosis*(*M. tuberculosis*)(Foye, W.O., *et al.*, 1995; Merck Manual, 1992). Hydrophobic properties are imparted to the organism, by the high lipid content of the cell wall with resistance to acid, alkali and alcohol, resulting in the classification as an aerobic acid-fast bacillus. The infection can involve almost any organ of the human body, but most commonly involves the lungs being almost always transmitted by the inhalation of airborne droplet nuclei containing the infective organism, or the desiccated airborne bacilli(Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991; Merck Manual, 1992). The infective droplet nuclei emanate from the respiratory tract of an individual who has tested sputum-smear positive for pulmonary tuberculosis, by the actions of sneezing, coughing or speaking, suspending these droplets in room air for several hours, increasing the chance of transmission (Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991). South Africa has one of the highest recorded incidence rates of TB in the world, with at least half of the 42 million people believed to be infected with *M. tuberculosis*. Normally, only one in ten of these would develop active TB, but today this proportion is increasing with the spread of human immunodeficiency virus(HIV) which weakens the immune system that can keep TB in check. In 1993 the World Health Organisation(WHO) declared TB to be a global emergency, so great was the concern about this modern epidemic. Similarly in November 1996, South Africa declared TB to be the country's top health priority, however, ineffective treatment strategies have led to fewer than half of all treated patients in South Africa being cured. The severity of the TB epidemic is such that it kills more of the youth and adults of the world than any other infectious disease, killing 2 to 3 million people every year, of which

at least 100 000 are children. This accounts for more than a quarter of all preventable adult deaths in developing countries, with 7 to 8 million people around the world, becoming symptomatic every year. The number of cases worldwide increased by 13% between the years 1993 and 1996(WHO TB Report, 1998).

1.2 Diagnosis and Treatment

A positive sputum smear for acid-fast bacilli is the most definitive test for the diagnosis of TB, with a tuberculin skin test being useful in confirming previous exposure to the organism(Herfindal, E.T., *et al.*, 1991). Culture results may take up to 6 weeks, so a decision to implement early therapy is usually based on clinical and radiographic findings. Hilar lymphadenopathy alone is sufficient evidence to initiate early treatment in children, even in the absence of a pulmonary infiltrate(Herfindal, E.T., *et al.*, 1991).

It is the use of rifampicin and isoniazid in combination, that has resulted in the usual course of TB treatment being shortened from the 18 to 24 month period. Treatment regimens are divided into intensive and continuation phases. The intensive phase bactericidal effect of treatment leads to bacteriological sputum conversion with a rapid improvement of the illness and a resultant negative sputum culture. During the continuation phase, fewer drugs are used, and the sterilising effect of treatment eliminates the remaining viable bacilli and prevents subsequent relapse(SAMF, 1997). Rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, *p*-aminosalicylate, ethionamide, cycloserine, capreomycin, kanamycin and newer agents such as rifapentine, ciprofloxacin, ofloxacin, rifabutine, clofazimine and gangamicin are all used in the treatment of TB. Which antimicrobial agents are employed at any one time depends on the culture and sensitivity testing to the agents.

Rifampicin(RIF), isoniazid(INH) and pyrazinamide(PZA) were chosen as the drugs of interest in this project, based on them being a prime feature in the WHO's Directly

Observed Treatment Short-course(DOTS) program, as well as being the currently recommended course of treatment listed in the South African Medicines Formulary(SAMF). The treatment of both new adult and paediatric cases employs a combination of all three of these agents in the initial 2 month intensive phase of treatment, followed by a discontinuation of PZA, with RIF and INH dosing continuing for a further 2 to 4 months. These courses of treatment are detailed in tables **1.1, 1.2** and **1.3**. Dosing is done under direct supervision, usually at a clinic, and comprises the DOTS program. Short-course treatment is an important advance in the treatment of TB, with once daily combination dosing with RIF and INH now being an established regimen, effective against pulmonary and extrapulmonary TB(Herfindal, E.T., *et al.*, 1991). Non-compliance with self-medication can, however, result in drug resistance.

Until 50 years ago, there were no drugs to cure TB, with strains that are resistant to a single drug or even a combination of the drugs used today having since emerged. At present there is no cure affordable to developing countries for some multi-drug resistant(MDR) strains. MDR-TB is defined as tubercular strains resistant to the two most important drugs, namely RIF and INH. This is a result of inconsistent or partial treatment due to patient non-compliance, incorrect prescribing of drugs and their combinations by health workers or if the drug supply is unreliable. 2000 new cases of MDR-TB are being recorded in South Africa every year, with between a fifth and a third of the total budget for TB control in some provinces already being committed to this problem(WHO TB Report, 1998).

As will soon become apparent, the focus is placed primarily on the treatment of TB in children, the results of which, lay the groundwork for the more effective treatment of geriatric TB patients. Treatment of TB in children is effectively the same as that for the treatment of adults, with the RIF, INH and PZA regimen having been found to have the same degree of effectiveness(Herfindal, E.T., *et al.*, 1991). Daily dosage with these agents, 5 days a week, is based on mg/kg body weight listed in tables **1.1, 1.2** and **1.3**, with

ethambutol having limited use in the treatment of TB in young children, due to the toxicity problem of retrobulbar neuritis and the difficulty in monitoring ocular toxicity.

Table 1.1 NEW ADULT CASES with primary TB and/or effusion (SAMF, 1997)

Intensive Phase 2 months	under 50 kg	over 50 kg
RIF/INH/PZA combination tablet 120/80/250mg	480/320/1000mg	600/400/1250mg
Ethambutol 400mg	800mg	1200mg
Continuation Phase 2 months		
RIF/INH		
combination tablet 150/100mg	450/300mg	-
combination tablet 300/150mg	-	600/300mg

Table 1.2 NEW CHILDREN with primary TB and/or effusion (SAMF, 1997)

Intensive Phase 2 months	5-10 kg	11-20 kg	21-30 kg
RIF/INH combination tablet 150/100mg	75/50mg	150/100mg	300/200mg
PZA 500mg	250mg	500mg	1000mg
Continuation Phase 2 months			
RIF/INH combination tablet 150/100mg	75/50mg	150/100mg	300/200mg

Table 1.3 NEW CHILDREN with progressive primary or cavitating TB or non-pulmonary TB (SAMF, 1997)

Intensive Phase 2 months	5-10 kg	11-20 kg	21-30 kg
RIF/INH combination tablet 150/100mg	75/50mg	150/100mg	300/200mg
PZA 500mg	250mg	500mg	1000mg
Continuation Phase 4 months			
RIF/INH combination tablet 150/100mg	75/50mg	150/100mg	300/200mg

1.3 Chemotherapeutic Agents

1.3.1 Rifampicin

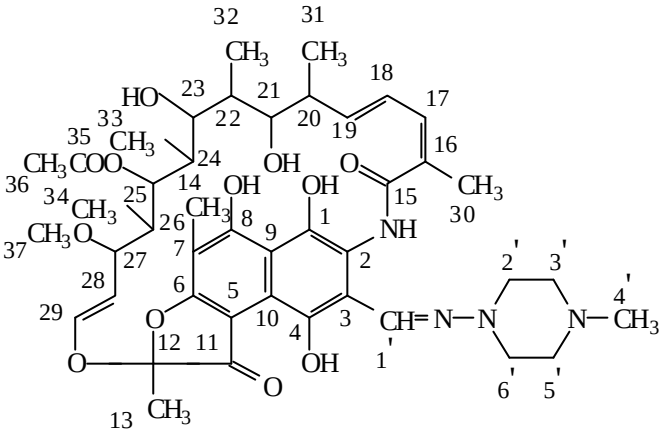
Rifampicin(Figure **1.1**) is a semisynthetic antibacterial agent, effective against a wide range of Gram negative and Gram positive organisms, but is specifically bactericidal against *M. tuberculosis.* It is highly active against rapidly dividing intracellular and extracellular bacilli and is effective against bacilli in caseous lesions. It is more active than INH against slow to intermittently growing organisms in macrophages and bacilli in caseous lesions(Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991; Merck Manual, 1992). RIF is a member of the ansamycin class of natural products, this class being characterised as molecules which possess an aliphatic chain forming a bridge between two non-adjacent positions of an aromatic moiety, the bridge forming the macro-ring of RIF(Figure **1.1**). Rifampicin differs from it's precursor, Rifamycin B, only in that it possesses a substituent in the C-3 position, which is produced by the introduction of a formyl group and then derived as a hydrazone, with a variety of hydrazines(Foye, W.O., *et al.*, 1995). This drug

exerts it's bactericidal action by inhibiting the bacterial DNA-dependent RNA polymerase

(DDRP), ie. preventing chain initiation, effectively inhibiting RNA synthesis. It was through the preparation of numerous derivatives of rifamycins, that the elucidation of the structure activity relationships(SAR) of RIF was possible. Free hydroxyl groups are required at C-1, 8, 21 and 23 for activity, and all must lie in a plane. These are important binding groups for attachment to DDRP(Foye, W.O., *et al.*, 1995). Acetylation of C-21 and C-23 produces inactive compounds and reduction of the double bonds in the macro-ring results in a progressive decrease in activity, while opening of the macro-ring produces inactive compounds. The reduction of the double bonds and opening of the macro-ring results in the effect of producing a conformational change in the drug structure which decreases binding to DDRP(Foye, W.O., *et al.*, 1995). Substitution at C-3 and C-4 produces compounds with varying degrees of antibacterial activity, by affecting transport across the bacterial cell wall. With reference to drug absorption, RIF is readily absorbed from the intestine following oral administration, with good tissue penetration due to the high lipid solubility of the drug, although absorption may be hindered by the concomitant intake of food which lowers and delays peak blood levels. The high lipid solubility of RIF results in extensive tissue penetration, including the lungs, liver, bone and central nervous system(CNS). Reports have indicated that INH can decrease RIF absorption, however, the opposite is not so. Extensive plasma protein binding of 60 to 90% is also a feature of the drug. RIF possesses the property of being a potent hepatic microsomal P450 enzyme inducer, thus potentiating it's own metabolism, as well as that of certain other drugs(Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991). The major route of metabolism(60 to 80% of RIF) is via the entero-hepatic circulation, by deacetylation at the C-25 acetate, producing desacetylrifampicin, an active metabolite. Hydrolysis also produces an active metabolite, although somewhat less active than RIF. RIF is prone to acid hydrolysis, being a zwitterionic molecule, the phenolic groups producing acidic properties (pK_a = 1.7) and the piperazine moiety producing basic properties (pK_a = 7.9).

Air oxidation of the *p*-phenolic groups in the naphthalene ring can occur, to produce the *p*-quinone(Foye, W.O., *et al.*, 1995). Rifampicin and it's metabolites are primarily excreted via the biliary and renal routes, with excretion in other body fluids (eg. tears, saliva, perspiration) producing the characteristic reddish discolouration(Foye, W.O., *et al.*, 1995).

Figure 1.1 Rifampicin structure



1.3.2 Isoniazid (Isonicotinicyl hydrazide)

Isoniazid(Figure **1.2**) is a synthetic antibacterial agent with bactericidal activity against *M. tuberculosis*. The action is bactericidal against replicating extracellular populations of organisms, but appears to be only bacteriostatic against non-replicating organisms(Foye, W.O., *et al.*, 1995). It is effective against intracellular and extracellular bacilli, as well as bacilli in caseous lesions, and is regarded as the single most useful and least expensive of all the antitubercular agents(SAMF, 1997). The exact mode of antitubercular action remains unknown, although several theories have been proposed. It is generally believed that INH exerts it's action by interfering with cell wall development in the organism by impairing the

synthesis of certain long-chain fatty acids that are important in cell wall development of mycobacteria. This has been demonstrated by the bacterium losing it's acid-fastness, after treatment with INH. Absorption of INH from the gastro-intestinal tract (GIT), following oral administration, is rapid and complete, protein binding is very low (10 - 15%) and a wide tissue distribution is achieved. The concomitant intake of food or antacids has been shown to decrease the absorption of the drug from the GIT(Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991). INH, being a simple synthetic molecule, led to the synthesis of an extensive series of derivatives, none of which produced any superior activity, with the activity of the drug appearing to be purely as a result of the parent INH chromophore.

Substitution at the N_3' position, in figure **1.3** results in active compounds (R_1 and R_2 = alkyl ; R_3 = H), whereas substitution of the N_2' hydrogen with alkyl groups destroys activity (R_1 and R_2 = H ; R_3 = alkyl)(Foye, W.O., *et al.*, 1995). The major route of metabolism has been found to be by acetylation and oxidation via hepatic P450 mixed oxidase, which accounts for 70 to 90% of the elimination of INH. Acetylation results in the production of inactive metabolites, the rate of which, is determined genetically, therefore dose adjustment may be necessary, depending on whether the individual is a fast or slow acetylator(Herfindal, E.T., *et al.*, 1991; Merck Manual, 1992). Slow acetylators have a relative lack of N-acetyltransferase and tend to be more prone to toxic side effects related to elevated serum concentrations. Hepatotoxicity is the most frequent side effect, although the incidence is small, appearing to be dose dependent and age related, but is not a problem for individuals under the age of twenty(Herfindal, E.T., *et al.*, 1991). Elimination of INH metabolites is primarily via renal excretion.

Figure 1.2 Isoniazid structure

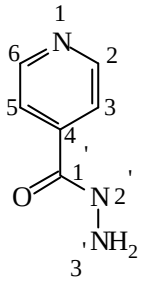
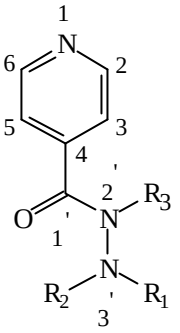


Figure 1.3 Isonicotinicyl hydrazide parent structure

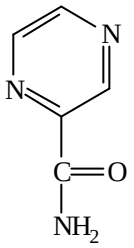


1.3.3 Pyrazinamide (Pyrazinecarboxamide)

Pyrazinamide(Figure **1.4**) is a synthetic bioisoster of nicotinamide, possessing bactericidal activity against intracellular bacilli of *M. tuberculosis*(Foye, W.O., *et al.*, 1995; Merck Manual, 1992). It is rapidly bacteriostatic and slowly bactericidal against the slow growing bacilli within the acidic pH of the macrophages(Foye, W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991). PZA can, therefore, penetrate and kill tubercle bacilli where other antitubercular agents cannot. It is these slow growing organisms that are responsible for the majority of recurrent cases of infection, with resistance to PZA developing rapidly. For these reasons, PZA is used as part of the first line therapy, in combination with RIF and INH(Herfindal, E.T., *et al.*, 1991; SAMF, 1997). The exact mode of action is still unknown, but may act in part as a prodrug. With regard to the SAR's, none of the derivatives of PZA that have been produced, have activity greater than that of the original structure. Substitution(Figure **1.4**) on the pyrazine ring, the use of alternate heterocyclic aromatic rings and the replacement of the amide moiety with similar functional groups, produces no increase in activity, with the exception of a carboxylic acid analogue. PZA is readily absorbed from the intestine, following oral administration, and has a wide tissue distribution, including the liver, lungs and cerebrospinal fluid(CSF). Elimination is primarily via renal and hepatic routes, with excretion(approx. 70% of PZA) being performed mainly by glomerular filtration(Foye,

W.O., *et al.*, 1995; Herfindal, E.T., *et al.*, 1991). The major metabolic route is via hydrolysis by hepatic microsomal pyrazinamide deaminidase to pyrazinoic acid, which then undergoes oxidation by xanthine oxidase to 5-hydroxypyrazinoic acid, the major excretory product. As in the case of INH, the major side effect of PZA is dose-dependent hepatotoxicity.

Figure 1.4 Pyrazinamide structure



1.4 Current Available Formulations

The antitubercular preparations of RIF, INH and PZA, that are currently available, were not initially designed for the treatment of paediatric TB patients, with preparations currently listed in the 1997 SAMF encompassing various dosage forms. RIF is available under several tradenames in either tablet, capsule, injectable or syrup form. Similarly, INH is available as a tablet or an elixir, with PZA only being available in tablet form. Combination products of these three agents are available on the market, with either RIF and INH together, or with PZA added to these, however, all are only available in tablet form. A list of current available formulations as stated in the October 1999 MIMS is given in table **1.4**. From a practical point of view, formulating each antitubercular agent in a separate dosage unit, is impractical when taking into account the administration of these agents.

Table 1.4 Current available formulations(MIMS, 1999; SAMF, 1997)

		Drug Dose (mg)					
Trade Name	**Dosage Form**	**RIF**	**INH**	**PZA**	**ETH**	**RBT**	**ETN**
Ethatyl	TABS						250
Myambutol	SYRUP				25/5ml		
Mycobutin	CAPS					150	
Mynah	TABS		100		300		
Myrin	TABS	150	75		300		
Pyrazide	TABS			500			
Rifadin	SYRUP TABS	100/5ml 600					
Rifater	TABS	120	80	250			
Rifcin	CAPS TABS	150 450/600					
Rifinah	TABS	150/300	100/150				
Rimactane	VIALS	600/vial					
Rozide	TABS			500			
Tuberol	TABS		100		300/365		

RIF(Rifampicin), INH(Isoniazid), PZA(Pyrazinamide), ETH(Ethambutol), RBT(Rifabutin), ETN(Ethionamide)

The treatment of TB involves the use of a combination of several antitubercular agents over a time period of several months. Although this process is simplified by the use of combination dosage forms available, at present, paediatric treatment remains a challenge, involving dosing the child with the rifampicin syrup and the isoniazid elixir, or alternatively, crushing tablets and mixing them with commercially available syrups. Cost and availability

of the agents are major factors affecting the treatment of TB in the underprivileged communities, particularly the paediatric population.

1.5 Motivation

For quite some time there has been a movement toward encouraging pharmaceutical manufacturers to develop additional paediatric dosage forms for already approved therapies, as paediatric formulation presents a major challenge. This is being implemented internationally, given recent initiatives implemented by the Food & Drug Administration (FDA) in the USA, and is now being mirrored by developments in other countries, including South Africa. The problems that have arisen in the past have been a result of the fact that manufacturers are not required to perform clinical trials on paediatric patients or to provide products formulated for children. Paediatric dosing has been left at the discretion of the doctor or pharmacist, with studies indicating that the majority of prescriptions written for children, involve medications only approved for adult use. This leads to inconsistency in dosing and the increased risk of adverse drug reactions among paediatric patients. The implementation of a paediatric program of formulation and testing, specifically aimed at this section of the population, is hoped to improve the safety and efficacy of drugs used in the treatment of illnesses among children. In the USA, growing demands are being placed on pharmaceutical manufacturers by the FDA and the public sector, for dose information on population groups such as the elderly, genders, ethnic groups and children(Wechsler, J., 1998). Hence, this pressure is being transferred to manufacturers elsewhere in the world, if their products are to meet with international criteria.

It is the development of dosage forms that are appropriate for each age group, that can be administered with relative ease, which is the greatest challenge to manufacturers. The ideal paediatric formulation should taste good, not require refrigeration and have a simple dosage

regimen. Shortcomings in any of these areas may lead to non-compliance, and hence failure of treatment. Children up to the age of twelve, tend to have great difficulty in swallowing tablets or capsules, with this being impossible in children under the age of one year. Previous attempts at developing intravenous concentrations for accurate administration to infants have proved to be extremely difficult and inconvenient. A suspension or liquid with an appealing taste, adequate stability and simple dosing, although ideal, is difficult to develop. As a result, there is often a tradeoff in the development of paediatric formulations, between stability, taste and product efficacy/bioavailability (Wechsler, J., 1998). The issues of taste, excipients, stability and toxicology are all issues that need to be addressed. Extemporaneous formulation of paediatric medication has been targeted as a compliance problem, with a lack of uniformity among products produced in this manner, being a major concern. The use of different excipients and manufacturing techniques both alter the quality and the efficacy of the final product. The inability of the manufacturer recommended preparation of RIF suspension for paediatric use to achieve the required 10mg/ml concentration, by incorporating RIF powder from the capsule form into syrup B.P. is an example of this(Krukenberg, C.C., *et al.*, 1986; Nahata, M.C., *et al.*, 1994 (a) & (b)). The development of a formulation for paediatric use is a complex undertaking, with extensive studies into the stability and aesthetic appeal of the product, not to mention paediatric pharmacokinetic studies and the assessment of efficacy and safety being required.

Drug absorption from a lipid medium in the gastro-intestinal tract is a function of the lipid digestion process in the body, in which bile release and pancreatic lipase play an important role. Bile and pancreatic lipase release into the duodenum are responsible for the emulsification, ie. lipid droplet dispersion, and complex triglyceride breakdown into fatty acids and monoglycerides(Charman, W.N., 1997). These smaller units form mixed micelles which aid in the absorption of lipophilic drugs. Emulsion droplet size can therefore be a determining factor in the rate and extent of drug absorption, due to pancreatic lipase only acting at the interface of the oil droplet(Carrigan, P.J., *et al.*, 1973; Craig, D.M.Q., *et al.*,

1995; Tarr, B.D., *et al.*, 1989). The result is a more rapid release of the drug from the triglycerides that carry it, with a more rapid breakdown of smaller lipid droplets. The hydrolysis of oil by pancreatic lipase is an important factor in drug release from the vehicle. This is demonstrated by the fact that a drug may have a solubility in excess of a 1000 times of that in aqueous media, with minimal partitioning, but unless the delivery vehicle can be broken down, absorption will be poor(Tarr, B.D., *et al.*, 1989). Micellar formation in the aqueous environment of the intestinal lumen aids the solubilisation of a drug and hence it's absorption, which is a function of lipid digestion. The more rapid the hydrolysis of the triglycerides, the more rapidly and the greater the quantity of micelles that are formed(Tarr, B.D., *et al.*, 1989). The micellar components are believed to aid the transport of lipid soluble drugs into the microvillar spaces of the intestinal wall, thus increasing the surface area for absorption of these drugs. In the case of microemulsions, due to the smaller droplet size and resultant increased surface area relative to macroemulsions, partitioning of drug from the lipid droplets into the aqueous environment is more significant, as is the lipase activity, with more rapid micellar formation. Microemulsions closely mimic the micelles that are ordinarily formed in the intestinal tract, with respect to composition and droplet size. This is an advantage in that the onset and rate of absorption will be faster, with the small droplet sizes resulting in more extensive absorption of the drug from the intestine having already been demonstrated, following the principles outlined here(Meinzer, A., 1995; Muranishi, S., 1997; Sarciaux, J.M., *et al.*, 1995; Tarr, B.D., *et al.*, 1989).

1.6 Microemulsion Science

The issue of developing a dosage form for the more effective delivery of RIF, INH and PZA in the treatment of paediatric TB patients is addressed in this project by looking at a novel formulation that has not received much attention in the past. Emulsions are generally only employed in pharmacy for the preparation of topical formulations or for intravenous preparations, with no pharmaceutical oral emulsions of note available, with the exception of

Sandimmune Neoral©. Traditionally, emulsions have been avoided due to problems associated with drug and formulation stability, not to mention aesthetic appeal. Microemulsions are gaining in popularity, ever since the release of Sandimmune Neoral©, a cyclosporine preparation, the first ever oral microemulsion preconcentrate approved for pharmaceutical application.

Emulsions are broadly defined as heterogeneous systems of dispersed macroscopic droplets of one liquid in another, having a droplet diameter which usually exceeds 100nm, when two immiscible liquids are mixed by mechanical agitation(Lieberman, H.A., *et al.*, 1988). They are characterised by little to no thermodynamic stability, having a turbid appearance, and unless a stabilising agent is present in the system, the droplets rapidly coalesce and separate to form definable layers of the two immiscible liquids(Ansel, H.C., 1981).

Microemulsions on the other hand, are defined as isotropic, transparent, thermodynamically stable microstructured mixtures consisting of oil and water systems stabilised by a surfactant, usually in conjunction with a cosurfactant, which may be a short chain alcohol, amine or other weakly amphiphilic molecule(Lawrence, M.J., 1994; Meinzer, A., *et al.*, 1995; Aboofazeli, R., *et al.*, 1991, 1993, 1994 (a) & (b), 1995; Malcolmson, C., *et al.*, 1993, 1995; Rushforth, D.S., *et al.*, 1986; Sarciaux, J.M., *et al.*, 1995 (a) & (b)). The resultant microemulsion generally has a dispersed phase of droplet size between 1 and 100nm, which forms spontaneously on preparation(Fubini, B., *et al.*, 1988; Lieberman, H.A., *et al.*, 1988; Lawrence, M.J., 1994; Meinzer, A., *et al.*, 1995; Sarciaux, J.M., *et al.*, 1995 (a)). The advantages of using microemulsions over common macroemulsions in pharmaceutical formulation, are defined as being the ease of preparation, clarity, stability, filtration sterilisation, improved bioavailability and delivery of lipophilic drugs, low viscosity and small droplet size(Lawrence, M.J., 1994; Aboofazeli, R., *et al.*, 1993, 1994 (a) & (b), 1995; Malcolmson, C., *et al.*, 1993, 1995; Mallon C., *et al.*, 1991).

The motivation for the development of a microemulsion dosage form, from the pharmacokinetic point of view, is(Meinzer, A., *et al.*, 1995):

- Fast release of drug from the formulation to utilise the entire absorption window.
- The development of a formulation which closely mimics the mixed micellar phase found during digestion, keeping the active drug in an absorbable form along the absorption site.
- A new formulation providing a stable micellar system which is minimally affected by different physiological states (bile flow, pH, food, etc.)

From all indications, Sandimmune Neoral© lives up to the specifications of a microemulsion. The improved solubilisation of cyclosporine and increased bioavailability of the drug, that has been produced, are proof that this method of drug delivery does hold advantages, and is reason enough why the biological microemulsion concept can be applied to the oral delivery of other lipophilic drugs(Friman, S., *et al.*, 1996; Kovarik, J.M., *et al.*, 1994; Meinzer, A., 1995; Sarciaux, J.M., 1995 (b)).

1.7 Aim

The aim of this project is therefore the development of a microemulsion dosage form for the oral delivery of RIF, INH and PZA in combination. The preliminary assessment of the stability of such a formulation, and of the drugs concerned, in the formulation and it's components is also addressed.

Chapter Two

Rifampicin, Isoniazid and Pyrazinamide Characterisation

2.1 Introduction

The characterisation of rifampicin, isoniazid and pyrazinamide is necessary to confirm the structure and purity of the compounds before the performance of any further stability or formulation studies(Carstensen, 1995). Spectroscopic techniques employed for the characterisation of these three drugs include infrared(IR), nuclear magnetic resonance(NMR), mass(MS) and ultraviolet-visible(UV) spectroscopy, all of which are extensively referenced for rifampicin, isoniazid and pyrazinamide(Furniss, B.S., 1978). The use of differential scanning calorimetry(DSC), melting range determination, chromatography and solubility determinations, have been made use of in this chapter to compliment the spectroscopic characterisation findings and to confirm the purity of the compounds.

2.2 Instrumentation and Equipment

2.2.1 Thermal Analysis

Differential Scanning Calorimetry was performed using a DSC 7 Calorimeter made by Perkin Elmer(UK). Visual melting point determination was performed using a 50Hz 220/240V Gallenkamp(UK) melting point apparatus following the open end glass capillary tube method.

2.2.2 Chromatography

Normal phase TLC F_{254} pre-coated aluminium plates were purchased from Merck, (Darmstadt, Germany). Glass chromatographic developing vessels with the dimensions of 100mmX50mmX30mm were used to run all TLC plates, which were visualised using a dual wavelength (254/356nm) UV light source, made by Raytech(USA).

2.2.3 Spectroscopy

A Spectrum 2000 FT-IR Spectrophotometer made by Perkin Elmer(UK) was used to perform all IR experiments. A GCQ-MS low resolution spectrometer using the electron ionisation mode, made by Finnigan(USA) was used for mass spectrometry of the drugs being characterised. NMR spectroscopy was performed using a 400MHz AMX NMR Spectrometer made by Bruker(Germany). A GBC UV/VIS 916 Double Beam Spectrophotometer made by GBC(Melbourne, Australia) was employed in UV-VIS spectral analysis, using GBC Spectral version 1.5 software.

2.2.4 Solubility

Model MH-4 magnetic stirrers manufactured by Fried Electric(Israel), were used in all solubility determinations. Concentrations were determined by high performance liquid chromatography, using the method and system developed and validated in chapters 2 and 3.

2.3 Solvents and Reagents

Rifampicin(RIF), isoniazid(INH) and pyrazinamide(PZA) were donated by the Druggists Group Reasearch Unit(Pharmacare-Lennon, RSA), as was sorbitol(70%m/v), glycerol,

propylene glycol and polyethylene glycol 400.

Methanol, acetonitrile, hexane, ethyl acetate and chloroform were purchased from BDH®, England(purity >99.9%). Dimethylsulfoxide-d_6 (DMSO-d_6) and chloroform-d_1 ($CDCl_3$-d_1) used to prepare samples for nuclear magnetic resonance spectroscopy, were purchased from Merck(Darmstadt, Germany). All aqueous fractions were prepared using double distilled deionised water, obtained from a Milli-RO 15 water purification system, manufactured by Millipore®(Massachusetts, USA). Miglyol 812 was supplied by Hüls, Southern Africa and corn, cottonseed, sunflower and soybean oil were all purchased from Sigma(St. Louis, USA).

2.4 Theory and Methodology

2.4.1 Thermal Analysis

Thermal analysis is broadly defined as the measurement of the physico-chemical properties of substances as a function of temperature(USP, 1995). The identity and purity of drugs can often be indicated by precisely determined thermodynamic events, such as a change of state. Melting and boiling temperatures of substances are transitions that are characteristic, hence the compendial standards that have long been established, contribute to the identification of the substances(USP, 1995). Transition temperature is most often used in thermal analysis, and is used in this chapter.

2.4.1.1 Differential Scanning Calorimetry (DSC)

As a sample is heated, its uptake or production of heat can be measured by DSC. Cooling the melted substance to room temperature and reheating, can often reveal polymorphism, a common phenomenon that can complicate formulation and bioavailability of some drug

substances. For RIF, INH and PZA, DSC was performed by heating 2mg samples of the individual drug from 50^0C, at a rate of 10^0C per minute until a maximum temperature of 250^0C was reached. The sample was cooled to room temperature and reheated to 250^0C. This procedure was performed in triplicate for each drug. DSC is indicative of the purity of a substance.

2.4.1.2 Visual Melting Range Determination

The melting range was determined for RIF, INH and PZA using the Gallenkamp apparatus specified, which adheres to the apparatus criteria stated in the USP(USP, 1995). The procedure for class **1a** substances in the USP was followed for the determination of the melting range for each substance. This involves size reducing the compound to a fine powder and then charging a glass capillary tube, sealed at one end, with the dry powder to a height of 2.5 to 3.5mm. The compound is then slowly heated and the temperature recorded at the point at which melting starts until the solid coalesces and is completely melted(BP, 1993; USP, 1995). Melting ranges were determined in triplicate.

2.4.2 Chromatography

The types of chromatography employed in compendial assays and tests are numerous, but the only two of interest in this work are high performance liquid chromatography(HPLC) and thin layer chromatography(TLC). HPLC is used for all qualitative and quantitative analysis detailed in chapters 3 to 7, with TLC being of interest for the identification and purity assessment of RIF, INH and PZA in this chapter.

2.4.2.1 Thin Layer Chromatography(TLC)

TLC is a simple and effective technique that is often employed for the assessment of drug

purity and for the development of efficient mobile phase combinations. Simple identification of a compound is possible by observing spots of identical R_f value and equal magnitude, when comparing a sample to a reference standard on the same plate(BP, 1993; USP, 1995). The R_f value is the ratio of the distance travelled on the medium, by a given compound, relative to the solvent front of the mobile phase.

The mobile phase combinations used for each drug differed, following compendial monographs. 30mg/ml solutions of RIF, INH and PZA was prepared using methanol as solvent, spotted onto the TLC plates and allowed to dry. For RIF an ethyl acetate:hexane mobile phase of 25:75(v:v) composition was employed, an ethyl acetate:acetone:methanol:hexane, in the proportions of 50:20:20:10(v:v:v:v) was employed for INH and a chloroform:methanol:conc. ammonia mobile phase in the ratio of 20:20:1(v:v:v) for PZA.

2.4.3 Spectroscopy

Ultraviolet, visible and infrared spectroscopy are generally classified as absorption spectrophotometry as they measure the interaction between electromagnetic radiation and the molecules or atoms of a chemical compound(USP, 1995).

2.4.3.1 Infrared Spectroscopy(IR)

Infrared spectra for RIF, INH and PZA were obtained using the stated apparatus, and by following the mull triturate method of sample preparation(BP, 1993). 10mg of drug was triturated with one drop of liquid paraffin to give a smooth paste, which was then compressed between two sodium chloride discs. The infrared spectrum was then recorded over a range of 600 to 4000.cm^{-1} in accordance with literature standards(Furniss, B.S., 1978; McMurry, 1992; USP, 1995) .

2.4.3.2 Mass Spectrometry(MS)

A mass spectrum is a representation of the relative abundance of each ionic species of a compound, sorted in order of their mass-to-charge ratio (m/e). Mass spectrometry makes the determination of the molecular mass of a compound possible, by generating the molecular ion(M^+), which results when one electron is removed from the molecule. Furthermore, fragment ions are generated from the molecular ion, by various bond cleavages, thus producing a fragmentation pattern for the compound(Furniss, B.S., 1978; USP, 1995). The fragmentation pattern is of use in the determination of possible degradation pathways of the compound in question. Mass spectra were generated for RIF, INH and PZA.

2.4.3.3 Nuclear Magnetic Resonance Spectrometry(NMR)

NMR is the most useful analytical procedure available to date, due to it's specificity, with every drug substance possessing a characteristic NMR spectrum that is unique(USP, 1995). Proton (^1H) nuclei are most frequently used in quantitative NMR analysis, with integration of the peaks giving an indication of the ratios of CH, CH_2 and CH_3 groups(Furniss, B.S., 1978; USP, 1995). Carbon (^{13}C) NMR is also of use in determining the number of carbon atoms in a compound. ^1H and ^{13}C NMR was performed for RIF, INH and PZA. Samples of INH and PZA were prepared by dissolving 15mg of drug in 0.8ml of DMSO-d_6, with the RIF sample being prepared by dissolving 10mg of drug in 0.8ml of $CDCl_3$-d_1.

2.4.3.4 Ultraviolet Spectroscopy(UV)

UV spectroscopy, serves it's purpose for quantitative assays, and remains useful as an additional method of identification for many substances, as is evident by the standards listed

in the compendia(BP, 1993; USP, 1995). 100 : g/ml solutions of RIF, INH and PZA, respectively, were prepared in a methanol:water solvent mixture of 20:80(v:v). The spectra for each drug were recorded over a wavelength range of 200 to 400nm.

2.4.4 Solubility

The solubility of RIF, INH and PZA in various solvents is listed in the compendia, however, these references do not cover all relevant solvents. For this reason, a solubility table for RIF, INH and PZA was constructed, with solubility determined in triplicate for each solvent following established procedures(Lund, W., 1994). An excess of each drug was added to 100ml of each solvent in a 150ml quickfit conical flask, which was purged with nitrogen, sealed and stirred for 24 hours at room temperature, protected from light. Samples were then drawn, appropriately diluted and determined by the HPLC method detailed in chapter 3. Samples of drugs in the oils listed in the subsequent tables in this chapter, underwent the extraction procedure detailed and validated in chapter 4, prior to HPLC analysis.

2.5 Results and Discussion

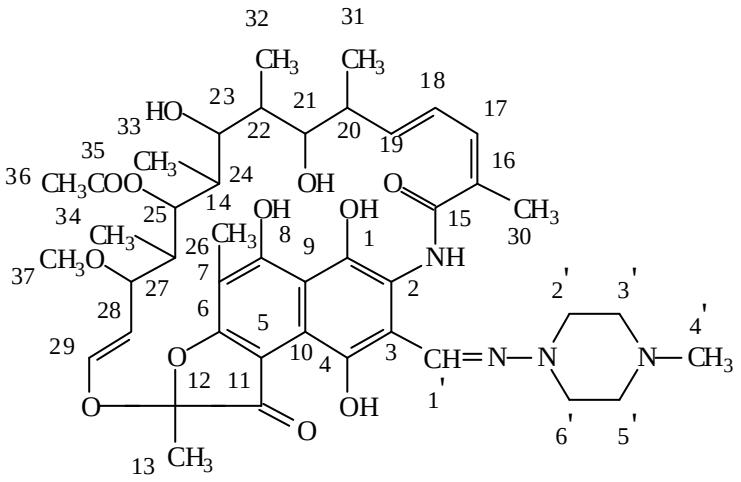
2.5.1 Rifampicin (RIF)

By IUPAC rules, Rifampicin, the nonproprietary name of rifampin, is designated as 2,7-(Epoxypentadeca[1,11,13]trienimino)naphthol[2,1-b]furan-1,11(2-h)-dione,5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formimidoyl]-21-acetate. According to the original nomenclature of rifamycins, however, rifampin is preferentially known as 3-[{(4-methyl-1-piperazinyl)imino}methyl]rifamycin SV(Gallo, G.G. *et al.*, 1974).

Commercially, RIF is available alone and in combination with other drugs, as Rifater®, Rifcin®, Rifadin® and Rimactane® in South Africa(SAMF, 1997; MIMS, 1999). It is a red-orange odourless, crystalline powder with $C_{43}H_{58}N_4O_{12}$ as the empirical formula and a molecular mass of 822.96g.mol^{-1}(BP, 1993; Gallo, G.G. *et al.*, 1974; Merck Index, 1983; USP, 1995). Packaging and storage conditions indicate preservation in air tight, light resistant containers, protected from excessive heat(BP, 1993; USP, 1995). RIF is reported to be freely soluble in DMSO and $CDCl_3$ (Merck Index, 1983).

RIF is a semisynthetic antibiotic, with 9 asymmetric carbons and 3 double bonds, and is believed to exist as only one isomer, as all the isomerogenic centres belong to the natural parent part of the molecule(Gallo, G.G., 1974). The more widely accepted USAN numbering is given in figure **2.1**.

Figure 2.1 Rifampicin (USAN Numbering)



2.5.1.1 Differential Scanning Calorimetry(DSC)

The DSC curve and the integrated results produced, indicate a melting endotherm starting at 180.70^{0}C, peaking at 194.53^{0}C and terminating as an exotherm at 203.12^{0}C. The melting peak corresponds closely to that stated in the monograph of 193^{0}C, with the exotherm corresponding to the recrystallisation of the melt and the subsequent exothermic decomposition of the drug structure at 240^{0}C also being in agreement with the literature(Gallo, G.G., 1974).

2.5.1.2 Visual Melting Range Determination

The melting range of RIF was found to be 184-190^{0}C, corresponding to the literature values of 183-188^{0}C(Gallo, G.G., 1974; Merck Index, 1983).

2.5.1.3 Thin Layer Chromatography

Visualisation under ultraviolet light(254nm) indicated a single orange spot compound with an R_f value of 0.68. Adjustment of mobile phase composition did not result in any spots, representing impurities, being made evident.

2.5.1.4 Infrared Spectroscopy

The IR results for RIF, tabulated in table **2.1** are consistent with literature findings(BP, 1993; Gallo, G.G., 1974; USP, 1995).

Table 2.1 Infrared absorption bands for rifampicin (Gallo, G.G., 1974)

Infrared Absorption Band (cm^{-1})		
Interpretation	**Literature**	**Experimental**
-OH	3300-3500	3449-3583
$-CH_3$	2930	2925
$-CH_3$-N	2800	2781
-NH, -NH bonded and -OH bonded	2300-3200	2500-3300
C=O (acetyl stretching)	1735	1734
C=O (furanone stretching)	1715	1712
C=N (amide 1)	1610 and 1670	1620 and1676
C=C	1570	1567
C=N (amide 2)	1520 and 1540	1332-1461
C-O-C (acetyl)	1020, 1050 and 1255	1021, 1048 and 1290

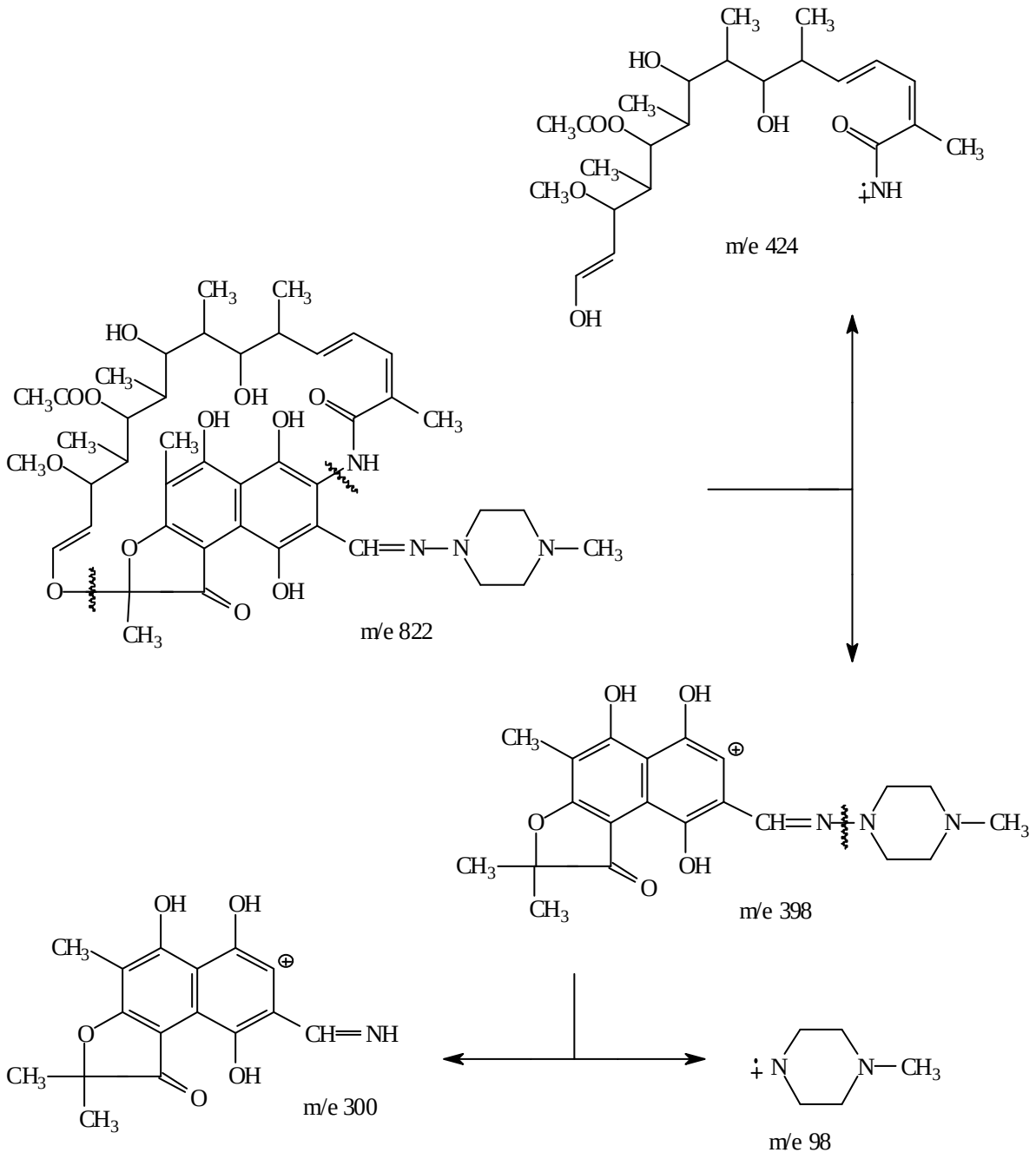
The region from 2000 to 2970cm^{-1} falls within the non-transparent region of the paraffin oil, hence, no peaks are predominant here(Gallo, G.G., 1974).

2.5.1.5 Mass Spectrometry

The mass spectrum obtained for RIF shows a molecular ion at m/e 822. Gallo *et al.* found that direct electron impact at elevated temperature did not produce the molecular ion, due to the compound decomposing in the ion source(Gallo, G.G., 1974). The spectrum yields the RIF chromophoric ion at m/e 398 and the base peak at m/e 98, which corresponds to the fragmentation pattern found in the literature(Gallo, G.G., 1974). The proposed fragmentation pattern for RIF, is displayed in figure **2.2**. Peaks at m/e 98, 300, 398 and

424 all correspond to fragments in the mass spectrum for RIF, as does the molecular ion at m/e 822, with corresponding relative abundance of 100, 30.24, 79.83, 2.43 and 3.21% respectively.

Figure 2.2 Proposed fragmentation pattern for rifampicin



2.5.1.6 Nuclear Magnetic Resonance Spectrometry

Both proton(^{1}H) and carbon(^{13}C) NMR was performed for RIF , as was a 2 dimensional NMR COSY experiment, with the results for the ^{1}H and ^{13}C NMR presented in tables **2.2** and **2.3**. The 2D COSY aids in the assignment of protons to specific carbons, which is particularly difficult with structures as large as RIF. As previously mentioned, USAN numbering is employed in the assignment of the RIF ^{1}H and ^{13}C spectra.

Table 2.2 Rifampicin ^{1}H spectral assignment (Gallo, G.G., 1974)

^{1}H Spectral Assignment			
		Chemical Shifts (ppm)	
Proton	**Multiplicity**	**Literature**	**Experimental**
NH	**s**	11.96	11.95
CH=N	**s**	8.22	8.23
CH_2 2' & 6'	**m**	2.9-3.3	2.9-3.3
CH_2 3' & 5'	**m**	2.4-2.8	2.5-2.8
N-CH_3	**s**	2.34	2.35
OH 1	**bs**	11.4-14.0	-
OH 8	**bs**	11.4-14.0	-
OH 4	**s**	13.16	13.17
CH_3 13	**s**	1.82	1.80
CH_3 14	**s**	2.22	2.22
H 17	**m**	6.3-6.8	6.5-6.7
H 18	**m**	6.3-6.8	6.5-6.7
H 19	**dd**	5.92	5.90

^{1}H Spectral Assignment

		Chemical Shifts (ppm)	
Proton	**Multiplicity**	**Literature**	**Experimental**
H 20	**ddq**	2.26	2.22
H 21	**dd**	3.78	3.77
OH 21	**bs**	3.2-4.2	3.2-4.2
OH 23	**bs**	3.2-4.2	3.2-4.2
H 22	**ddq**	1.70	1.70
H 23	**dd**	3.04	3.05
H 24	**ddq**	1.52	1.52
H 25	**dd**	4.96	4.95
H 26	**ddq**	1.22	1.22
H 27	**dd**	3.58	3.75
H 28	**dd**	5.00	5.05
H 29	**d**	6.20	6.20
CH_3 30	**s**	2.10	2.10
CH_3 31	**d**	0.88	0.88
CH_3 32	**d**	1.01	1.00
CH_3 33	**d**	0.58	0.58
CH_3 34	**d**	-0.33	-0.33
CH_3 36	**s**	2.06	2.05
CH_3 37	**s**	3.05	3.05

s = singlet **m** = multiplet **d** = doublet

dd = doublet of doublets **ddq** = doublet of doublets of quartets

bs = broad signal

Table 2.3 Rifampicin ^{13}C spectral assignment (Gallo, G.G., 1974)

^{13}C Spectral Assignment					
	Chemical Shift			Chemical Shift	
Carbon	Literature	Experimental	Carbon	Literature	Experimental
1	138.6	138.6	**23**	76.7	76.8
2	105.9	105.8	**24**	37.6	37.5
3	110.8	111.0	**25**	74.4	74.5
4	147.8	147.2	**26**	39.5	39.5
5	112.8	112.8	**27**	76.7	76.7
6	174.3	174.2	**28**	118.7	118.8
7	120.3	120.3	**29**	142.6	142.5
8	169.3	169.3	**30**	20.7	20.7
9	104.4	104.5	**31**	17.8	17.8
10	117.8	117.8	**32**	10.9	11.0
11	195.3	195.0	**33**	8.5	8.5
12	108.7	108.7	**34**	8.8	8.8
13	21.5	21.5	**35**	171.9	172.0
14	7.6	7.5	**36**	20.7	20.8
15	169.6	169.6	**37**	57.0	57.0
16	129.4	129.5	**38**	134.4	135.5
17	135.0	135.0	**39**	50.2	50.2
18	123.2	123.2	**40**	53.9	54.0
19	142.6	142.5	**41**	53.9	54.0
20	38.6	38.7	**42**	50.2	50.0
21	70.7	70.7	**43**	45.8	45.8
22	33.4	33.4			

The assigned proton spectrum corresponds to the literature values, confirming the

identity of RIF(Gallo, G.G., 1974). From table **2.3**, the number of carbon atoms can be seen to correspond with the literature values, as well as the empirical formula of RIF(Gallo, G.G., 1974). NMR is a highly specific analytical technique, with the RIF structure being effectively confirmed by MS, ^{1}H, ^{13}C and COSY NMR.

2.5.1.7 Ultraviolet Spectroscopy

The UV spectrum obtained for RIF, produced three characteristic maxima, at 235.3, 254.1 and 333.4nm, corresponding to the literature values of 237, 255 and 334nm(Gallo, G.G., 1974; Merck Index, 1983). The experimental molar absorptivity(,) values of 31.55, 31.08 and 27.90 correspond to the literature values of 33.20, 32.10 and 27.00(Gallo, G.G., 1974; Merck Index, 1983).

2.5.1.8 Solubility

The solubility of RIF in selected solvents is tabulated in table **2.4** together with the literature values(Gallo, G.G., 1974; Merck Index, 1983).

Table 2.4 Rifampicin solubility table (Gallo, G.G., 1974; Merck Index, 1983)

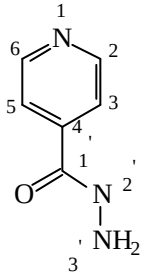
Rifampicin Solubility (mg/ml)		
Solvent	**Literature**	**Experimental**
Water 25^{0}C	2.50	1.50
Methanol	16.00	16.30
Acetone	14.00	15.00
Chloroform	349.00	360.00
Ethyl Acetate	108.00	105.00
Rifampicin Solubility (mg/ml)		

Solvent	Literature	Experimental
Glycerol	practically insoluble	<0.078
Polyethylene Glycol 400	-	19.73
Sorbitol (70%m/v)	-	<0.078
Corn oil	-	2.98
Cottonseed oil	-	1.70
Miglyol 812	-	10.76
Soybean oil	-	2.26
Sunflower oil	-	2.32

2.5.2 Isoniazid (INH)

Isoniazid is the most commonly recognised name of 4-pyridinecarboxylic acid hydrazide, but is also known as isonicotinic acid hydrazide or isonicotinylhydrazide. It is a colourless or white crystalline powder, which is odourless and has a sweet to bitter taste(Brewer, G.A., 1977; BP, 1993; USP, 1995). The empirical formula is $C_6H_7N_3O$, with a molecular mass of 137.15g.mol^{-1}(Brewer, G.A., 1977; Merck Index, 1983; USP, 1995). It is commercially available in South Africa either alone or in combination with other drugs, as Isoniazid®, Norstan Isoniazid® or Rifater®. Packaging and storage requires air tight, light-resistant containers(BP, 1993; USP, 1995).

Figure 2.3 Isoniazid numbering



The numbering system employed in

figure **2.3**, will be used in all subsequent INH spectral assignments.

2.5.2.1 Differential Scanning Calorimetry

DSC performed for INH showed an onset of the endotherm at 171.62^{0}C, peaking at 175.20^{0}C and ending at 179.85^{0}C. This sharp peak confirms the purity of the drug substance, with no noticable impurities present, corresponding to the literature value of 170^{0}C(Brewer, G.A., 1977).

2.5.2.2 Visual Melting Range Determination

The melting range for INH found to be 171-174^{0}C, corresponds to literature values of 170-174^{0}C, as well as the onset and peak of the DSC endotherm(Brewer, G.A., 1977; Merck Index, 1983; USP, 1995).

2.5.2.3 Thin Layer Chromatography

The mobile phase combination employed, produced an R_f value of 0.70 for INH with no other impurities evident after visualisation under ultraviolet light(254nm). Variation of mobile phase composition did not resolve any spots, representing impurities.

2.5.2.4 Infrared Spectroscopy

The results of the IR spectrum are represented in table **2.5**.

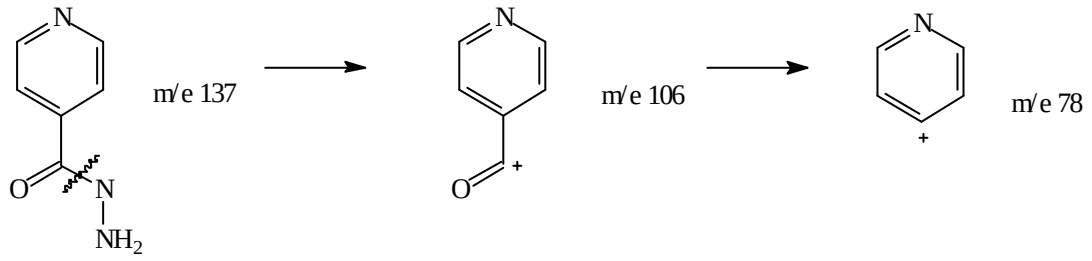
Table 2.5 Infrared absorption bands for isoniazid (Brewer, G.A., 1977)

Infrared Absorption Band (cm^{-1})		
Interpretation	**Literature**	**Experimental**
-NH bonded & -NH	3000-3300	3000-3300
aromatic C-H	-	3174
-C=O	1670	1667
NH_2 deformation	1640	1661
ring C=C and C=N	1610	1635
aromatic ring	-	1465, 1500 &1560

The functional groups identified from the spectrum correspond to the stated literature values, confirming the identity of INH(Brewer, G.A., 1977).

2.5.2.5 Mass Spectrometry

Figure 2.4 Proposed fragmentation pattern for isoniazid



In the mass spectrum of INH, the molecular ion at m/e 137 and relative abundance of 16.85%, corresponds to the molecular mass of INH. Cleavage of the side chain in two

steps yielding the ions at m/e 78 and 106 with corresponding relative abundance of 41.62 and 100%, is represented in figure **2.4**. Further cleavage of the pyridine ring may occur to yield a m/e 51 fragment with relative abundance of 36.18%. These fragmentations correspond to those reported in the literature(Brewer, G.A., 1977).

2.5.2.6 Nuclear Magnetic Resonance Spectrometry

The sample of INH for ^{1}H and ^{13}C NMR was prepared as described using DMSO-d_6 as solvent. The spectra were assigned, the results of which are tabulated in tables **2.6** and **2.7**.

Table 2.6 Isoniazid ^{1}H spectral assignment (Brewer, G.A., 1977)

^{1}H Spectral Assignment			
		Chemical Shifts (ppm)	
Proton	**Multiplicity**	**Literature**	**Experimental**
CO-NH	**s**	10.10	10.15
Hydrazine H	**s**	4.60	4.60
Aromatic H	**dd**	7.73, 8.70	7.73, 8.70

s = singlet **dd** = doublet of doublets

Both ^{1}H and ^{13}C NMR spectra identified the carbon and hydrogen atoms stated in the empirical formula of INH and adhere to the findings in the literature(Brewer, G.A., 1977). Together with the MS and IR results, the complete structural assignment of INH was possible.

Table 2.7 Isoniazid ^{13}C spectral assignment (Brewer, G.A., 1977)

^{13}C Spectral Assignment		
	Chemical Shift	
Carbon	**Literature**	**Experimental**
Hydrazine	164.4	164.0
Heterocyclic	121.6, 140.0, 150.0	121.5, 140.0, 150.2

2.5.2.7 Ultraviolet Spectroscopy

The UV spectrum of INH produced a single absorption maximum at 262.6nm with a molar absorptivity of 4215. These values agree with the literature values of a maximum at 265nm with a molar absorptivity of 4300(Brewer, G.A., 1977; Merck Index, 1983).

2.5.2.8 Solubility

The solubility table **2.8** was constructed for INH. As is evident, the solubility is insignificant in the oils listed, as opposed to the solubility of RIF in the same oils. INH is very hydrophilic, having a low affinity for the oils, which may be beneficial when looking at formulation of INH with RIF in chapters 6 and 7.

Table 2.8 Isoniazid solubility table (Brewer, G.A., 1977; Merck Index, 1983)

Isoniazid Solubility (mg/ml)		
Solvent	**Literature**	**Experimental**
Water 25^{0}C	140.00	130.50
Methanol	-	-
Acetone	-	-
Chloroform	1.00	0.85
Isoniazid Solubility (mg/ml)		

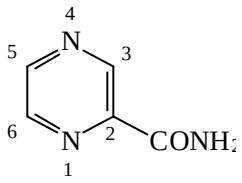
Solvent	Literature	Experimental
Ethyl Acetate	-	-
Glycerol	-	24.92
Propylene Glycol	-	32.90
Polyethylene Glycol 400	-	23.74
Sorbitol (70%m/v)	-	37.68
Corn oil	-	0.24
Cottonseed oil	-	0.23
Miglyol 812	-	0.32
Soybean oil	-	0.21
Sunflower oil	-	0.21

2.5.3 Pyrazinamide (PZA)

Pyrazinamide is the international nonproprietary name for pyrazinecarboxamide or pyrazine-2-carboxamide. It is a white crystalline powder, odourless, with a slightly bitter taste(BP, 1993; Felder, E., 1983; USP, 1995). It has a molecular mass of 123.11g.mol^{-1} and empirical formula of $C_5H_5N_3O$, and is commercially available in South Africa either alone or in combination with other drugs, as Isopas®, Pyrazide®, Rozide® or Rifater®(BP, 1993; Felder, E., 1983; SAMF, 1997; USP, 1995).

The structure of PZA and the numbering employed in figure **2.5** is used in all references and assignment of spectra that follow.

Figure 2.5 Pyrazinamide numbering



2.5.3.1 Differential Scanning Calorimetry

DSC performed on PZA provided an onset of the melting endotherm at 189.1^{0}C, peaking at 191.7^{0}C and ending at 192.9^{0}C. The peak is sharp, indicating the purity of the compound being used. No literature values for DSC of PZA were available for comparison. The onset of the melting endotherm does relate to the visual melting range of PZA, determined.

2.5.3.2 Visual Melting Range

The visual melting range determined for PZA was 189-192^{0}C, which is in agreement with the onset and end of the melting endotherm, found by DSC, as well as matching the melting range of 188-191^{0}C stated in the literature(Felder, E., 1983; Merck Index, 1983).

2.5.3.3 Thin Layer Chromatography

TLC of PZA yielded a single spot on visualisation under UV light(254nm). The compound has an R_f value of 0.50, with the single spot serving as an indication of the absence of impurities.

2.5.3.4 Infrared Spectroscopy

The IR spectral data is displayed in table **2.9**.

Table 2.9 Infrared absorption bands for pyrazinamide (Felder, E., 1983)

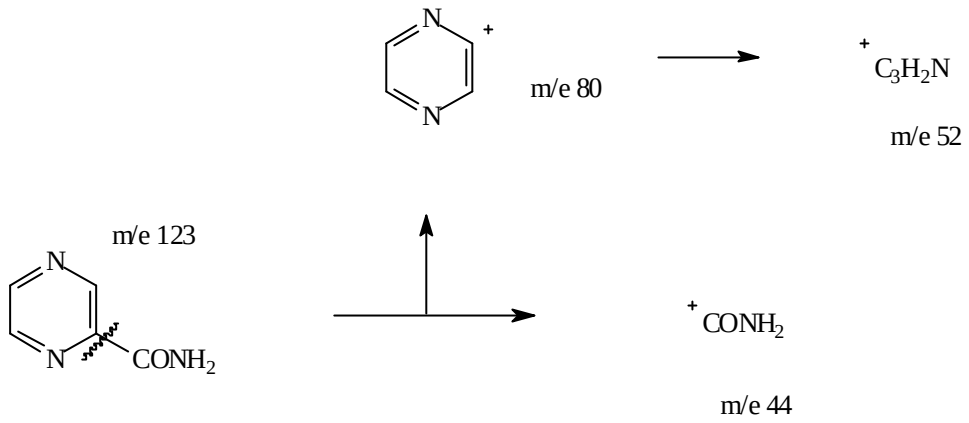
Infrared Absorption Band (cm^{-1})		
Interpretation	**Literature**	**Experimental**
-NH & -NH bonded	**3160, 3290, 3425**	**3161, 3290, 3414**
NH & CN (amide 2)	**1614**	**1611**
C = O (amide 1)	**1716**	**1711**
C = N	**1528, 1585**	**1539, 1581**
aromatic ring	**1382**	**1378**
NH_2 & CH out of plane	**782-1183**	**787-1183**

The bond stretching and absorption bands interpreted are in agreement with the literature values, confirming the identity of PZA(Felder, E., 1983).

2.5.3.5 Mass Spectrometry

The molecular ion at m/e 123 and relative abundance of 19.84% corresponds to the literature value and molecular mass of PZA, with the base peak at m/e 80 corresponding to the chromophoric ion(Felder, E., 1983). Cleavage of the side chain from the chromophore yields the m/e 44 and 80 fragments with the corresponding relative abundance of 4.80 and 100%, respectively. The chromophore cleaves again to yield a C_3H_2N fragment at m/e 52 with relative abundance of 33.13%. The proposed fragmentation pattern is represented in figure **2.6**.

Figure 2.6 Proposed fragmentation pattern of pyrazinamide



2.5.3.6 Nuclear Magnetic Resonance Spectrometry

The ^{1}H and ^{13}C NMR are represented in tables **2.10** and **2.11**.

Table 2.10 Pyrazinamide ^{1}H spectral assignment (Felder, E., 1983)

^{1}H Spectral Assignment			
		Chemical Shifts (ppm)	
Proton	**Multiplicity**	**Literature**	**Experimental**
$CONH_2$	**s**	7.88, 8.25	7.85, 8.25
H5	**q**	8.71	8.70
H6	**d**	8.85	8.85
H3	**d**	9.21	9.19

s = singlet **q** = quartet **d** = doublet

Carbons C3, C4 and C5 are assigned on the basis of selective decoupling. The ^{1}H and ^{13}C NMR spectra assigned for PZA correspond to the stated literature findings, which together with IR and MS enables the precise assignment of the PZA structure(Felder, E., 1983).

Table 2.11 Pyrazinamide ^{13}C spectral assignment (Felder, E., 1983)

^{13}C Spectral Assignment		
	Chemical Shifts (ppm)	
Carbon	**Literature**	**Experimental**
2	145	145
3	143.6	143.5
5	143.2	143.2
6	147.3	147.5
C = O	165	165

2.5.3.7 Ultraviolet Spectroscopy

The UV spectrum obtained for PZA gave absorption maxima, observed at 208, 267.7 and 310.4nm. These results correspond to the literature values for UV analysis of PZA in aqueous medium, the maxima being 209, 269 and 310nm respectively(Felder, E., 1983; Merck Index, 1983). The molar absorptivities of the three peaks, were 8900, 8100 and 600, respectively, corresponding to the literature values of 8765, 8036 and 611(Felder, E., 1983).

2.5.3.8 Solubility

A solubility profile for PZA was constructed and listed in table **2.12**, together with the literature values(Felder, E., 1983; Merck Index, 1983). The solubility of PZA in the oils investigated is less than that of RIF, but only slightly more than INH. The similarity in solubility is due to the similar chemical structure of PZA and INH. A noticable difference is seen for the aqueous solubility of PZA, being significantly more soluble in water and the glycols tested than RIF, but not as soluble as INH.

Table 2.12 Pyrazinamide solubility table (Felder, E., 1983; Merck Index, 1983)

Pyrazinamide Solubility (mg/ml)		
Solvent	**Literature**	**Experimental**
Water 25^{0}C	26.5	25.44
Methanol	16.3	20.42
Acetone	-	-
Chloroform	2.8	5.30
Ethyl Acetate	7.0	10.03
Glycerol	-	15.71
Propylene Glycol	-	16.20
Polyethylene glycol 400	-	26.32
Sorbitol (70%m/v)	-	10.06
Corn oil	-	0.18
Cottonseed oil	-	0.18
Miglyol 812	-	0.50
Soybean oil	-	0.18
Sunflower oil	-	0.18

2.6 Conclusion

The purpose of sample characterisation, as outlined at the beginning of this chapter, is the certification that the drug substances that are to be investigated and extensively used in later studies, are sufficiently pure and uncontaminated to avoid unpredictable variation in the results obtained. The four principle spectrometric techniques employed, allow for the accurate elucidation of the structures of RIF, INH and PZA. IR, MS and NMR jointly produce an accurate interpretation of the structures of the RIF, INH and PZA samples provided by the sponsor. UV spectra, melting range, DSC and TLC confirm the purity of

the drug substances, complementing the results of the other spectrometric techniques. UV maxima, melting range and DSC are specific to a compound, any significant variation in these results would indicate the presence of impurities, which would also be visible by TLC. The UV spectra for RIF, INH and PZA indicate a common maximum at approximately 260nm. It is for this reason that the UV detectors used in HPLC analyses in this project are set at this wavelength for the simultaneous determination of the three drugs. RIF, INH and PZA were adequately characterised with reference to the monograph standards already referenced and were found to be sufficiently pure to be used in subsequent work. Manufacturer documentation on the purity of all solvents and surfactants used in this project was provided on purchase and donation of the substances.

Chapter Three

Analytical Method Development and Validation

3.1 Introduction

The analysis of drug substances and finished products within the pharmaceutical industry, both in South Africa and internationally, is carried out to satisfy the manufacturer and regulatory authorities of the quality, integrity and stability of the medicinal agent or product to be administered to the patient(Clarke, G.S., 1994).

The first step in the analysis of a product is the development of an appropriate analytical method that is preferably simple to perform, and that will produce accurate and reproducible results(Dadgar, D. *et al.*, 1995). In developing the method of analysis, the drugs being investigated need to be taken into account, as must whether the aim of the analysis is qualitative or quantitative in nature. It is the primary focus of this work to determine the stability of the medicinal agents: Rifampicin(RIF), Isoniazid(INH) and Pyrazinamide(PZA). The stability is assessed alone and in combination in various formulation components and ultimately in selected liquid dosage forms developed for the delivery of these drugs. Due to the versatility of high performance liquid chromatography (HPLC), and the high degree of precision associated with quantitative analysis using this chromatographic technique, as opposed to others(Paper Chromatography, Thin Layer Chromatography and Gas Chromatography), it is widely employed in the pharmaceutical industry. For this reason, and due to the types of formulations investigated in this project, HPLC has been selected for the stability assessment of RIF, INH and PZA.

Liquid chromatographic techniques have previously been reported for the analysis of INH and RIF alone and in combination, but these have failed to separate RIF, INH and PZA

when in combination(Jindal, K.C. *et al.*, 1994; Prankerd, R.J. *et al.*, 1992; Saxena, S.J. *et al.*, 1977; Shah, Y. *et al.*, 1992). Other researchers have approached this problem, however, the methods employed require complex mobile phases, specific elution criteria and specialised columns(Argekar, A.P. *et al.*, 1996; Gaitonde, C.D. *et al.*, 1991). In developing an HPLC method that will be of use for the routine assessment of these drugs in the pharmaceutical industry, a more simple and cost effective method is thus sought. The method developed must be reproducible, precise and accurate, all of these criteria are addressed by validation of the method produced.

3.2 Method Development

The purpose of the method development is to optimise the chromatographic system, conditions and mobile phase for the simultaneous determination of RIF, INH and PZA. Based on previous work done on the analysis of these drugs, several chromatographic columns have been selected for evaluation. Chromatographic conditions are chosen from previously successful reports as a starting point for the evaluation and selection of a set of optimum conditions for this work(Gharbo, S.A., *et al.*, 1989; Jindal, K.C. *et al.*, 1994; Prankerd, R.J. *et al.*, 1992; Saxena, S.J. *et al.*, 1977; Shah, Y. *et al.*, 1992). Experimentation with mobile phase composition and pH is then undertaken to produce the optimum composition, that will result in acceptable retention times and peak resolution for RIF, INH and PZA.

3.2.1 Instrumentation and Equipment

A Model 6000A solvent delivery system, Model 710B Waters Intelligent Sample Processor (WISP), Model 481 Lambda-Max multiwavelength LC Spectrophotometer and Model 730 Waters Data Module integrator and chart recorder, comprise the chromatographic system manufactured by Waters Associates®, Massachusetts, a division of Millipore®. **(System 1)**

This system was not used in the method development, but was validated later on in this chapter and used in the stability studies detailed in chapters 6 and 7.

A manual HPLC system was used for the method development to identify the column and conditions optimal for the simultaneous determination of RIF, INH and PZA. This system comprised a SpectraSERIES P100 isocratic high pressure pump (Thermal Separation Products, Florida, USA), a SpectraSERIES UV100 UV Detector (Thermal Separation Products, Florida, USA), a Rikadenki Chart Recorder (Rikadenki Kogyo Co. Ltd., Tokyo) and a model 7125 20 : L fixed loop Rheodyne (Rheodyne Inc., California, USA).**(System 2)**

An HPLC filter system using a 0.45 : m type HVLP filter (Millipore®, Massachusetts), and an M32 Digital pH Meter(Beckman, USA) were used for mobile phase preparation and in pH optimisation.

3.2.2 Reagents and Solvents

All solvents used were of HPLC grade. All aqueous fractions were prepared using double distilled deionised water, obtained from a Milli-RO 15 water purification system, manufactured by Millipore®(Massachusetts, USA). Methanol (MeOH) and acetonitrile (MeCN) used as the organic solvent components of mobile phases and orthophosphoric acid (H_3PO_4) were purchased from BDH®, England. Disodium orthophosphate (Na_2HPO_4) and tetrabutylammonium hydroxide (tBAH or $(Bu)_4NOH$) were purchased from Merck®(Darmstadt, Germany).

3.2.3 Chromatographic System Conditions

The following settings apply to the chromatographic systems employed in each evaluation,

varying only the chromatographic column used and the mobile phase composition:

System 1

Flow Rate	=	1.0ml/min
Volume of Injection	=	20∶L
Wavelength of Detection	=	260nm
Detector A.U.F.S.	=	1.0
Data Module Instrumental Noise Rejection	=	1000 units
Data Module Integrator Area of Rejection	=	100000 units
Data Module Recorder Chart Speed	=	0.3cm/min

System 2

Flow Rate	=	1.0ml/min
Volume of Injection	=	20∶L
Wavelength of Detection	=	260nm
Detector A.U.F.S.	=	1.0
Rikadenki Chart Recorder Speed	=	0.3cm/min

The wavelength of 260nm was selected for the detection of RIF, INH and PZA, as all three drugs have a common absorbance maximum at or very near this wavelength, as indicated in chapter 2.

3.2.4 Chromatographic Column and Mobile Phase Evaluation

Isocratic HPLC was used for all analyses of RIF, INH, PZA and their decomposition products(Prankerd, R.J. *et al.*, 1992). The changing of selectivity in separations can often be accomplished by a change in bonded phase chemistry of the column. Generally, short-chain bonded phases are more unstable and irreproducible than bonded phases with longer chains, due to increased susceptibility to hydrolysis as the hydrophobicity of the bonded

phase decreases. This can reduce the ability to obtain robust methods(Ricker, R.D. *et al.*, 1995). In this research gradient elution is not employed and temperature is ambient. Resolution of the drug peaks and degradants is therefore obtained by the type of column and mobile phase composition used.

3.2.4.1 Method

1mg/ml aqueous stock solutions of RIF, INH and PZA were prepared. The stock solutions were diluted to 0.100mg/ml and it is these individual solutions that are injected onto the chromatographic system using the stated conditions, during the evaluation of each column and mobile phase composition.

A 0.02M buffered solution of Na_2HPO_4 was obtained by dissolving 0.0568g of Na_2HPO_4 in 1000ml of water. A 0.0002M buffered solution of $(Bu)_4NOH$/tBAH was obtained by the dilution of 2.5ml of 20% v/v $(Bu)_4NOH$ up to 1000ml with water. All mobile phases prepared, used one of the buffered solutions listed as the aqueous component and either MeOH or MeCN as the organic component.

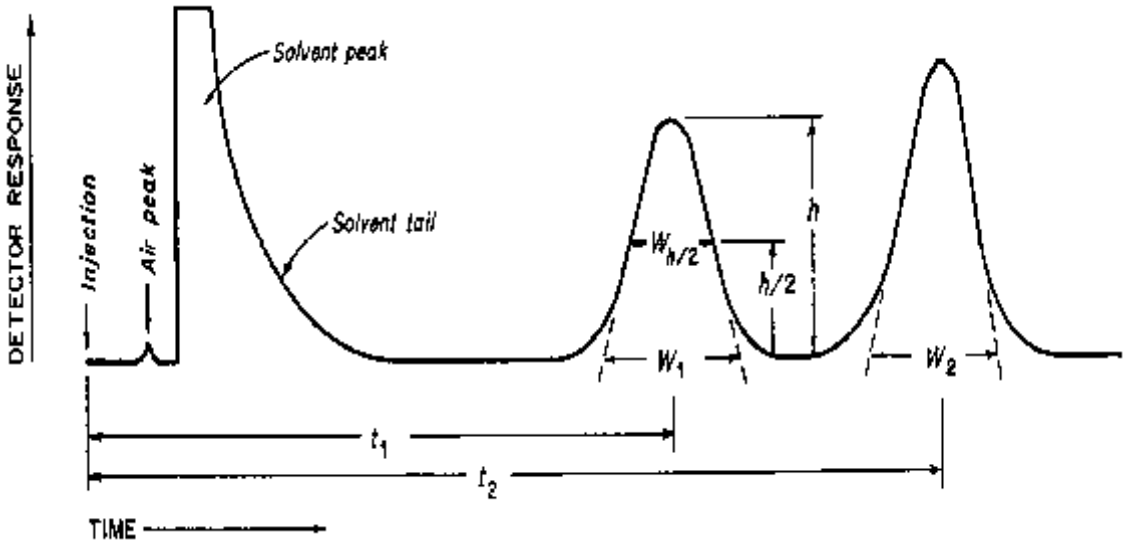
Mobile phase composition was accurately calculated and prepared by % v/v of organic phase to aqueous phase, with orthophosphoric acid used for the final adjustment of the mobile phase to the required pH. The prepared mobile phase was then filtered and degassed prior to HPLC analysis using the system stated.

The same mobile phase compositions were evaluated for each of the chromatographic columns selected.

3.2.4.2 Results and Discussion

Two system suitability tests usually discussed as part of the method validation, are addressed here, as they are of significant importance in the selection of an appropriate chromatographic column and mobile phase. These two criteria are retention time and resolution. Figure **3.1** is the schematic representation of the chromatographic separation of two substances 1 and 2. h is the height, h_2 is the half-height and $W_{h/2}$ is the peak width at half-height. Chromatographic retention times, although characteristic of the compounds that they represent, are not unique.

Figure 3.1 Chromatographic separation of two substances (USP, 1994)



For the separation of two components in a mixture, the resolution, R, is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1} \quad \text{----------Equation 3.1}$$

in which t_1 and t_2 are the retention times of the two components, and W_1 and W_2 are the corresponding widths at the bases of the peaks obtained by extrapolating the straight sides

of the peaks to the baseline. Peak areas and peak heights are usually proportional to the quantity of compound being eluted, although peak areas are generally used, they may be less accurate if peak interference occurs. For manual measurement, the chart recorder was run at a speed of 1cm per minute (faster than that used for analysis) to minimise error in the measurements taken. Accurate quantitative work requires the components measured to be separated from any interfering components, with peak tailing and fronting and the measurement of peaks on solvent tails to be avoided(USP, 1994).

The resolution, R, is a system suitability test that is a function of column efficiency, *N*, and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system, and ensuring that the internal standards are resolved from the drug(USP, 1994). Column efficiency and peak symmetry are criteria addressed in the method validation, once a suitable chromatographic column and mobile phase have been identified.

3.2.4.2.1 Nova-pak® C18 (Waters Associates®, Massachusetts)

A Nova-pak®, 3.9mm i.d. x 150mm C18 steel cartridge chromatographic column, with 4.6 μm particle diameter octadecylsilane and a resultant 60Å pore diameter was used. The column was evaluated at various mobile phase compositions and the resulting retention times(Rt) of RIF, INH and PZA, as well as the phase compositions, listed in tables **3.1**, **3.2**, **3.3** and **3.4**. The MeOH:Na_2HPO_4 buffer mobile phase combination and Nova-pak® C18 column listed in this section were used in previous work done by Shah *et al.* on the simultaneous determination of RIF and INH, and thus served as the starting point for this method development(Shah, Y. *et al.*, 1992). Unfortunately, these workers did not investigate the determination of PZA. From the results tabulated and the R values calculated using equation **3.1**, it is evident that baseline peak resolution of INH and PZA is poor, despite significant alteration of the mobile phase composition.

Table 3.1 MeOH : Na_2HPO_4 mobile phase with the Nova-pak® C18

MeOH : Na_2HPO_4 Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
80:20	**4.5**	1.73	0.83	0.83
75:25	**4.5**	2.40	0.91	0.92
70:30	**4.5**	2.90	1.03	1.04
65:35	**4.5**	3.32	1.06	1.08
60:40	**4.5**	6.05	1.11	1.12

This column and mobile phase composition is thus not suitable for the simultaneous determination of these three drugs. MeCN commonly employed by other researchers was thus used to replace MeOH in further development, being a less polar solvent it was hoped to produce better resolution of RIF, INH and PZA(Prankerd, R.J. *et al.*, 1992; Ricker, R.D. *et al.*, 1995). Table **3.2** indicates that MeCN produced a shorter Rt for RIF and a very slight increase in separation of INH and PZA, but did not improve the baseline peak resolution of INH and PZA significantly.

Table 3.2 MeCN : Na_2HPO_4 mobile phase with the Nova-pak® C18

MeCN : Na_2HPO_4 Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
60:40	**3.0**	2.14	0.95	1.04
50:50	**3.0**	3.92	1.02	1.06
40:60	**3.0**	5.65	1.09	1.11

Table 3.3 MeOH : tBAH mobile phase with the Nova-pak® C18

MeOH : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
75:25	**3.7**	3.34	1.20	1.39
70:30	**3.7**	2.10	0.99	0.99
65:35	**3.7**	4.11	1.51	1.70

The Na_2HPO_4 aqueous buffer component, suggested by Shah *et al.* was replaced by tBAH. This produced the results tabulated in table **3.3**, with greater resolution between INH and PZA, while resulting in RIF maintaining a reasonably short Rt and good peak resolution at the greater aqueous composition of the mobile phase of 65:35. Although this was an improvement, the separation achieved between INH and PZA was still insufficient.

Table 3.4 MeCN : tBAH mobile phase with the Nova-pak® C18

MeCN : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
40:60	**3.7**	1.71	1.30	1.34
45:55	**3.7**	1.44	1.12	1.15
50:50	**3.7**	1.60	1.05	1.10
60:40	**3.7**	1.24	1.05	1.06

By looking at the results achieved by substituting MeCN for MeOH and tBAH for Na_2HPO_4, combining MeCN and tBAH should produce improved resolution between INH and PZA while shortening the Rt of RIF. This was, however, not the case for this particular column, where baseline peak resolution remained poor. The mobile phases

evaluated here were then tested on a cyano (Nova-Pak® CN) and two other reversed- phase columns (Bondex® C18 & : Bondapak™ C18) of differing column length, internal diameter, particle size and pore diameter. The results are listed in the tables that follow, for each column type.

3.2.4.2.2 Bondex® C18 (Phenomenex®, California)

A Bondex®, 3.9mm i.d. x 300mm C18 steel cartridge chromatographic column packed with 10 : m particle diameter octadecylsilane with a pore diameter of 60D was evaluated. The mobile phase compositions examined are listed in the following tables **3.5**, **3.6** and **3.7**.

Table 3.5 MeCN : Na_2HPO_4 mobile phase with the Bondex® C18

MeCN : Na_2HPO_4 Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
60:40	**3.0**	4.93	2.61	2.75
50:50	**3.0**	7.44	2.53	2.78
40:60	**3.0**	> 20.00	2.66	2.93

The Rt of RIF is greater than that for the same mobile phase compositions using the Nova-Pak® C18 column, with INH and PZA resolution improving further, but still not resolved**.** Although this Bondex column is also a C18 column with a pore diameter of 60D and internal diameter of 3.9mm, the column is twice the length of the Nova-Pak® previously evaluated, thus producing the longer retention times indicated in table **3.5**. This effect was even more significant at the mobile phase compositions in table **3.6**, with resolution of INH and PZA very poor, but retention times that are longer.

Table 3.6 MeOH : tBAH mobile phase with the Bondex® C18

MeOH : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
75:25	**3.7**	7.89	3.44	3.45
70:30	**3.7**	10.22	3.33	3.36
65:35	**3.7**	14.22	3.15	3.26

Table 3.7 MeCN : tBAH mobile phase with the Bondex® C18

MeCN : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
40:60	**3.7**	9.87	2.94	3.18
45:55	**3.7**	6.99	3.00	3.10
50:50	**3.7**	5.15	2.55	2.81
60:40	**3.7**	4.02	2.12	2.18

As for the evaluation of the Nova-pak® C18 column, the MeCN:tBAH mobile phase produced the best resolution and retention times for the drugs under investigation, with a 50:50 mobile phase composition being optimal for this column. Further work was done to find a column which would produce better resolution between INH and PZA, as RIF resolution from the other peaks, has not posed a problem. The effect of the column on RIF is purely an effect on it's retention time, with this drug peak being too far removed to interfere with INH and PZA. This column could be of use, however, more precise adjustment of mobile phase composition and pH would be necessary to optimise drug peak separation.

3.2.4.2.3 Nova-Pak® CN (Waters Associates®, Massachusetts)

A Nova-Pak®, 3.9mm i.d. x 150mm CN-HP chromatographic column with 5 µm particle diameter packing material and a 60Å pore diameter was evaluated. The mobile phase compositions evaluated on this column and the resulting retention times are listed in tables **3.8**, **3.9** and **3.10**. The pore diameter and column length is the same size and length as the preceding Nova-pak® C18 column tested, differing in the cyano column being more polar. In table **3.8**, the retention times are much shorter for RIF, INH and PZA, when

Table 3.8 MeCN : Na_2HPO_4 mobile phase with the Nova-Pak® CN

MeCN : Na_2HPO_4 Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
60:40	**3.0**	1.78	1.30	1.27
50:50	**3.0**	2.12	1.32	1.32
40:60	**3.0**	2.87	1.34	1.35

compared to the results listed in tables **3.2** and **3.5** at the same pH and mobile phase compositions, this effect being due to the polarity of the column.

Table 3.9 MeOH : tBAH mobile phase with the Nova-Pak® CN

MeOH : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
75:25	**3.7**	3.46	1.29	1.26
70:30	**3.7**	2.88	1.17	1.18
65:35	**3.7**	3.45	2.10	2.78

Table 3.10 MeCN : tBAH mobile phase with the Nova-Pak® CN

MeCN : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
40:60	**3.7**	2.51	1.60	1.65
45:55	**3.7**	2.22	1.56	1.58
50:50	**3.7**	1.77	1.35	1.35
60:40	**3.7**	1.53	1.16	1.17

The MeCN:tBAH mobile phase is not suited for use on this column. For the Nova-Pak® CN column, the MeOH:tBAH mobile phase in table **3.9** at a ratio of 65:35 produced the best results with regard to resolution and retention time. INH and PZA were sufficiently separated as not to produce any peak overlap, with RIF having a short retention time, thus facilitating the efficient use of time for replicate HPLC determinations. The problem here is that the degradants of these three drugs elute at similar retention times to each other and the drugs as is demonstrated in the validation section of this chapter and the lack of resolution between the degradants and the drugs, thus negating the usefulness of this column. The difference in retention times for all mobile phases evaluated on this column, is a combined result of the bonded phase chemistry and length of this column. For the drugs under investigation, and the mobile phases being evaluated, this column is not suitable.

3.2.4.2.4 : Bondapak™ C18 (Waters Associates®, Massachusetts)

A : Bondapak™, 4.6mm i.d. x 250mm steel cartridge C18 chromatographic column with 10 : m particle diameter octadecylsilane and 125 D pore diameter was investigated, including a 20mm : Bondapak™ C18 guard column of the same specifications.

The results of the mobile phase compositions evaluated and the resulting retention times are listed in tables **3.11**, **3.12** and **3.13**.

Table 3.11 MeCN : Na_2HPO_4 Mobile Phase with the : Bondapak™ C18

MeCN : Na_2HPO_4 Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
60:40	**3.0**	6.20	2.80	3.15
50:50	**3.0**	11.59	2.77	3.28
40:60	**3.0**	13.27	2.89	3.37

Table 3.12 MeOH : tBAH mobile phase with the : Bondapak™ C18

MeOH : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
75:25	**3.7**	8.12	3.75	3.68
70:30	**3.7**	10.56	3.42	3.46
65:35	**3.7**	14.45	3.46	3.58

Tables **3.11** and **3.12** indicate significantly different retention times for the drugs on this column, compared to those previously evaluated. The Rt of RIF was significantly longer for both of these mobile phases, removing it from the area of elution of the RIF, INH and PZA degradants demonstrated by the retention times and resolution factors calculated in section **3.3.2.4**. The MeCN:Na_2HPO_4 mobile phase combinations resolved the INH and PZA peaks, where the MeOH:tBAH mobile phase combinations did not.

Table 3.13 MeCN : tBAH mobile phase with the ⁚Bondapak™ C18

MeCN : tBAH Mobile Phase Composition				
Mobile Phase		**Retention Time (min)**		
Ratio	**pH**	**RIF**	**INH**	**PZA**
40:60	**3.7**	13.49	3.15	3.71
42.5:57.5	**3.7**	9.02	3.19	3.70
45:55	**3.7**	8.15	3.08	3.63
47.5:52.5	**3.7**	7.27	3.02	3.37
50:50	**3.7**	6.14	2.72	3.30
60:40	**3.7**	4.28	2.61	3.42

The most successful combination of column and mobile phase, of all those evaluated, produced the results listed in table **3.13**. The ⁚Bondapak™ C18 column using MeCN:tBAH as the mobile phase produced the best retention times for all three drugs with the INH and PZA peaks resolved from each other by the R values calculated. Peak resolution of INH and PZA has been a problem throughout the method development, due to the structural similarity and polarity of these two compounds, and hence the relatively similar elution times.

Optimisation of the mobile phase composition indicated that a 42.5:57.5%v/v (MeCN:tBAH) ratio produced optimum separation at the fixed pH stated. The peak tailing factor approached unity with all peaks symmetrical for RIF, INH or PZA, which will be confirmed in the method validation.

3.2.4.3 pH Optimisation

With the desired chromatographic system for analysis selected, and an appropriate mobile

phase identified, the next step is the optimisation of the mobile phase pH, in an attempt to improve baseline peak resolution and to finely adjust the Rt of the drugs under investigation.

The optimum mobile phase composition identified consists of 42.5:57.5%v/v (MeCN:tBAH). Using this mobile phase composition, the ∶Bondapak™ C18 column and previously stated chromatographic system, the Rt of RIF, INH and PZA were determined at the pHs listed in table **3.14**.

Replicate injections (n = 6) at each pH, produced an optimum pH range of 3.10 to 3.40, with the Rts of INH and PZA decreasing and that of RIF increasing as pH decreased. For the given mobile phase composition and chromatographic system, mobile phase pH within this stated range produces adequate baseline peak resolution and Rt for the simultaneous determination of RIF, INH and PZA. The Standard Deviation calculated for the replicate injections of each drug was less than 1% in the 3.10 to 3.40 pH range for all three drugs. A mobile phase pH of 3.10 was used for all subsequent HPLC stability studies of these three drugs.

Table 3.14 pH Optimisation results

MeCN : tBAH (42.5%:57.5%v/v)			
	Retention Time (min)		
pH	**RIF**	**INH**	**PZA**
3.70	9.02	3.19	3.70
3.50	9.39	3.17	3.69
3.40	9.85	3.11	3.63
3.20	10.68	2.92	3.56
3.10	10.97	2.90	3.54
3.00	11.68	2.95	3.47
2.85	12.00	2.89	3.45

3.2.5 Conclusion

The chromatographic system and conditions selected as being optimal for HPLC analysis of RIF, INH and PZA in combination, is **System 1** using the : Bondapak™ C18 column with MeCN:tBAH(0.0002M) mobile phase in a ratio of 42.5:57.5%v/v, within a pH range of 3.10 to 3.20. It is evident that in this work, bonded phase particle size, pore size and column length play a more significant role in separating these three drugs, than the mobile phase compositions evaluated.

3.3 Method Validation

Method validation is one of the most important areas of method development, and consists of a series of experiments which confirm that the method produces results which have the desired degree of accuracy and precision for the intended use(Edwardson, P.A.D. *et al.*, 1990; Wahlich, J.C. *et al.*, 1990). Method validation parameters and system suitability tests routinely assessed for chromatographic techniques are ruggedness and robustness, accuracy, precision, selectivity, stability, linearity, limit of detection and limit of quantitation (Dadgar, D. *et al.*, 1995; Edwardson, P.A.D. *et al.*, 1990; ICH Guidelines, 1996; Salo, J-P., *et al.*, 1996; Wahlich, J.C. *et al.*, 1990). Method development has only been completed when the method has been stringently tested and shown to demonstrate acceptable analytical performance(Edwardson, P.A.D. *et al.*, 1990).

3.3.1 Chromatographic System

3.3.1.1 Instrumentation and Equipment

The column selected is detailed in section **3.2.4.2.4** with the selected analytical conditions stated in section **3.2.5**. Method validation was performed using **System 1** and **System 2**

detailed in sections **3.2.1** and **3.2.3**. System suitability tests were done on **System 1**,which is used in all subsequent stability studies.

3.3.1.2 Solvents and Reagents

The solvents and reagents are listed in sections **3.2.2** and **3.2.4.1** in the method development.

3.3.1.3 Chromatographic Conditions

The chromatographic conditions for **System 1** and **System 2** are detailed in sections **3.2.1** and **3.2.3**. The : Bondapak™, 4.6mm i.d. x 250mm steel cartridge C18 chromatographic column with 10 : m particle diameter octadecylsilane and 125D pore diameter, incorporating a 20mm : Bondapak™ C18 guard column of the same specifications, was the column selected for validation and subsequent use.

3.3.1.4 Mobile Phase

MeCN : tBAH(0.0002M) in a ratio of 42.5% : 57.5%v/v, within a pH range of 3.10 to 3.20.

3.3.2 Validation Tests and Criteria

3.3.2.1 Column Efficiency and Peak Symmetry

Column Efficiency is measured in terms of the number of theoretical plates of a column. As indicated, this is purely a qualitative assessment of column efficiency, and the usefulness of this system suitability test in deciding on the suitability of a system is not absolute.

Column efficiency and peak symmetry rely heavily on the peak that is used for their calculation, with an inefficient column being characterised by a low column efficiency, but also by an inability to separate the components of interest(Wahlich, J.C. *et al.*, 1990).

It is the 5F method that is used to calculate the number of theoretical plates.

$$N = 25(d/W_{0.05})^2 \quad \text{----------Equation 3.2}$$

N = number of theoretical plates

d = distance from injection to peak (mm)

$W_{0.05}$ = peak width at 5% of the peak height (mm)

For the calculation of column efficiency and peak symmetry, recorder chart speed was set to 1cm per minute for both HPLC systems, to reduce error. According to literature reviewed, the 5F method provides a more accurate estimation of column efficiency and errs on the side of a lower plate count(BP, 1993; USP, 1994). It is stated that a theoretical plate count in excess of 6000 is indicative of an adequately efficient column.

As determined for the : Bondapak™ C18 column being used,

N = 14000 **(System 1)**

N = 13950 **(System 2)**

at the beginning of this project.

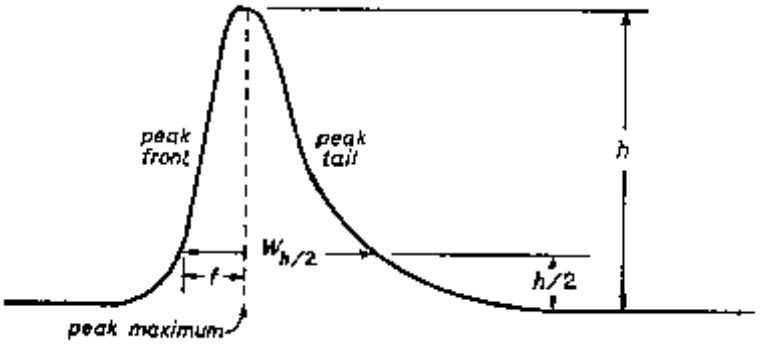
N = 13250 **(System 1)**

N = 14000 **(System 2)**

at the end of this project.

System 2 used in the method development, does not employ an integrator to calculate the area under the curve(AUC) as does **System 1**, but employs peak height as a measure of the response. The tailing factor, T, is a measure of peak symmetry, approaching unity for perfectly symmetrical peaks and increasing as tailing becomes more pronounced (Figure **3.2**). An asymmetric peak results in integration and therefore, precision becoming less reliable(USP, 1994).

Figure 3.2 Asymmetric peak



$$T = W_{h/2}/2f \qquad \text{----------Equation 3.3}$$

T = Tailing/Symmetry Factor

$W_{0.05}$ = peak width at 5% of peak height(mm)

f = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of peak height(mm)

Acceptable limits of symmetry are within 0.95 to 1.05. The tailing/symmetry factor calculated for each peak that appeared in the chromatogram approached unity (0.98 to 1.00) for RIF,INH and PZA on **System 1** and **System 2.**

3.3.2.2 Linearity

The linearity of a method is tested to demonstrate a proportional relationship of detector response to analyte concentration over the working range of the calibration curve, and is usually investigated over a wide range of 10 to 200% of the nominal analyte response(Edwardson, P.A.D. *et al.*, 1990; Clarke, G.S., 1994). The nominal analyte response for RIF, INH and PZA for the chromatographic system and conditions used, occurs at a concentration of 0.10mg/ml.

Aqueous stock solutions of RIF, INH and PZA were prepared for the construction of the standard curves. Solutions of 1.00mg/ml were prepared for INH and PZA and a solution of 0.50mg/ml for RIF. These solutions were used for the preparation of all dilutions used for the determination of linearity, precision and accuracy.

Standard curves were constructed for the analytical HPLC system (**System 1**) as well as for the method development HPLC system (**System 2**). The standard curves for **System 1** were constructed by plotting area under the curve (AUC) versus drug concentration (mg/ml), while those for **System 2** were constructed by plotting the peak height versus drug concentration (mg/ml). Each point on the curve was determined in triplicate and linear regression plots for each curve constructed. The concentrations of drug used to plot the standard curves are listed for each drug for each system, in tables **3.15** and **3.16**.

Table 3.15 Standard curve concentrations for System 1

Drug	Concentration (mg/ml)									
RIF	0.20	0.16	0.14	0.12	0.10	0.08	0.06	0.04	0.02	0.01
INH	0.20	0.16	0.14	0.12	0.10	0.08	0.06	0.04	0.02	
PZA	0.20	0.16	0.14	0.12	0.10	0.08	0.06	0.04	0.02	

Table 3.16 Standard curve concentrations for System 2

Drug	Concentration (mg/ml)					
RIF	0.14	0.10	0.08	0.05	0.04	0.02
INH	0.20	0.14	0.10	0.06	0.04	0.02
PZA	0.12	0.10	0.06	0.04	0.02	0.01

For the purpose of constructing a standard curve a linear relationship is desired between concentration and the response of the detector. The linearity of a standard curve is expressed by the equation:

$$Y = a.X + c \quad \text{----------Equation 3.4}$$

Y = detector response (AUC or peak height, depending on chart recorder/integrator)

a = slope of the line

X = concentration (mg/ml)

c = constant/Y-intercept

and the results of linear regression analysis for each curve are expressed in table **3.17.**

Ideally, the constant/Y intercept should equal zero, hence the standard curve should pass through the origin. This does not occur in any of the standard curves constructed here, however, the Y intercepts approach zero. The Correlation Coefficients (r) calculated for each curve are all greater than 0.999, approaching unity, which is an indication of the linearity of the curve.

Table 3.17 Linear regression output

		Drug		
System	**Regression Output**	**RIF**	**INH**	**PZA**
System 1	**Slope**	192716608.17	107708215.66	229206933.60
	Constant	-429954.64	972754.82	821755.88
	r value	0.9995	0.9993	0.9994
System 2	**Slope**	55.26	115.23	169.44
	Constant	0.11	-0.15	0.13
	r value	0.9997	0.9995	0.9996

(r value = Correlation Coefficient)

Linearity, precision and accuracy were performed for chromatographic **System 1** and **System 2**. All other method validation testing was performed using chromatographic **System 1**.

3.3.2.3 Precision and Accuracy

Precision gives a measure of a method's reproducibility, with any increase of the relative standard deviation(RSD) greater than 2% warranting revalidation(ICH Guidelines, 1996). Precision monitors the RSD of responses of replicate samples on application in the chromatographic system(Edwardson, P.A.D. *et al.*, 1990; Wahlich, J.C. *et al.*, 1990). Accuracy is a measure of how close the amount/value determined is to the theoretical amount/value expected. For the determination of precision and accuracy, 6 replicate determinations were made at the upper and lower limits of each of the standard curves. For RIF, INH and PZA using **System 1**, this was done at 0.16mg/ml and 0.04mg/ml, in **System 2**, this was done at 0.10mg/ml and 0.04mg/ml for RIF, 0.14mg/ml and 0.04mg/ml

for INH, and 0.10mg/ml and 0.02mg/ml for PZA. Accuracy is measured by the mean concentrations of the replicate injections, while precision is measured by the RSD at each concentration(ICH Guidelines, 1996; Jenke, D.R., 1996).

The results for the precision and accuracy are listed in tables **3.18** and **3.19**.

Table 3.18 Precision and accuracy results for RIF, INH and PZA for System 1

System 1				
Drug	**Theoretical Concentration (mg/ml)**	**Mean (n=6) Concentration Determined (mg/ml)**	**Percentage Relative Standard Deviation**	**Percentage of Concentration t**
RIF	0.160	0.162	0.270	101.25
	0.040	0.041	0.310	102.00
INH	0.160	0.158	0.381	98.75
	0.040	0.039	0.454	98.00
PZA	0.160	0.157	0.122	98.13
	0.040	0.039	0.073	98.00

t Concentration of drug determined as a percentage of the theoretical concentration. A measure of accuracy.

Precision and accuracy for the method of analysis employed, were demonstrated over the concentration ranges used for both chromatographic systems. The RSD is below the 2% stated in the literature and the percentage of mean concentration within the 98 to 102% stated limit of the theoretical concentration, in all cases(Jenke, D.R., 1996).

Table 3.19 Precision and accuracy results for RIF, INH and PZA forSystem 2

System 2				
Drug	**Theoretical Concentration (mg/ml)**	**Mean (n = 6) Concentration Determined (mg/ml)**	**Percentage Relative Standard Deviation**	**Percentage of Concentration t**
RIF	0.100	0.102	0.260	102.00
	0.040	0.039	0.421	98.00
INH	0.140	0.140	0.173	100.00
	0.040	0.041	0.150	102.00
PZA	0.100	0.099	0.392	99.00
	0.020	0.020	0.391	100.00

t Concentration of drug determined as a percentage of the theoretical concentration. A measure of accuracy.

3.3.2.4 Specificity

The principle reason for selecting a chromatographic method is for it's ability to resolve the analytes from other interfering species(Lang, J.R., 1991; Wahlich, J.C. *et al.*, 1990). The interfering species can take the form of degradation products, synthetic impurities, contaminated solvents or samples, or mixtures of analytes. In the case of the work being performed in this project, the interfering species is not available in every instance for the purpose of performing a baseline resolution check. For this reason it is necessary to specify a resolution criterion of > 1.5(Wahlich, J.C. *et al.*, 1990). Stressing of the analytes by exposure to oxidative, heat, light and acid/base hydrolysis conditions, was performed to aid the identification of as many possible interfering species as possible(Edwardson, P.A.D. *et al.*, 1990). The use of a photodiode array detector has also made it possible to determine

the selectivity of the method by evaluating homogeneity of the analyte peak. This is done by comparing the spectra of the upslope, apex and downslope of the peak. 0.20mg/ml aqueous stock solutions of RIF, INH and PZA were prepared and used in the preparation of solutions for oxidative, acid/base, heat and light stressing.

3.3.2.4.1 Oxidation

0.10mg/ml aqueous solutions of RIF, INH and PZA in 30% hydrogen peroxide were prepared using the stock solutions prepared, water and 100% hydrogen peroxide. These solutions were allowed to stand at room temperature, protected from light, for 24hrs, after which samples were drawn and analysed. The results are listed in table **3.20**.

Table 3.20 Major oxidation degradants

Drug	Drug Retention Time(min)	Major Degradants Retention Time(min)
RIF	10.81	2.40(1), 3.80(2), 4.25(3) and 5.54(4)
INH	2.85	-
PZA	3.55	2.57(5) and 3.19(6)

Table 3.21 Resolution between drug and degradants

	Degradant					
Drug	**1**	**2**	**3**	**4**	**5**	**6**
RIF	28.03	23.37	21.87	17.57	27.47	25.40
INH	3.00	6.33	9.33	17.93	1.87	2.26
PZA	7.67	1.67	4.67	13.27	6.53	2.40

The parent drugs were sufficiently resolved from the oxidation degradants, so that quantitative analysis would not be affected. The resolution factors between the drugs and the numbered degradants specified in table **3.21** were calculated using equation **3.1**, as are all subsequent resolution calculations. After 24hrs, no oxidation degradants were found for INH, the solution was allowed to stand for a further 24hrs, however, still no INH degradants became evident upon HPLC analysis. Photodiode array comparison of the upslope, apex and downslope for each drug peak indicated UV spectra that mirrored the standards reported in chapter 2, confirming that there is no overlap between the oxidation degradants and the parent drugs.

3.3.2.4.2 Heat

0.10mg/ml aqueous solutions of RIF, INH and PZA were prepared using the stock solutions previously mentioned. The samples were sealed in ampoules, half of which were sealed under nitrogen and half under air. The samples were then subjected to 60^0C dry heat for a period of 30 minutes. The results are listed in table **3.22**.

Table 3.22 Major heat degradants

Drug	Drug Retention Time(min)	Major Degradants Retention Time(min)
RIF	10.97	2.40(1) and 5.54(2)
INH	2.85	2.56(3) and 3.18(4)
PZA	3.54	2.57(5) and 3.18(6)

As is evident from the retention times of the drugs and major thermal degradation products, the degradant peaks do not elute at times similar to RIF, INH and PZA, although they do co-elute with other degradant peaks. Resolution calculations in table **3.23** indicate that the

degradant peaks are sufficiently resolved from the parent drugs as not to interfere with the analysis of the quantity of drug.

The results obtained from the photodiode array analysis, agree with that demonstrated in table **3.23**, in that no overlap in the elution of the thermal degradants occurs with the parent drugs.

Table 3.23 Resolution between drug and degradants

	Degradant					
Drug	**1**	**2**	**3**	**4**	**5**	**6**
RIF	28.57	18.10	28.03	25.97	28.00	25.97
INH	3.00	17.93	1.93	2.20	1.87	2.20
PZA	7.60	13.33	6.53	2.40	6.47	2.40

3.3.2.4.3 Acid/Base

5.0M HCl or NaOH was diluted using the aqueous stock solutions of RIF, INH and PZA to produce 0.1M solutions containing the drugs. Some solutions were stored at room temperature, while others were refluxed in a water bath at 86 ^{0}C for 30 minutes with nitrogen flushing and under air. The results are listed in table **3.24**.

Degradation of RIF in both acidic and basic conditions was significant, while INH and PZA were more stable. Resolution calculations in table **3.25** indicate that the degradant peaks are sufficiently resolved from the parent drugs as not to interfere with their quantitative determination. The only failure to comply, is observed for INH and a degradant of PZA in basic solution.

Table 3.24 Major Acid/Base degradants

Acid/Base	Drug	Drug Retention Time(min)	Major Degradants Retention Time(min)
0.1M HCl	**RIF**	10.97	5.14(1), 7.33(2) and >15.0
	INH	2.82	5.17(3) and 5.32(4)
	PZA	3.50	5.15(5) and 5.27(6)
0.1M NaOH	**RIF**	10.97	2.66(1) and 7.73(2)
	INH	2.90	-
	PZA	3.60	3.05(3)

Table 3.25 Resolution between drug and degradants

		Degradants					
	Drugs	**1**	**2**	**3**	**4**	**5**	**6**
HCl	**RIF**	19.43	12.13	19.33	18.83	19.40	19.03
	INH	15.47	30.07	15.67	16.67	15.53	16.33
	PZA	10.93	25.53	15.67	12.13	11.00	11.80
NaOH	**RIF**	19.40	10.80	26.40	-	-	-
	INH	1.60	32.20	1.00	-	-	-
	PZA	6.27	27.53	3.67	-	-	-

These resolution results are confirmed by photodiode array analysis, including the overlap

of the INH with the PZA degradant peak. Since the studies performed in this project are in neutral to acidic media, this will not affect the results obtained.

3.3.2.4.4 Ultraviolet Radiation

0.100mg/ml aqueous solutions of RIF, INH and PZA were prepared using the stock solutions previously mentioned. The samples were sealed in ampoules, half of which were sealed under nitrogen and half under air. The samples were exposed to ultraviolet light (15W) at 254nm for a period of 1 week. The HPLC results are listed in table **3.26**.

Table 3.26 Major UV degradants

Drug	Drug Retention Time(min)	Major Degradants Retention Time(min)
RIF	10.95	-
INH	2.84	-
PZA	3.56	2.30(1) and 3.99(2)

After a period of one week, the only degradants that emerged, were trace amounts of PZA degradants at the retention times in table **3.26**. On standing for an extra week, no degradants of RIF and INH emerged.

The resolution between RIF and the degradant peaks 1 and 2 is greater than 10, with that for INH being 1.53 and 4.60 respectively and 2.52 and 1.72 for PZA**.** The resolution of the parent drugs from the degradants and each other meet stated criteria, this being confirmed by the photodiode array analysis.

3.3.2.5 Ruggedness and Robustness

Ruggedness refers to the effect of operational parameters on a method's suitability, while robustness refers to it's capacity to be transferred to another identical system. Compliance of the method with precision and accuracy criteria acts as confirmation that ruggedness and robustness are within their defined ranges(Wahlich, J.C. *et al.*, 1990). Ruggedness is evaluated by looking at column effect, ie. analysis of standards on two identical columns and the effect of small variations in mobile phase composition (less than 2% of each component) and variations in buffer pH. This gives an indication of the method's ability to maintain critical separation under expected mobile phase variations and column to column variability(Dadgar, D. *et al.*, 1995). Another criterion of ruggedness acceptability is that the limit of quantitation must demonstrate a reproducible response which is distinguishable from background noise.

The results of mobile phase and pH variation detailed during the method development are listed in tables **3.13** and **3.14**. They provide evidence that the ruggedness of the method meets accepted criteria within a pH range of 3.10 to 3.40 producing a variation in retention time of 1.12, 0.21 and 0.09 minutes for RIF, INH and PZA respectively, and changes in mobile phase composition of up to 2.5% having no significant effect on peak resolution, despite the respective 5.34, 0.07 and 0.08 minute variation for RIF, INH and PZA in retention time. The effect of pH and mobile phase variation, although more noticable for RIF, is not a concern due to it's adequate resolution from the other drugs and degradant peaks. Quantitation of RIF, INH and PZA was conducted using 0.10mg/ml stock solutions of these drugs. Five replicate 20∶L samples of the freshly prepared standards were injected onto a ∶Bondapak™ C18 column using **System 1** and **System 2** and the concentrations of the drugs determined. The same volumes of the standard solutions were injected onto another ∶Bondapak™ C18 column of the same specification and the

concentrations determined. The results are listed in table **3.27**.

Table 3.27 Column effect

		Concentration (mg/ml)		
System	**Drug**	**Theoretical**	**Analytical Column**	**Duplicate Column**
System 1	**RIF**	0.10	0.101	0.10
	INH	0.10	0.099	0.101
	PZA	0.10	0.099	0.099
System 2	**RIF**	0.10	0.099	0.098
	INH	0.10	0.103	0.099
	PZA	0.10	0.098	0.101

The concentrations and % RSD calculated for all three drugs on **System 1** and **System 2**, for the analytical and the duplicate columns, varied from 98% to 103%, depending on the drug, column and system used. These values approach unity and adhere to accepted limits of between 95 and 105% of the stated concentration. % RSD was less than 2% for all determinations.

For the analytical method developed to be valid for use in this project, it is necessary to prove that RIF, INH and PZA will be stable for a sufficient period of time at room temperature. This is to allow for HPLC determination of the drug concentrations, before any significant degradation occurs due to the analytical conditions. This test serves to confirm that for the WISP used in **System 1**, the drugs are stable on standing in the auto-injector, an indicator of system ruggedness.

Figure 3.3 Rifampicin stability

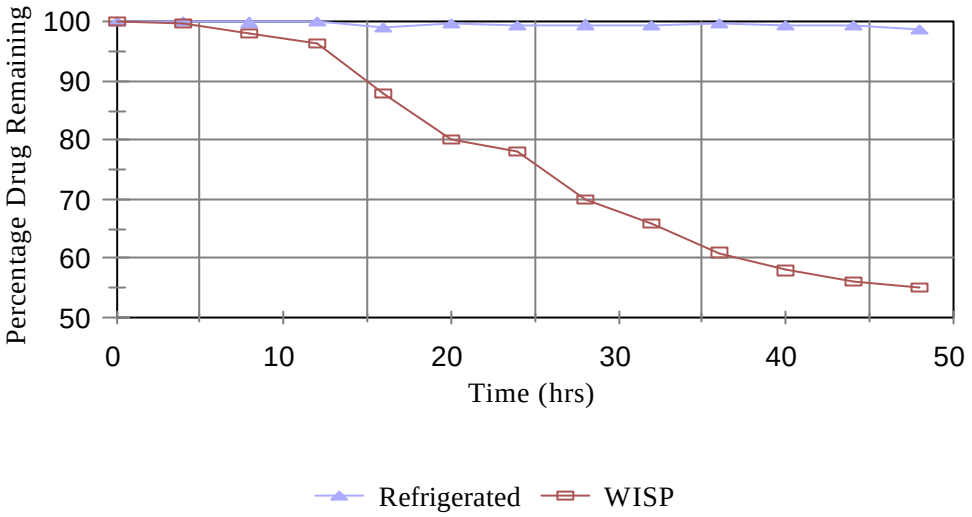
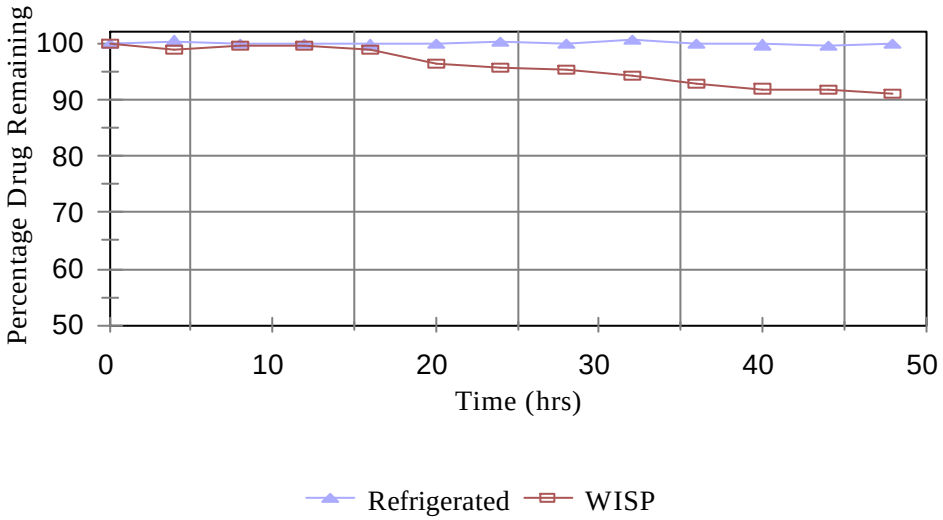


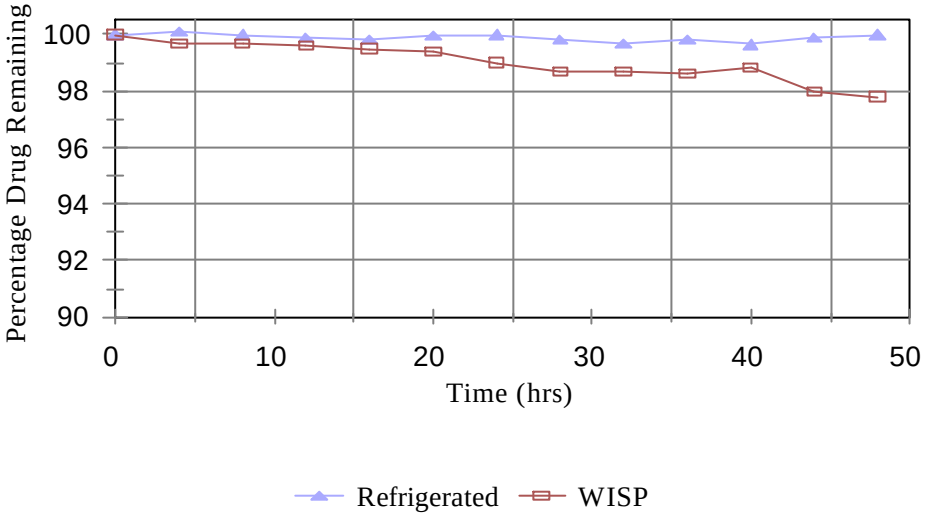
Figure 3.4 Isoniazid stability



A mixed stock solution of 0.10mg/ml of RIF, INH and PZA each, was prepared. WISP sample vials were filled and placed in the auto-injector of **System 1** and the sampler programmed to inject samples onto the column at four hour intervals, for a period of forty eight hours. This was also performed for a stock solution prepared and refrigerated at 5^{0}C.

In the case of the refrigerated samples, a vial was removed from the fridge at four hour intervals and placed into the auto-injector for immediate analysis. The results of this forty eight hour stability trial are displayed in graphs **3.1**, **3.2** and **3.3**, where plots are of percentage drug remaining versus time(hrs).

Figure 3.5 Pyrazinamide stability



RIF, INH and PZA are all very stable under refrigerated conditions, with negligible degradation occuring, as is evident in graphs **3.1**, **3.2** and **3.3.** Over the entire forty eight hour period, INH degraded less than 10% at room temperature in the WISP, with PZA being most stable, degrading less than 2.5%. RIF proved to be a problem, with approximately 45% of the drug degrading in the forty eight hour period at room temperature. It is evident that INH and PZA stability is not a concern, but rather, RIF is the deciding factor. It can be concluded from these plots, that drug concentrations obtained from HPLC analysis using **System 1**, will only be accurate if analysis is carried out within 12 hours of drawing samples.

3.3.2.5.1 Limit of Quantitation and Limit of Detection

The Limit of Detection(LOD) and the Limit of Quantitation(LOQ) were determined for **System 1.** This is often determined from the signal:noise ratio, but can also be done by sequential dilution and determination of an observable chromatographic peak, which was done in this case(Clarke, G.S., 1994). The LOD is the smallest concentration that can be distinguished from the background noise, with the LOQ being at least twice the response of the LOD and falling within the predefined boundaries of precision and accuracy, ie. the lowest concentration that can be reproducibly quantitated above background noise(Dadgar, D, *et al.*, 1995; Edwardson, P.A.D, 1990). 0.20mg/ml stock solutions of RIF, INH and PZA were prepared, and used for the determination of the LOD and LOQ. Sequential dilution of these stock solutions and HPLC analysis at the chromatographic conditions specified in section **3.3.1.3** produced the LOD and LOQ results listed in table **3.28**. Three replicate injections were performed, when the LOD was reached and ten for the LOQ. The LOQ and LOD confirm the degree of sensitivity of this analytical technique with the mean concentration determined and RSD falling within accepted limits(ICH Guidelines, 1996; Jenke, D.R.,1996). All HPLC determinations in subsequent stability studies, are performed at sample concentrations of approximately 0.10mg/ml, falling in the centre of the standard curves constructed, exceeding the LOD and LOQ determined for these drugs.

Table 3.28 LOD and LOQ results

Drug	Mean LOD (∶g/ml)	LOD RSD (%)	Mean LOQ (∶g/ml)	LOQ RSD (%)
RIF	0.10	4.53	0.20	1.80
INH	0.075	3.95	0.150	1.26
PZA	0.075	4.11	0.150	1.63

3.3.3 Conclusion

In section **3.2** an analytical method was developed for the HPLC analysis of RIF, INH and PZA in combination. This method in turn has adhered to specific criteria and is valid. The criteria outlined and subsequently tested, comprise the method validation. Column efficiency and peak symmetry are two system suitability tests which in turn have a bearing on peak resolution and precision respectively, both of which proved to be acceptable. Linearity, performed for the set of standard curves of each drug, met the stated criteria, for both **System 1 and 2.** The precision and accuracy parameters calculated, confirmed the reproducibility of the method, as did the ruggedness and robustness of the system. Specificity of the system for the detection of the three drugs was exhibited, confirmed by peak resolution calculations and photodiode array detection.

In conclusion, **System 1** using the chromatographic column and mobile phase outlined in section **3.2.2.4**, and under the conditions specified, has demonstrated that it adheres to all criteria detailed as part of the method validation. This justifies the use of this system for the further stability assessment of RIF, INH and PZA in combination in the drug and formulation stability studies which follow in chapters 6 and 7.

Chapter Four

Lipid-Aqueous Extraction - Method Development and Validation

4.1 Introduction

The drugs of interest in this project are to be analysed both in lipid and aqueous media, thus posing a unique challenge. An HPLC method has been developed and validated for the simultaneous determination of RIF, INH and PZA in aqueous and aqueous miscible/soluble media in chapter 3. This is convenient as the samples being analysed can be readily diluted/dissolved and injected onto the HPLC column. This is not so in the case of degradation studies conducted with RIF, INH and PZA dissolved, dispersed or suspended in lipid media. Despite an extensive literature survey, no reference could be found that related to HPLC analyses of analytes in lipids. Work has been done on the development of a method for the HPLC analysis of certain lipids, which could be adapted for the analysis of some lipophilic drugs in lipid by direct injection. The method and system employed, however, was complex and expensive, not facilitating the reliable and reproducible method and system desired for the day-to-day analysis of RIF, INH and PZA. The vastly differing hydrophilic and lipophilic properties of RIF, INH and PZA is another factor counting against the adaptation of this method. This was demonstrated in the solubility profiles constructed in Chapter 2, as well as by other workers(Brewer, G.A., 1977; Felder, E. *et. al.*, 1983; Gallo, G.G. *et. al.*, 1976).

This prompted the development and validation of a method for the extraction of the three drugs from lipid media, into an aqueous fraction for analysis by HPLC.

4.2 Method Development

The identification and optimisation of a simple, reproducible method for the extraction of the drugs being analysed from lipid media into an aqueous fraction became an important goal. This aqueous fraction could then be diluted and injected directly onto the HPLC column. Whether a normal or reverse phase column is used, direct injection of a lipid sample onto a chromatographic column would result in the ultimate shortening of the column life. This is a result of the lipid accumulating on the chromatographic column, effectively reducing the number of theoretical plates and hence, the column efficiency at separating the drugs and degradants. This has been confirmed a member of the Waters® technical division, Southern Africa(Verbal Communication, Howard Markham, August 1998).

4.2.1 Instrumentation and Equipment

Thin Layer Chromatographic (TLC) RP-18 F_{254S} pre-coated aluminium plates and normal phase TLC F_{254} pre-coated aluminium plates were purchased from Merck(Darmstadt, Germany). A forced circulation oven, type FSOH, made by Labcon(USA), at 60^0C was used to activate reverse phase TLC plates. 100mmX50mmX30m glass chromatographic vessels were used to run all TLC plates, which were visualised using a dual wavelength (254/356nm) UV light source, made by Raytech(USA).

A GBC UV/VIS 916 Spectrophotometer made by GBC(Melbourne, Australia) was employed in UV-VIS spectral analysis, using GBC Spectral version 1.5 software. UV-VIS was employed for the extraction medium optimisation and the evaluation of filter efficiency.

A vortex mixer made by Gemmy Industrial Corporation, Taiwan R.O.C. was used to accelerate drug partitioning between the lipid and aqueous phases. Disposable 0.22∶m

Cameo® hydrophilic filters and 5ml plastic syringes(Promex) were used to filter all aqueous fractions prior to injection onto the HPLC column. The chromatographic system used for the analysis of all aqueous fractions was the same as that developed and validated in Chapter 3.

4.2.2 Solvents and Reagents

Methanol, Acetonitrile, Hexane, Ethyl Acetate and Chloroform were purchased from BDH®, England(>99.9% purity). Miglyol 812 was supplied by Hüls, Southern Africa. Corn oil, cottonseed oil, sunflower oil and soybean oil were all purchased from Sigma(St. Louis, USA).

4.2.3 Method

Several stages were involved in the development of an efficient lipid-aqueous extraction method for the analysis of RIF, INH, PZA and their degradants in lipid media. The stages of development are discussed in the sections that follow.

4.2.3.1 Lipid-Drug Separation

Determination of a mobile phase that would adequately separate RIF, INH and PZA chromatographically from the lipids under investigation was the first step. This was performed by TLC of the drugs and the lipids of interest at mobile phase combinations and ratios listed. The lipids investigated were miglyol 812, corn, cottonseed, sunflower and soybean oil.

Saturated stock solutions of RIF, INH and PZA in the lipids listed in table **4.1** were prepared by agitation for 3 hours under nitrogen and in the absence of light. This was done

for the length of time stated, due to the expected poor lipid solubility of INH and PZA, reported in literature solubility profiles(Brewer, G.A., 1977; Felder, E. *et. al.*, 1983). 0.10mg/ml solutions of RIF, INH and PZA in methanol were prepared. Activated TLC plates were spotted with the saturated solutions of drug in lipid, drug in methanol solution and pure lipid. TLC plates were run using the specified vessels at the mobile phase compositions and ratios listed in tables **4.1, 4.2** and **4.3,** and the Rf values calculated**.** Normal phase TLC was also performed following the method detailed here, with the results listed in table **4.4**.

Table 4.1 TLC Rf values using acetonitrile:water mobile phase combinations

Acetonitrile : Water					
	Mobile Phase Ratio				
Substance	**80:20**	**60:40**	**50:50**	**40:60**	**20:80**
Rifampicin	0.510	0.282	0.112	0.031	0.010
Isoniazid	0.782	0.743	0.699	0.672	0.574
Pyrazinamide	0.784	0.714	0.661	0.632	0.431

Table 4.2 TLC Rf values using acetonitrile:tBAH mobile phase combinations

Acetonitrile : tBAH					
	Mobile Phase Ratio				
Substance	**80:20**	**60:40**	**50:50**	**40:60**	**20:80**
Rifampicin	0.702	0.732	0.546	0.283	0.042
Isoniazid	0.849	0.803	0.754	0.723	0.693
Pyrazinamide	0.849	0.794	0.701	0.596	0.513

Table 4.3 TLC Rf values using methanol:water mobile phase combinations

Methanol : Water					
	Mobile Phase Ratio				
Substance	**80:20**	**60:40**	**50:50**	**40:60**	**20:80**
Rifampicin	0.460	0.240	0.090	0.025	0
Isoniazid	0.732	0.788	0.722	0.611	0.500
Pyrazinamide	0.732	0.775	0.667	0.500	0.426

In no instance did any of the pure lipids spotted on the plates leave the baseline, at the mobile phase compositions and ratios stated in tables **4.1**, **4.2** and **4.3.** The free drug remaining on the plate after the evaporation of the methanol did, however, produce the Rf values listed. Conversely, using normal phase TLC, the lipids all moved at the more polar solvent combinations, producing the Rf values listed in table **4.4**, while the drugs, did not move.

Table 4.4 TLC Rf values using hexane:ethyl acetate mobile phase combinations

Hexane : Ethyl Acetate					
	Mobile Phase Ratio				
Substance	**80:20**	**60:40**	**50:50**	**40:60**	**20:80**
Miglyol 812	0.394	0.306	0.245	0.104	0.014
Corn oil	0.304	0.292	0.203	0.086	0.030
Cottonseed oil	0.312	0.285	0.216	0.099	0
Sunflower oil	0.298	0.251	0.156	0.059	0.021
Soybean oil	0.273	0.237	0.167	0.031	0

Normal phase separation of the lipids and drugs was poor, as indicated, and for the purpose of extraction of the drugs into an aqueous fraction for injection onto the HPLC system at hand, the normal phase extraction solvent combination, will also not suffice.

In the case of each lipid solution of drug, in no instance did RIF, INH or PZA achieve adequate separation from the lipid purely by moving with the mobile phase solvent front. Elongated spots stretching from the baseline toward the solvent front, were visualised under the ultraviolet lamp at 254nm for RIF, INH and PZA. This is believed to be due to the slow partitioning of drug from the lipid and subsequent movement up the TLC plate with the mobile phase. This compares to the reverse phase TLC of the pure lipids, where the lipids all remained on the baseline, while the free drug moved up the plate. TLC indicated that chromatography of RIF, INH and PZA can produce adequate retention time separation from the lipids, but not if the drug is in solution in the lipid. This reinforces the decision not to inject the lipid solutions of the drugs directly onto the column, as well as the necessity of an extraction step prior to HPLC analysis of the drugs.

Originally, it was intended to use 1ml Waters Sep-Paks to separate the aqueous and lipid fractions, once the drugs had partitioned between the phases. For this reason, mobile phases for both normal phase and reverse phase TLC plates were evaluated for the extraction process. The aim of this stage was the identification of a mobile phase combination that would produce a significant separation of all three drugs, from the lipids under investigation. The reverse phase TLC produced favourable results with RIF, INH and PZA all moving with the solvent front, relative to the lipids which remain on the baseline. Solvent ratios in tables **4.1**, **4.2** and **4.3**, of 80:20 and 60:40 produced the best separation of the drugs from the lipids, at these higher organic to aqueous ratios. The acetonitrile:tBAH combination produced the best separation at 80:20 and 60:40 relative to the acetonitrile:water and the methanol:water combinations. For the purpose of cost effectiveness, considering the volumes of solvent that will ultimately be used in this extraction process, a compromise was

made, with the methanol:water extraction medium being selected for further development.

4.2.3.2 Extraction Medium Optimisation

The extraction medium selected was evaluated in terms of lipid solubility at various ratios of methanol:water. An excess of each lipid was added to 10ml portions of methanol:water at the ratios listed in table **4.3**, and agitated using a vortex mixer for a period of 10 minutes. On allowing the excess lipid globules to sediment, samples were drawn and analysed by UV/VIS Spectrophotometry. UV spectral scans were conducted on all solutions over a wavelength range of 200 to 400nm. This was possible for all lipids except for those at the 80:20 solvent ratio, which remained turbid. Increased sensitivity in absorption spectra over the 200 to 400nm wavelength range of analysis was encountered for the lipids of natural origin. Corn, cottonseed, soybean and sunflower oils, which are complex mixtures of varying chain length fatty acid esters, will absorb UV light due to the shear size of the molecules, however, absorption spectra are unreadable due to the complex nature of the mixtures. Miglyol 812, a synthetic lipid produced the only readable spectrum, as a greater degree of control over chain length exists. This lipid was selected as the model for further method development. A significant amount of miglyol 812 was soluble in the methanol:water medium, increasing as the methanol content increased. This could be problematic if the the methanol:water extraction medium (aqueous fraction) is injected directly onto the HPLC column, as discussed earlier.

4.2.3.3 Lipid Extraction Filter Efficiency

It is necessary to determine the quantity of lipid dissolved in the aqueous fraction prior to injection onto the HPLC. The efficiency of the filtration unit at removing the lipid from the aqueous fraction is therefore of great importance. A 6%v/v solution of miglyol 812 was

prepared and subjected to UV spectral analysis over the range of 200 to 400nm. The identified maxima were at 208.9, 214.8, 219.1 and 231.9nm with a λ_{max} at 240.4nm for the lipid. A minor peak at 273.7nm also existed.

A standard curve of absorbance versus concentration(%v/v) using chloroform as the solvent was constructed over a range of 10%v/v to 0.2%v/v of Miglyol 812, with absorbance measured at 240nm(table **4.5**).

0.2ml samples of lipid were accurately pipetted into 5ml volumetric flasks and made up to volume with methanol:water(60:40). The samples were agitated and at intervals over a period of 15 minutes, passed through 0.22 µm hydrophilic syringe filters and analysed. Different agitation times were used to determine if this would play a significant role in the amount of Miglyol 812 dissolving in the extraction medium, and hence the filter efficiency. Absorbance was measured at 240nm as well as over a 200 to 400nm wavelength range. At the various mixing times, the filtered fraction was found to have a maximum lipid concentration of 0.163%v/v.

4.2.4 Conclusion

Considering the fact that the volume injected onto the HPLC is only 20 µL, the amount of lipid injected onto the column is an insignificant 0.00326% of the volume stated in section **4.2.3.3**. The concern of the accumulation of lipid on the column, with time, is therefore minimal. Miglyol 812 does not absorb at the 260nm wavelength selected for analysis of RIF, INH and PZA and will not interfere with HPLC results. Agitation time investigated in section **4.2.3.3** indicated no significant increase of lipid dissolving in the aqueous fraction with time, with only a 1% increase in miglyol 812 concentration in the extraction medium at time greater than 2 minutes. The result is that a medium composed of 60:40 methanol:water can be used for the extraction of the drugs under investigation from a lipid,

with a minimal quantity of the lipid being present in the final filtrate. The purpose of this extraction procedure is to separate RIF, INH and PZA from the lipid, for HPLC analysis, without injecting the lipid directly onto the chromatographic column. The filter efficiency evaluation will be used as a periodic system check during later HPLC studies.

4.3 Method Validation

4.3.1 Linearity, Precision and Accuracy

Following principles previously outlined (Wahlich, J.C. *et. al.*, 1990; Edwardson, P.A.D. *et. al.*, 1990), a standard curve for Miglyol 812 was constructed over the concentration range listed in table **4.5** for UV/VIS Spectrophotometry.

Table 4.5 Standard curve concentrations for miglyol 812

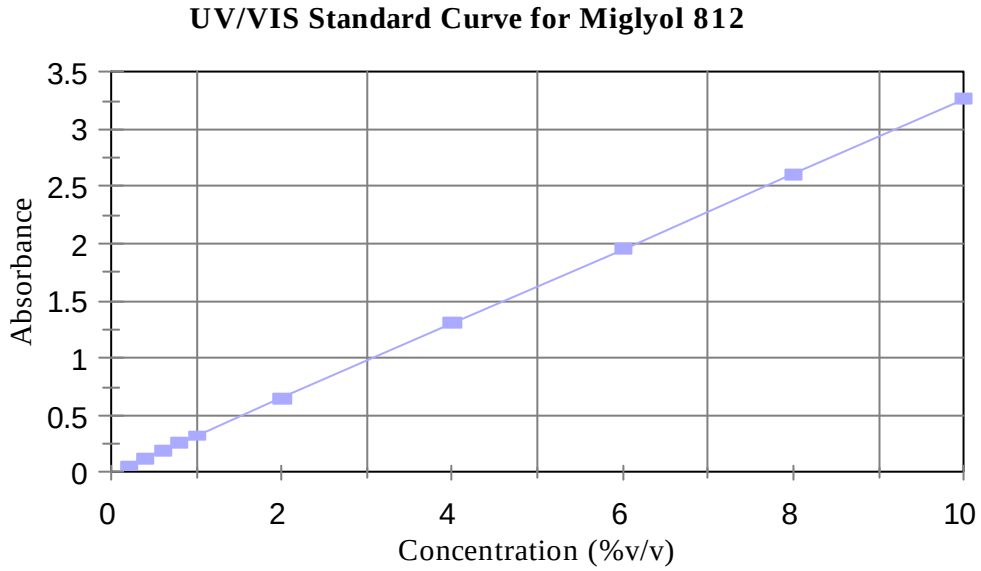
Lipid	**Concentration (%v/v)**									
Miglyol 812	10	8	6	4	2	1	0.8	0.6	0.4	0.2

A stock solution of miglyol 812 was prepared and diluted, to obtain the standard curve concentrations listed in table **4.5**. All determinations were performed in triplicate and a plot of absorbance versus concentration(%v/v) at 240nm wavelength constructed(figure **4.1)**. Miglyol 812 demonstrated an absorbance maximum at 240nm. The construction of this curve and the handling of the results was in adherance with ICH guidelines regarding range and linearity, covering more than the minimum 5 concentrations required for linearity and 70 to 130% of the test concentration(ICH, 1996).

Linear regression analysis produced the following results, in adherance with the stated ICH guidelines:

Slope	=	0.326355
Y-intercept	=	0
R^2 value	=	0.9982

Figure 4.1 Standard curve for miglyol 812



The Y-intercept passes through the origin, adhering to the ideal. The correlation coefficient (**R^2**) was greater than 0.998, which is an indication of the linearity of the curve and falls within acceptable statistical limits. Precision and accuracy were calculated following the previously outlined method in Chapter 3. Six replicate determinations were performed at concentrations at each end of the standard curve adhering to ICH specifications, the results of which, are listed in table **4.6.** RSD provides a measure of the precision of the analytical method and percentage of concentration a measure of it's accuracy, both of which are within acceptable statistical limits for the data listed in table **4.6**.

Table 4.6 Precision and accuracy

Precision and Accuracy Results				
Lipid	**Theoretical Concentration (%v/v)**	**Mean Concentration Determined (n=6)**	**Percentage Relative Standard Deviation**	**Percentage Concentration**
Miglyol 812	8.0**0**	8.01	0.01000	100.13
	0.4**0**	0.39	0.00009	97.5**0**

4.3.2 Drug-Filter Adsorption

The filter efficiency at removing lipid from the aqueous fraction is important, with it being desirable that the drugs being analysed do not adsorb onto the filter membrane, thus requiring the syringe filters to be evaluated.

0.100mg/ml stock solutions of RIF, INH and PZA were prepared, being made up to volume using the extraction medium (methanol:water). Samples of these solutions were injected onto the HPLC before and after being passed through a 0.22∶m hydrophilic syringe filter, the results being listed in table **4.7**. Standard curves previously constructed for RIF, INH and PZA for the analytical HPLC system were used to calculate drug concentrations(chapter 3).

As is evident from table **4.7** almost complete recovery was indicated for each drug, hence loss of drug from the final filtrate will not be significantly affected by the filter used. A small variation in percentage recovery can, however, be seen in order of drug hydrophilicity. The order of hydrophilicity being INH > PZA > RIF, which adheres to literature solubility profiles for these drugs(Brewer, G.A., 1977; Felder, E. *et. al.*, 1983; Gallo, G.G. *et. al.*,

1976).

Table 4.7 Drug recovery after filtration

Drug Recovery After Filtration			
	Concentration (mg/ml)		
Drug	**Before**	**After**	**Percentage Recovery**
RIF	0.0996	0.0972	97.661
INH	0.0995	0.0986	99.095
PZA	0.0980	0.0967	98.665

4.3.3 Drug Partition Coefficients

In order for the HPLC results obtained for the analysis of the aqueous fraction to be meaningful, the partition coefficients of RIF, INH and PZA between the miglyol 812 and the extraction medium need to be determined. A 2.00mg/ml stock solution of RIF in miglyol 812 was prepared, while 0.10mg/ml stock solutions of INH and PZA were prepared. 0.2ml samples of the solutions were accurately pipetted into 5ml volumetric flasks and made up to volume using the extraction medium. These samples were then subjected to agitation and analysis at time intervals of 1,2,3,4,5 and 10 minutes to examine the effect of agitation time on partitioning, the results of which are listed in table **4.8**.

The partition coefficients calculated approach unity for RIF, INH and PZA, with almost complete partitioning of the drugs into the extraction medium. The effect of agitation time has little effect on the degree of drug partitioning between the lipid and extraction medium, with complete partitioning for RIF and INH after 1 minute and complete partitioning for all three drugs after 2 minutes.

Table 4.8 Partition coefficients

Partition Coefficients				
		Concentration (mg/ml)		
Drug	**Agitation Time (min)**	**Lipid [j]**	**Aqueous [t]**	**Partition Coefficient**
RIF	**1**	2.000	1.998	0.999
	2	2.000	2.035	1.018
	3	2.000	2.005	1.003
	4	2.000	2.035	1.018
	5	2.000	2.000	1.000
	10	2.000	2.013	1.007
INH	**1**	0.100	0.098	0.980
	2	0.100	0.102	1.020
	3	0.100	0.094	0.940
	4	0.100	0.101	1.010
	5	0.100	0.099	0.990
	10	0.100	0.097	0.970
PZA	**1**	0.100	0.077	0.770
	2	0.100	0.100	1.000
	3	0.100	0.088	0.880
	4	0.100	0.100	1.000
	5	0.100	0.099	0.990
	10	0.100	0.098	0.980

t Concentration of drug in aqueous fraction after partitioning.

j Concentration of drug in lipid before partitioning.

4.3.4 Conclusion

An accurate, reproducible, indirect method was developed and validated for the determination of RIF, INH and PZA in miglyol 812. As will be made clear in chapters 2,5,6 and 7, miglyol 812 is the lipid selected from the literature, in which RIF is most soluble of all those tested, and hence will be the prime focus of all subsequent work. The method developed and validated involves the partitioning of the drug from 0.2ml of the lipid sample into a 60:40 methanol:water aqueous fraction after 2 minutes of agitation, with subsequent filtration through a 0.22 μm Cameo® hydrophilic syringe filter. This method produced optimum partitioning of RIF, INH and PZA into the aqueous fraction, while minimising the amount of lipid that appears in the final aqueous filtrate.

A successful UV Spectrophotometric method has been developed and validated, to be used as a system check in later lipid degradation studies and final formulation studies, prior to the injection of aqueous filtrate samples onto the chromatographic column.

Chapter Five

Microemulsion Phase Mapping

5.1 Introduction

It is argued that a microemulsion is a single optically isotropic and thermodynamically stable liquid solution, excluding aggregate structures, eliminating thermodynamically unstable emulsion systems and therefore should not be considered an emulsion(Gautier, J.C., 1995; Lieberman, H.A., *et al.*, 1988). This conundrum is not to be solved in this work and is left to the authorities to debate. The principles that apply to the process of emulsification are the same whether formulating macro or microemulsions, with the physical differences being, the size of the droplets formed, stability and appearance, as defined and outlined in chapter 1.

In the presence of a stabilising agent, two possible types of emulsion can be formed. Oil-in-water type emulsions (o/w) have the lipid phase dispersed as droplets throughout the continuous aqueous phase. A water-in-oil (w/o) emulsion has the aqueous phase dispersed as droplets throughout the continuous lipid phase. An emulsion represents a highly unstable system, with the tendency of the system to reduce the interfacial surface free energy until the minimum possible value is attained, achieved by decreasing the interfacial surface area through the coalescence of the droplets(Ansel, H.C., 1981; Lieberman, H.A., *et al.*, 1988). The surface free energy arises due to the cohesive forces between similar molecules being greater than the adhesive forces between two immiscible liquids. It is the cohesive forces which result in coalescence of the droplets. Thermodynamic stability is concerned with the free energy of the system. Reduction of exposed surface area and hence free energy of the system, approaches thermodynamic stability. The common macroemulsion is a system which is not thermodynamically stable, as once the phases separate, the emulsion will

not reform on it's own(Florence, A.T, *et al.*, 1981; Lieberman, H.A., *et al.*, 1988). For thermodynamic stability to be a reality, the interfacial free energy must be extremely small, approaching 10^{-3} mN/m(Ho, H-O., *et al.*, 1996; Lieberman, H.A., *et al.*, 1988). Interfacial free energy of common macroemulsions varies from 1 to 10 mN/m. Thermodynamic stability means reversibility(Lieberman, H.A., *et al.*, 1988). Unless interfacial tension between the lipid and aqueous phases is zero, there will always be a tendency for the oil or water droplets to reduce the area of oil and water contact(Florence, A.T, *et al.*, 1981). It is by observing the forces involved in the emulsification process that it is possible to determine an appropriate method of stabilisation and hence an appropriate and viable, case specific theory of emulsification.

Reduction of surface tension in the common emulsion, has little effect on stability, while for microemulsions, the ultralow interfacial tension reaches the level making thermodynamic stability possible. Emulsifiers, as a result of their structure, are attracted to both oil and water phases tending to reside at the interface. Their presence reduces surface tension, but does not always lead to a stable system, as very low interfacial tensions can lead to instability(Lieberman, H.A., *et al.*, 1988). An emulsifier consists of a polar head group and a hydrocarbon chain, with the hydrocarbon chain soluble in the oil and the polar group in water, resulting in the preferentially orientating at the oil-water interface. Pharmaceutical emulsions intended for oral administration are typically of the o/w type, which are of particular use when looking at the antitubercular agents intended to be formulated. In terms of an o/w emulsion, emulsifiers stabilise the dispersed oil droplets by their presence at the interface, with the dispersed droplets generally of a diameter large enough to refract light, giving macroemulsions a turbid appearance. The type of emulsion formed is dependent on the phase ratio of the aqueous and lipid phases, together with the type, concentration and preferred solubility of the emulsifying agents used. On the basis of surfactant molecular structure, portions of the same molecule can act independently, when it comes to surface action. The portion of a surfactant molecule rich in hydroxyl groups will

have an affinity for the aqueous phase, due to hydrogen bonding interactions, whereas the hydrocarbon chain portion does not. Hence, the surfactant molecules are not only squeezed to the surface, but also align in such a manner that the hydroxyl groups face the aqueous phase, and the hydrocarbon chains face away. As hydrocarbon chain length increases, the tendency to concentrate at the surface increases, this effect decreases surface tension. If the concentration of the surfactant in solution increases beyond the level at which it can be accommodated at the surface, micelles(associated structures) form(Lawrence, M.J., 1994).

The Oriented Wedge theory of emulsification assumes monomolecular layers of emulsifying agent curved around a droplet of the internal phase of the emulsion. The theory assumes that certain emulsifying agents orient themselves around and within a liquid, in a manner reflecting their solubility in the particular liquid(Ansel, H.C., 1981). In a system containing two immiscible liquids, the emulsifying agent will be preferentially soluble in the one and not the other, hence, embedded more deeply and tenaciously in that phase. Depending on the shape and size of the molecules, solubility characteristics and thus their orientation, the wedge-shape arrangement will form, surrounding the oil or water droplets. A surfactant with greater hydrophilic character will form o/w emulsions, while greater lipophilic character will form w/o emulsions(Ansel, H.C., 1981). This theory, however does not accurately depict the molecular arrangement of the emulsifying agent.

Hydrophile-lipophile balance, phase inversion temperature and the critical packing parameter are three criteria commonly employed in the selection of a surfactant combination to stabilise a particular system.

5.1.1 Hydrophile-Lipophile Balance (HLB)

The Hydrophile-Lipophile Balance (HLB) is a system that has been devised to classify surfactants, by calculating this value(Ansel, H.C., 1981; Lund, W., 1994). The choice of surfactant to be used as the emulsifier, is determined by the average HLB requirement of the proposed emulsion system, while a combination of surfactants may also be used to obtain the desired HLB(Aboofazeli, R., 1993). The HLB number is calculated according to an empirical formula, for nonionic surfactants, assigning a value from 0 to 20, along an arbitrary scale. Surfactants with HLB values between 8 and 18 generally form o/w emulsions and are used as detergents and solubilising agents, while those between 3 and 6 form w/o emulsions(Ansel, H.C., 1981; Aboofazeli, R., 1993; Bhargava, H.N., 1987; Lund, W., 1994). There are numerous methods for the calculation of the HLB, depending on the type of substance, with the value assigned indicative of the polarity of the agent, with lipophilic agents having low HLB values and hydrophilic agents having high values. The HLB system, does not take into account the effect of additives or temperature. The presence in an emulsion of agents which salt-in or salt-out nonionic surfactant chains, will respectively increase and decrease the effective HLB values. Generally, it is believed that the optimal HLB of the system should be close to that of the oil(Magdassi, S., 1985).

5.1.2 Phase Inversion Temperature (PIT)

The Phase Inversion Temperature (PIT) of a system depends on the oil, the surfactant and any additives present. The PIT is the temperature at which the hydrophile-lipophile properties just balance, and can be considered a measure of the HLB of a surfactant in a given system(Lieberman, H.A., *et al.*, 1988). For o/w emulsions, phase inversion to a w/o emulsion occurs at elevated temperature. If the phase inversion temperature of a system is greater than 30^0C above the intended temperature range for use of an o/w emulsion, then the emulsion will be stable to temperature fluctuations, with the reverse being true for w/o

emulsions. Phase inversion is commonly encountered during the sterilisation or manufacturing process, but may also be used to produce a more stable and finer emulsion(Lieberman, H.A., *et al.*, 1988). A useful guideline is that as the lipid solubility of the surfactant increases, the PIT decreases. Electrolytes possess the ability to alter the PIT of nonionic surfactants such as the polyoxyethylene ethers which are commonly used in pharmaceutical preparations.

5.1.3 Critical Packing Parameter (CPP)

The Critical Packing Parameter (CPP) is defined as the ratio of the alkyl chain volume of a surfactant molecule, to the area occupied at the interface by the polar head group and the critical alkyl chain length. The ratio defines the spontaneous curvature of a particular surfactant, and is used in conjunction with the HLB for the purpose of selecting an appropriate emulsifier for a system. The geometric theory of surfactant aggregation predicts the formation of an o/w microemulsion when the surfactant structural properties result in a CPP between 0.5 and 1.0. A value greater than 1 causes the formation of reverse aggregates, whereas values lower than 0.5 indicate the presence of normal aggregates(Israelachvili, J.N., *et al.*, 1976; Lawrence, M.J., 1994). Although amendment of the CPP equation is advocated, due to the interpenetration of the oil into the hydrophobic region of the surfactant film, it is not readily quantified. Penetration of the oil molecule into the hydrocarbon portion of the surfactant interface, has the effect of increasing the alkyl chain volume of the interface and, therefore, increases the effective CPP(Aboofazeli, R., 1993; Lawrence, M.J., 1994; Lieberman, H.A., *et al.*, 1988). This effect may be compensated if the oil penetrates as far as the head group, where it will also increase the area occupied by the polar head at the interface. The CPP characterises the shape of the average volume per molecule in a given phase and the area occupied at the interface by the polar head, with the length of the hydrocarbon chain relating to parameters of the phase, and not to the intrinsic properties of the molecule. The CPP of a molecule is strictly a function of

phase, making it easy to see why the CPP fails to predict which surfactant will form a microemulsion.

In order for a proposed theory to be of any use, it must explain the type of emulsion formed and the stability of that system. While the use of the HLB of a surfactant and the CPP have been advocated, they are too simplistic to be of any great use in microemulsion formulation(Israelachvili, J.N., *et al.*, 1976; Lawrence, M.J., 1994). This has been proven in work done using nonionic polyoxyethylene ether surfactants, where surfactants of similar HLB and CPP values did not both form microemulsion systems. Other factors such as fluidity of the hydrophobic chains are important in determining whether or not a microemulsion will form. The HLB system does not take into account the surfactant concentration, the oil:water ratio or the temperature, and lacks sensitivity to the structure of the surfactant. Some believe that the HLB system has been superceded by the CPP, however, the same limitations that apply to the HLB system, apply to the CPP. It also fails to explain why polyoxyethylene ether surfactants with high CPP values fail to produce microemulsions.

5.1.4 Practical emulsification

No single theory can be applied to the process of emulsification, that can adequately predict and explain the phase behaviour of these systems, due to the fact that different emulsifiers produce stable emulsions, based on principles unique to the emulsifier type.

Schulman *et al.*, in previous papers, have outlined the following essential conditions for the formation of a microemulsion(Lawrence, M.J., 1994):

1 - The production of a transient negative interfacial tension at the oil/water interface.

2 - The formation of a highly fluid interfacial surfactant film.

3 - The penetration and association of the molecules of the oil phase with the interfacial surfactant film.

These observations may be considered an over simplification, but they provide a useful starting point for microemulsion formulation. A small, rather than a transient negative interfacial tension is now recognised as one of the conditions for microemulsion formulation, in most cases requiring a cosurfactant. The exception to this rule is the nonionic surfactants, which at their PIT also exhibit ultralow interfacial tensions in the order of 10^{-1} to 10^{-2} mN/m(Lawrence, M.J., 1994; Sarciaux, J.M., 1995 (b)). This explains why nonionic surfactants at their PIT frequently form microemulsions over a wide range of compositions. At temperatures below the PIT any microemulsions formed by nonionic surfactants are of the o/w type, above the PIT, the microemulsion will be water dispersed in oil. For this reason, the focus of this work is on the nonionic imwitor, Brij and cremophor surfactants and the zwitterionic ovothin 200(lecithin).

5.2 Instrumentation and Equipment

A vortex mixer made by Gemmy Industrial Corporation(Taiwan, R.O.C.) was used to homogenise the surfactants, lipid and aqueous phases. A 1ml auto-pipette manufactured by Eppindorf(Germany) was used to pipette all aqueous fractions.

5.3 Solvents and Reagents

Cremophor RH, isopropyl myristate(IPM) and sorbitol(70%m/v) were donated by the Druggists Group Reasearch Unit(Pharmacare-Lennon, RSA). Miglyol 812, imwitor 308 and imwitor 742 were donated by Hüls, Southern Africa, with ovothin 200, epikuron 200 and epikuron 170 donated by the Lucas Meyer Company, Germany. Brij 97 was purchased from Sigma, USA, and crillet 3 donated by Croda, Southern Africa. Absolute

ethanol was purchased from BDH, England. All aqueous fractions were prepared using double distilled deionised water, obtained from a Milli-RO 15 water purification system, manufactured by Millipore®(Massachusetts, USA).

5.4 Methodology

Ternary and pseudo-ternary phase diagrams were constructed by titrating a series of mixtures (lipid:cosurfactant and/or surfactant) with water at room temperature. Surfactant:cosurfactant ratios of 9:1, 7:3, 5:5, 3:7 and 1:9 were prepared. Mixtures of lipid and surfactant(SAA), with or without cosurfactant, were prepared to produce 3g fractions of SAA mixture:lipid in the ratios of 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 5.5:4.5, 5:5, 4.5:5.5, 4:6, 3.5:6.5, 3:7, 2.5:7.5, 2:8, 1.5:8.5, 1:9 and 0.5:9.5. Water was added to each fraction in 2.5% portions, followed by agitation on a vortex mixer with the mixture allowed to stand and stabilise. The phases formed were visually assessed after each addition and classified as isotropic, liquid crystalline or coarse emulsion. Isotropic regions in each phase diagram are transparent fluid microemulsion and mixed micellar solution regions in areas of low oil or low water composition and are designated by the notations **L** and $\mathbf{L_a}$. Liquid crystalline regions are designated as **LC**, being defined as birefringent gel-like phases, tending to be a region of transition between high lipid and high water content phases. Only stable single phase regions were recorded in the ternary and pseudo-ternary phase diagrams mapped including coarse emulsions that proved stable with time, these designated by **E** and $\mathbf{E_a}$. The distinction between the compositions of regions **L** and $\mathbf{L_a}$ or **E** and $\mathbf{E_a}$ was not conclusively defined. Based on the aqueous and lipid composition of each region, the distinction between o/w and w/o emulsions and microemulsions was made. Titration with water was performed until near 90% of the total mixture composition, effectively covering the entire range of single phase regions that can form. The phase diagrams were constructed with the top apex representing 100% water content, the left apex representing 100% surfactant/cosurfactant and the right apex representing 100% lipid.

5.5 Results and Discussion

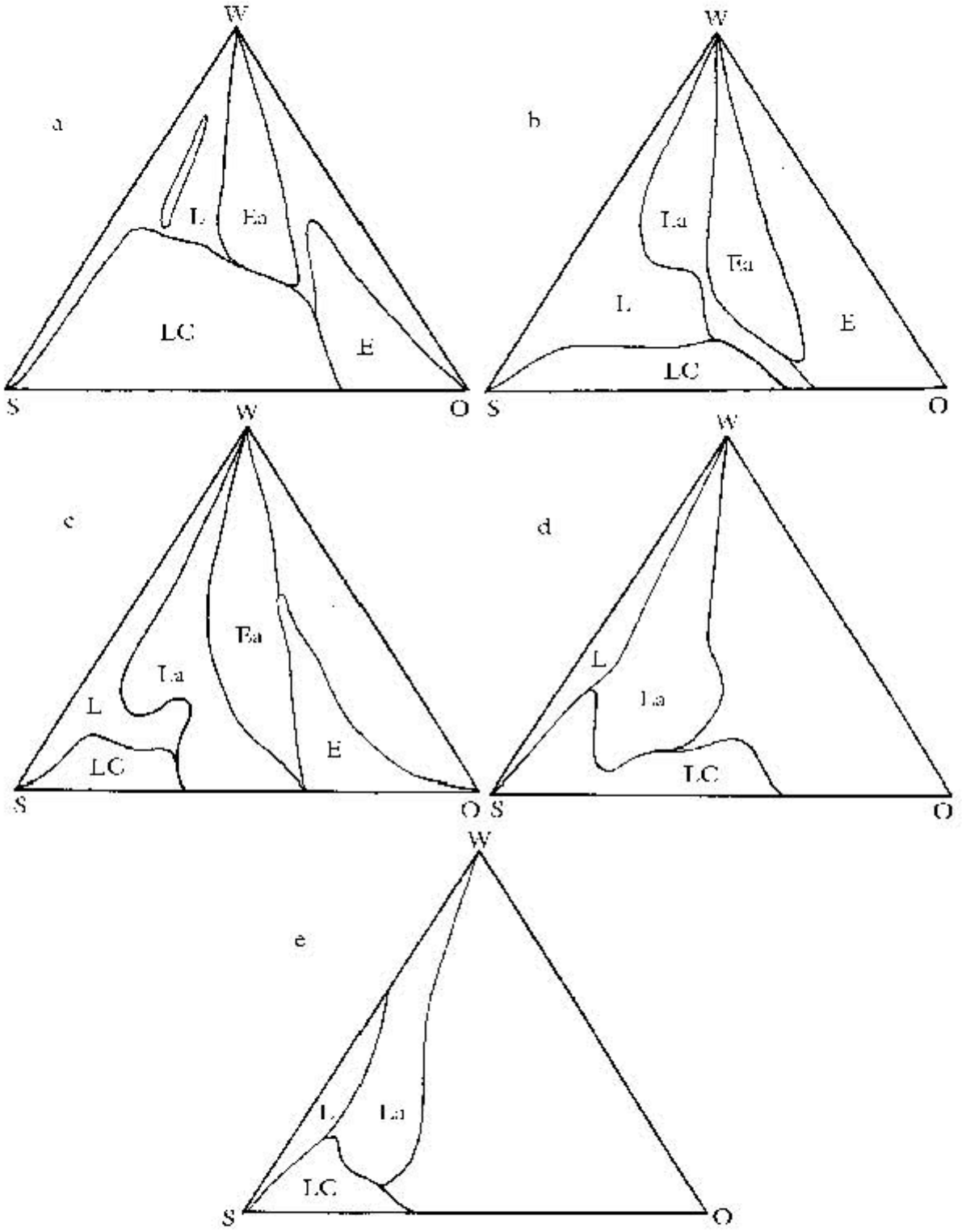
The initial goal of this chapter was to map as many microemulsion systems as would be possible. This had to be limited, due to the number of combinations, given the components at hand. Miglyol 812 was selected as the lipid of choice in the phase mapping due to the solubility profiles of RIF, INH and PZA, for it's accepted use in oral pharmaceutical preparations and for the reasons justified in this section. Corn oil and isopropyl myristate(IPM) were used in the mapping of selected systems, with this route abandoned due to the poor RIF solubility in them and IPM not being suitable for ingestion.

Ovothin 200, epikuron 200 and epikuron 170 (with sorbitol) systems were mapped using IPM as the lipid phase. These systems did not produce microemulsion phases of any use, with the difficulty of working with the sticky semisolid lecithins being a hinderance. Brij 97 and sorbitol systems mapped using IPM as lipid produced some extensive well defined microemulsion regions. Due to this promise, the same surfactant combinations were used to map phase diagrams with corn oil and miglyol 812. The results for the miglyol 812 are reported in this chapter. Corn oil did not produce the same results as were achieved for the Brij system using IPM or miglyol 812, with small isotropic regions only formed at high Brij content. Ovothin 200 and absolute ethanol as cosurfactant were used to map a series of phase diagrams which produced some favourable results, despite the reluctance expressed in the use of ethanol as a formulation component.

5.5.1 Cremophor/sorbitol combination

The phase diagrams mapped for the cremophor and sorbitol surfactant combination, are

Figure 5.1 Pseudo-ternary phase diagrams for cremophor RH and sorbitol(70%m/v) as surfactants and miglyol 812 as lipid

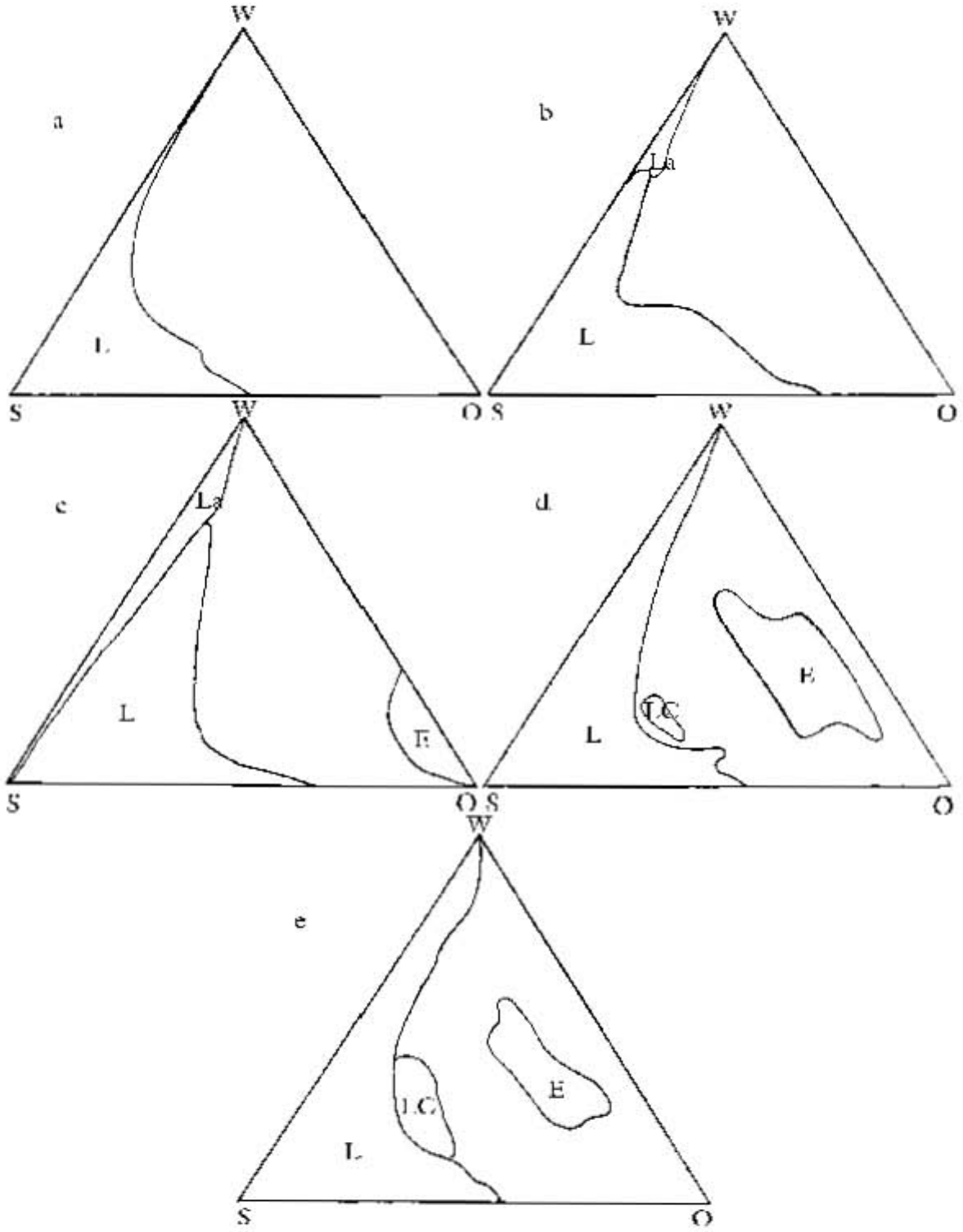


(a)Cremophor RH:sorbitol(9:1), (b)Cremophor RH:sorbitol(7:3), (c)Cremophor RH:sorbitol(5:5), (d)Cremophor RH:sorbitol(3:7), (e)Cremophor RH:sorbitol(1:9)

displayed in figure **5.1.** Cremophor RH has been known to form o/w microemulsions without the aid of a cosurfactant, due to it's hydrophilicity (HLB 13). In this work, a 70%m/v sorbitol solution is employed as a cosurfactant to the cremophor, the sorbitol being a polyhydric alcohol. At high cremophor concentration and respective low sorbitol concentrations, several formulation types were evident. Initially a large liquid crystalline (**LC**) region exists as well as two separate coarse emulsion regions **E** and $\mathbf{E_a}$, which subsequently regress as the proportion of cremophor decreases and the transparent isotropic o/w microemulsion region designated by **L** increases. At a ratio of cremophor:sorbitol of 7:3 in figure **5.1**(b), a second more viscous opalescent region designated by $\mathbf{L_a}$ emerges, with the size of **L** peaking here and decreasing as the proportion of sorbitol increases. Due to the structure of cremophor(polyoxyl 40 hydrogenated castor oil) possessing a large polar head and long hydrophobic tail, it tends to form microemulsions over a wide concentration range, being stable to dilution, up to a point. The area of existence of **L** is greater when sorbitol is used in low concentration, due to the added fluidisation of the interfacial film and the decreased CPP produced by it's dissolution in the aqueous continuous phase and incorporation into the film. It must be noted that assessments are made purely on a visual basis, with the isotropic and liquid crystalline phases having compositions of normal and reverse microemulsion droplets or weakly structured aggregates.

In work using n-alkyl polyoxyethylene ether surfactants, such as Brij 97, and various polyhydric alcohols as cosurfactants, it has been found that the type of cosurfactant and the relative concentration of the surfactant and cosurfactant have a pronounced effect on the region of existence of microemulsions(Lawrence, M.J., 1994). As the concentration of the polyhydric alcohol cosurfactant increases, so the microemulsion area of existence decreases, being due to the opposing effects of the surfactant and cosurfactant on the interfacial film(Kale, N.J., *et al.*. 1989). The surfactant causes the interfacial film to condense, while addition of the polyhydric alcohol cosurfactant causes the film to expand.

Figure 5.2 Pseudo-ternary phase diagrams for imwitor 308 and crillet 3 as surfactants and miglyol 812 as lipid



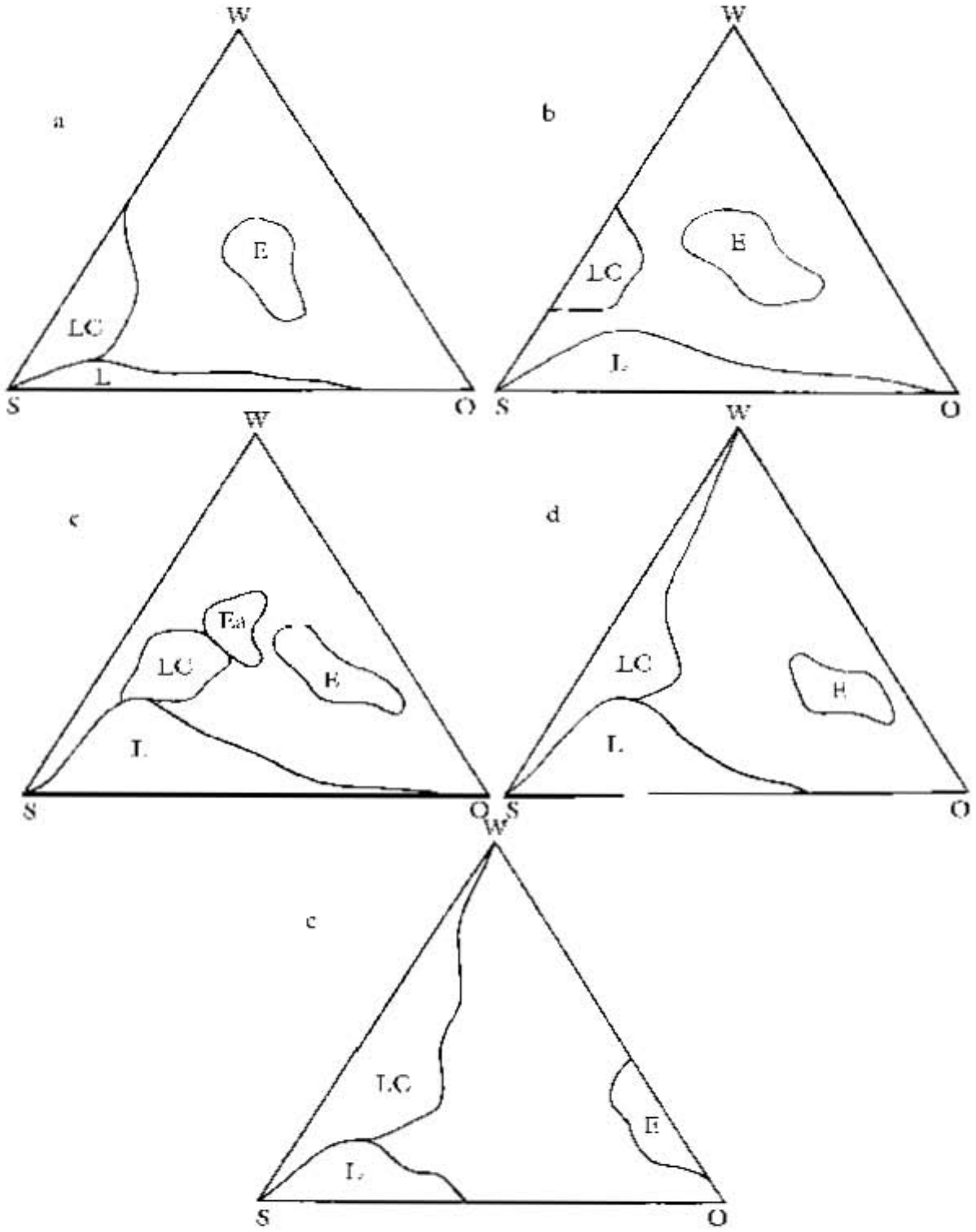
(a)Imwitor 308:crillet 3(9:1), (b)Imwitor 308:crillet 3(7:3),
(c)Imwitor 308:crillet 3(5:5), (d)Imwitor 308:crillet 3(3:7),
(e)Imwitor 308:crillet 3(1:9)

Polyhydric alcohols exerting their effect in microemulsification by incorporation into the interfacial film, is still under dispute. The presence of polyhydric alcohols such as glycerol and sorbitol, which are of interest and great use in pharmaceutical formulation, can decrease the PIT, resulting in decreased stability.

5.5.2 Imwitor 308/crillet 3 combination

Imwitor 308 consists of a mixture of 80-90% monoglycerides of stearic and palmitic acids and variable quantities of di- and triglycerides, more commonly known as glyceryl monostearate(Martindale, 1989). It is a nonionic surfactant with an HLB value of approximately 6, being very lipophilic. Due to the lipophilic nature of the imwitor 308, it will not form an o/w microemulsion on it's own, requiring a cosurfactant. Crillet 3(Polyoxyethylene 20 sorbitan monooleate), is a hydrophilic nonionic surfactant with an HLB value of 15, and is commonly used in the production of stable o/w emulsions and as a solubility enhancing agent(Martindale, 1989). The synergistic effect of these two agents is displayed in the pseudo-ternary phase diagrams constructed in figure **5.2**. The combined effect of the two surfactants is manifested by a larger area of existence of the isotropic region **L**, than in any other systems mapped. The size of the region increases as the crillet 3 composition increases, with the largest and most dilutable microemulsion region existing in figure **5.2**(c), capable of solubilising up to 25-30% miglyol 812. The fluid complex interfacial film formed by the small polar head, long hydrophobic chained imwitor 308 and the large polar head, shorter chained crillet 3, is stable to disruption, with leaching of the water soluble crillet 3 not occuring to any significant extent on dilution. It is this system that is selected for formulation in chapter 7. A single coarse emulsion region appears in figure **5.2**(c), increasing in size as the composition of crillet 3 increases. An isotropic region $\mathbf{L_a}$ exists in figures **5.2**(b) and **5.2**(c) , but it is not known if it is part of the o/w microemulsion region **L** or not, with this region being more fluid. The liquid crystalline region is small, only emerging at high crillet 3 composition as indicated by

Figure 5.3 Pseudo-ternary phase diagrams for imwitor 742 and crillet 3 as surfactants and miglyol 812 as lipid



(a)Imwitor 742:crillet 3(9:1), (b)Imwitor 742:crillet 3(7:3),
(c)Imwitor 742:crillet 3(5:5), (d)Imwitor 742:crillet 3(3:7),
(e)Imwitor 742:crillet 3(1:9)

figures **5.2**(d) and **5.2**(e). Other systems mapped can incorporate larger quantities of lipid, but do not maintain the degree of dilutability and stability expressed by the microemulsion formed in this system.

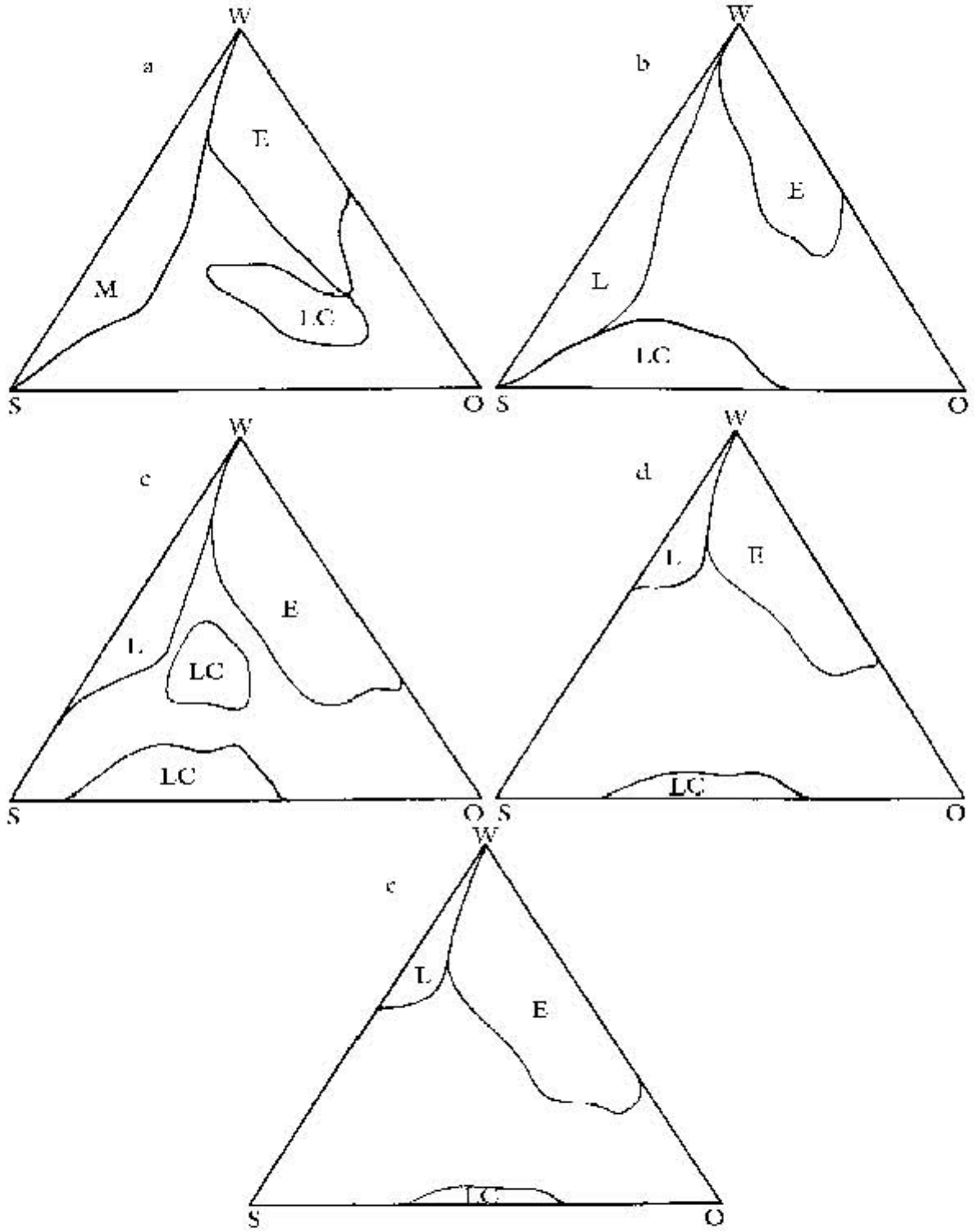
5.5.3 Imwitor 742/crillet 3 combination

The phase diagrams constructed for imwitor 742 are reported in figure **5.3**. Imwitor 742 is a mixture of mono- and diglycerides of capric and caprylic acid esters, being more lipophilic than imwitor 308, possessing an HLB of approximately 3.5. This is demonstrated by the phase diagrams mapped. Despite the use of the hydrophilic crillet 3 as cosurfactant, the interfacial film formed incorporating imwitor 742 is very rigid, forming the microemulsion region **L** over a range of high to low surfactant and lipid composition, but at very low water content. As the proportion of crillet 3 increases relative to imwitor 742, **L** migrates toward the surfactant apex, with the amount of lipid solubilised decreasing, but with the amount of water that can be incorporated increasing. This microemulsion region never reaches a point of dilutability, with a gel-like liquid crystalline region **LC** emerging, as water composition increases at higher crillet 3 and lower imwitor 742 proportions. In figure **5.3**(e) **LC** reaches a point where it is dilutable with water, with a maximum of 25% miglyol 812 that can be solubilised. A small coarse emulsion region, **E**, forms, but is not stable to dilution with water. At the ratio of imwitor 742:crillet 3 in figure **5.3**(c), a second coarse emulsion region $\mathbf{E_a}$ emerges, which is believed to be a disjointed portion of **E**.

5.5.4 Brij 97/sorbitol combination

Figure **5.4** illustrates the pseudo-ternary phase diagrams obtained for the Brij 97 and 70%m/v sorbitol systems mapped. As is evident from the diagrams, the microemulsion region **L** is quite substantial at high Brij 97 composition. This region decreases in size,

Figure 5.4 Pseudo-ternary phase diagrams for Brij 97 and sorbitol(70%m/v) as surfactants and miglyol 812 as lipid



(a)Brij 97:sorbitol(9:1), (b)Brij 97:sorbitol(7:3),
(c)Brij 97:sorbitol(5:5), (d)Brij 97:sorbitol(3:7),
(e)Brij 97:sorbitol(1:9)

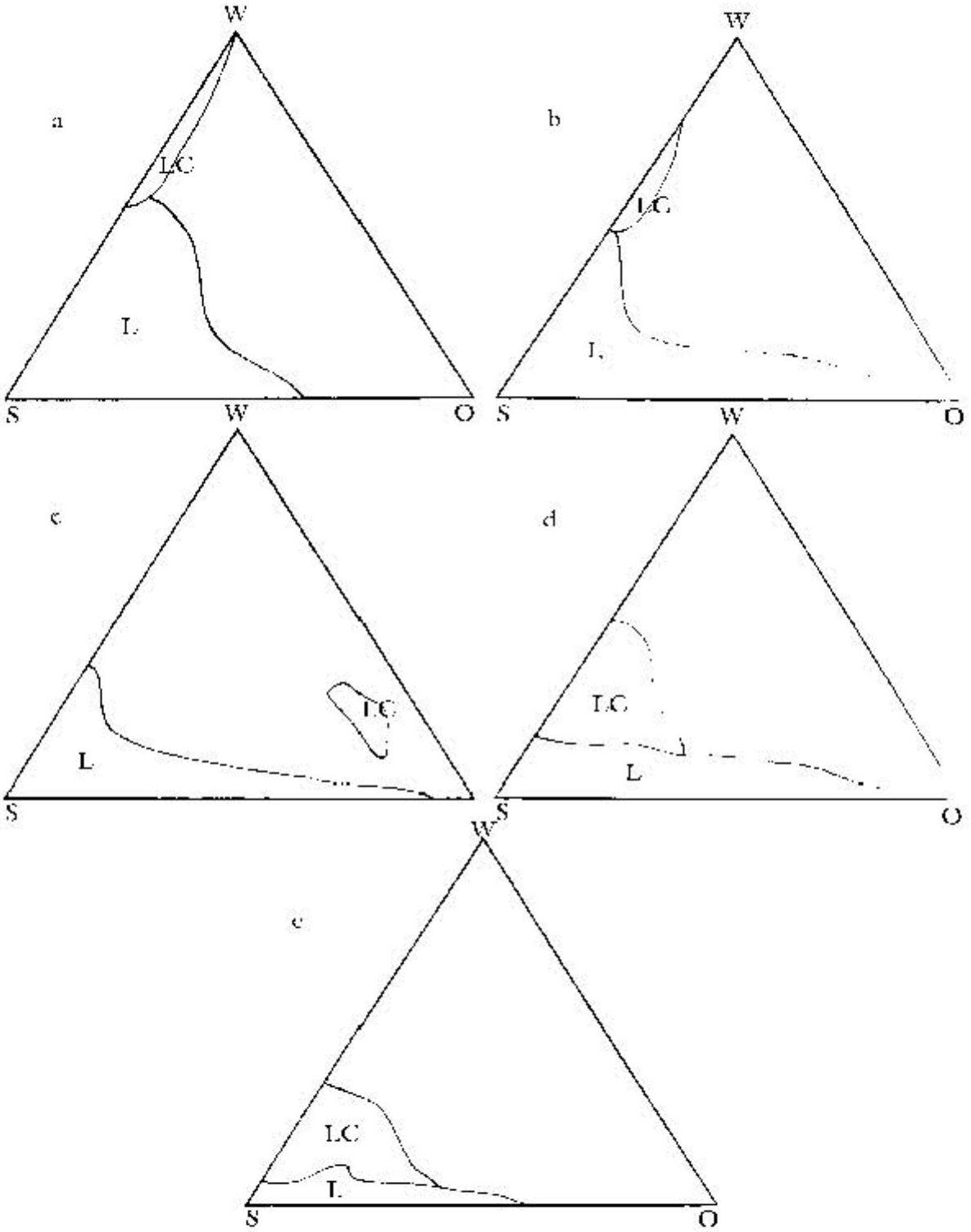
due to the reason previously mentioned when discussing the cremophor system employing sorbitol as a cosurfactant. This confirms the tendency of Brij 97 to form microemulsions in the absence of a cosurfactant, as **L** increases in size, approaching complete dilutability with water, as the sorbitol content decreases. The effect noted with an increase in sorbitol content is an increase in the amount of miglyol 812 solubilised, however, this occurs with a decrease in water content and size of the microemulsion phase.

The polyoxyethylene ether surfactants are particularly attractive, in that they can produce a microemulsion without the need for the addition of a cosurfactant(Lawrence, M.J., 1994). Work has shown that it is possible to incorporate certain lipophilic drugs into microemulsions using a vegatable oil and the polyoxyethylene surfactant Brij 97, without using a cosurfactant, although the structure of the drug to be incorporated may affect the stability of the microemulsion(Lawrence, M.J., 1994). The presence of a *cis* double bond in the hydrocarbon chain of Brij 97 introduces a degree of disorder, forming a less tightly packed film, adding fluidity to the interface which results in the formation a microemulsion, where other surfactants of like HLB and CPP do not. Brij 97 also possesses the desirable property in that it is much less sensitive to the size of the oil incorporated in it's ability to form a microemulsion. The systems mapped for Brij 97 and sorbitol although quite desirable, forming a large area of existence for the microemulsion, incorporating up to 55% miglyol 812 and being dilutable, proved to be unusable in this project. At the high Brij 97 content that this occurs at, when RIF is incorporated into it, a semi-solid gel is formed, this probably due to the large structure of the drug complexing with the long chain surfactant molecules forming an unpourable matrix.

5.5.5 Ovothin 200/ethanol combination

Figure **5.5** illustrates the phase diagrams mapped for the ovothin 200 and absolute ethanol surfactant combination. Ovothin 200 is an egg lecithin composed of greater than 92%

Figure 5.5 Pseudo-ternary phase diagrams for ovothin 200 and ethanol as surfactants and miglyol 812 as lipid



(a)Ovothin 200:sorbitol(9:1), (b)Ovothin 200:sorbitol(7:3), (c)Ovothin 200:sorbitol(5:5), (d)Ovothin 200:sorbitol(3:7), (e)Ovothin 200:sorbitol(1:9)

phosphatidylcholine. Epikuron 170 and epikuron 200 are lecithins of soybean origin with phosphatidylcholine content of greater than 68% and 94% respectively, with epikuron 170 also containing 10% phosphatidylethanolamine. The difference between soybean and egg lecithin being the nature of the component fatty acids. Previous work found there to be no significant difference in the microemulsion forming ability between the different lecithins, confirmed in the early stages of this project(Aboofazeli, R., *et al.*, 1994 (a)). These findings are, however, disputed by Attwood *et al.* in an earlier publication(Attwood, D., 1992).

A transparent isotropic region **L** was formed at high ovothin composition relative to ethanol. As the ovothin content decreased, this region shifted towards the water apex, forming a microemulsion at lower surfactant content, but also with a reduced lipid solubilising capacity. A liquid crystalline region **LC** emerged at low water content, decreasing in size as the ethanol content increased. The maximum miglyol 812 incorporated into the microemulsion of 15% occured at the ovothin:ethanol composition in figure **5.5**(a). The ability of this system to form a microemulsion is purely as a result of the fluidity introduced to the interfacial film by the ethanol. A fluid interfacial film is produced by the addition of a cosurfactant, this is demonstrated particularly well in the case of the ovothin 200 systems mapped in figure **5.5**. Lecithin on it's own is too lipophilic to form a stable microemulsion, tending to form lamellar phases and bilayers over a limited range, due to it's high CPP of approximately 0.8(Israelachvili, J.N., *et al.*, 1976; Shinoda, K., *et al.*, 1991; Trotta, M., *et al.*, 1995). This strong hydrophobicity is exhibited due to the two long hydrocarbon chains and the lipophilicity of the zwitterionic polar head groups that are also strongly hydrated(Saint Ruth, H., *et al.*, 1995). The presence of the ethanol, a short chain cosurfactant, functions by dissolving in the aqueous phase thus reducing it's hydrophilicity and therefore the CPP. One implication of the need for a fluid interfacial film, is that the surfactants used should not possess very long hydrophobic chains, and if they do, should contain sufficient unsaturated bonds to ensure that the hydrophobic chains are in a fluid state, hence the hydrophobe should not have a melting point above the temperature at

which the microemulsion is to be used. Incorporation of the ethanol also increases the fluidity of the interfacial film, reducing the rigidity imparted by the long hydrophobic chains of the lecithin, allowing the interfacial film to adopt the curvatures required to form stable microemulsions(De Gennes, P.G., *et al.*, 1982; Shinoda, K., *et al.*, 1991). It has been noted in previous work and confirmed in figure **5.5**, that as the proportion of ethanol decreases, the size of the microemulsion region decreases(Saint Ruth, H., *et al.*, 1995).

Unfortunately, microemulsions which incorporate a cosurfactant into the interfacial film tend to be unstable, in that they are destroyed on dilution. The cosurfactant partitions into the interfacial film, the water and oil phases, hence on dilution, the cosurfactant leaves the interface entering the continuous phase to restore the equilibrium, which destroys the film. This was particularly demonstrated for the ovothin 200 and imwitor 742 microemulsions produced.

The size of the oil molecules is important in determining whether a microemulsion is formed or not. When selecting an oil for emulsification, the general rule is that greater solubilisation is achieved with smaller oils (Lawrence, M.J., 1994). No microemulsion is formed if the chain length of the oil is too great, this effect being demonstrated for both o/w and w/o microemulsions if the hydrophobic chains of the surfactant are not longer than those of the oil to be incorporated(Aboofazeli, R., *et al.*, 1995; Florence, A.T., *et al.*, 1982). This is probably due to greater penetration of shorter oils into the surfactant film, or due to solubilisation of long hydrocarbon chain oils requiring a higher temperature for maximum solubilisation. It has been shown that at room temperature in systems using certain nonionic surfactants, when short to medium chain oils are employed as the dispersed phase, the area of microemulsion existence can be extended into areas of lower surfactant concentration, showing higher oil incorporation as can be seen for all the systems illustrated in this chapter using miglyol 812(Aboofazeli R., *et al.*, 1995). The logical deduction is that in order to maximise solubilisation and the area of existence of a microemulsion, it is

necessary to use short chain oils. It is unfortunately not that simple(Lawrence, M.J., 1994). In the case of the nonionic surfactants and zwitterionic lecithins, the increase in the area of existence of the microemulsions formed, using short chain oils, decreases the amount of drug incorporated. The short chain oils significantly penetrate the interfacial surfactant film, altering one of the major sites of solubilisation in a microemulsion, namely the hydrophobic chain portion of the surfactant, closest to the hydrophobic core(Aboofazeli R., *et al*., 1995). A compromise needs to be sought between the amount of oil solubilised and the degree of disruption of the interfacial surfactant layer, thus the use of medium to long chain oils is advocated, from the point of view of the amount of drug incorporated. In lipid selection the solubility of the drug in the oil forming the dispersed phase of an o/w microemulsion is another factor to be considered, in that the amount of drug incorporated will be greater than that in a micellar solution. Drugs generally show improved solubility in polar oils such as medium to long chain triglycerides when compared to nonpolar oils, but if polarity is too great a microemulsion will not form. Unfortunately pharmaceutically acceptable oils tend to be large and semipolar(Aboofazeli, R., *et al.*, 1995). From this, if the oil selected as the dispersed phase in the formulation of an o/w microemulsion, is a polar long chain molecule, then the corresponding surfactant required to incorporate these oils must possess long alkyl chains, which in turn must contain numerous unsaturated bonds or other fluidising groups to ensure the ability to produce a microemulsion.

Due to the size of triglyceride oils such as miglyol 812, it is not expected to alter the CPP as it doesn't penetrate the surfactant film to any significant extent. It can therefore be assumed that when used in o/w microemulsion formulation, the core will consist entirely of the oil. Work done on IPM, indicates that there is no significant hinderance to the formation of clear systems at ambient temperature, with microemulsion formation possible at relatively low polyoxyethylene ether surfactant concentration, as demonstrated for miglyol 812 as well, in figure **5.4.** Some researchers believe that although isopropyl myristate is a large molecule, penetration of the oil into the surfactant film cannot be discounted. The

triglyceride oil, miglyol 812 has proven effective as dispersed phases in microemulsion formulation, in previous work, with corn oil having also been used successfully(Aboofazeli, R., *et al.*, 1995; Constantinides, P.P., *et al.*, 1995). Vegetable oils have, however, been found to not form balanced microemulsions at low emulsifier compositions(Engström, S., 1995). No synergistic effect, has yet been noticed from the use of two oils of varying chain lengths in combination, making this avenue of investigation of little value.

5.8 Conclusion

The selection of the surfactants to be used to find microemulsion regions was the criterion which was given the most attention, given the lack of specificity reported in section **5.1** for the HLB and CPP in aiding in this selection. The criteria for the use of a surfactant in a pharmaceutical preparation for human use, particularly in emulsion formulation, include compatability with other formulation components, stability to deterioration, non-toxic with respect to the intended use and the ability to promote emulsification and to maintain the stability of the emulsion for the intended shelf life(Ansel, H.C., 1981). It is the nature of the hydrophilic head group that provides the most useful means of categorising surfactants.

All the surfactants employed in this chapter were nonionic in nature, used for their lack of toxicity and predisposition towards the formation of stable emulsion systems. Ionic surfactants are frequently toxic and are thus unsuitable for pharmaceutical formulation, as are aliphatic and aromatic oils, which some investigators have used in macro and microemulsion formulation. Of the nonionic esters, it is the glyceryl, polyglyceryl, sorbitan, sucrose and ethoxylated esters that are most useful as emulsifiers. The ethoxylated esters are of particular use as solubilisers, with molecules of varying HLB values being possible to synthesise. When using nonionic surfactants, a fluid interfacial film is required. This is demonstrated by the n-alkyl polyoxyethylene ethers, of which Brij 97 is an example, with the

introduction of a *cis* double bond into the long hydrocarbon chain, which fluidises the film sufficiently to allow the formation of an o/w microemulsion at ambient temperature. The corresponding saturated chain surfactant does not form an o/w microemulsion under the same conditions, requiring an elevation of temperature to facilitate the fluidisation of the chains, to enable microemulsion formation. The systems mapped indicate the necessity of a cosurfactant to stabilise the interfacial film and to introduce the degree of flexibility required for microemulsification to occur. The only two exceptions to the rule are Brij 97 and cremophor RH, which can form microemulsion regions without the need for a cosurfactant. Cremophor RH and sorbitol produced a stable, dilutable microemulsion, as did Brij 97, although neither of these are of use in this project. The amount of lipid incorporated by the cremophor microemulsion is insufficient to carry the quantity of RIF required in formulation, with the Brij 97 forming a semi-solid gel when RIF is added.

Since the development of the DLVO Theory, the use of nonionic surfactants has increased. This has led to the acceptance that there must be additional stabilising forces involved, other than electrostatic repulsive forces(Florence, A.T, *et al.*, 1981). Even though reduction of interfacial tension lowers the interfacial free energy produced on dispersion, the role of the emulsifying agents as interfacial barriers is most important, which is particularly true for nonionic surfactants. The presence of a closely packed surfactant film at the interface acts as a mechanical barrier to the flocculation and coalescence of the dispersed droplets. Nonionic surfactants stabilise emulsions via this interfacial film mechanism. The approach of emulsified droplets with adsorbed surfactant molecules at the oil-water interface results in repulsion on interaction(Florence, A.T, *et al.*, 1981). This steric repulsion arises due to the long chains of the surfactant molecule being restricted on contact, which results in a loss of entropy. This loss of entropy contributes to the positive free energy change, as well as to the change in solute-solvent interactions in the overlap region. The theory developed by E.W. Fischer is of particular interest, as it encompasses parameters which are of theoretical value(Florence, A.T, *et al.*, 1981). When adsorbed layers form, to produce the dispersed

phase droplets, a region of high concentration of surfactant molecule extends outward from the droplet surface, into a region of lower concentration of the surfactant. The layer forms a gradient of polymer concentration, which is a function of the length of the adsorbed surfactant molecules, the degree of surface coverage and the conformation of the polymer chains in the environment. If two identical spherical droplets, with identical adsorbed layers of surfactant, collide in a common medium, the overlap volume of the adsorbed layers, results in an increase in the local surfactant chain concentration, which is expressed as an excess chemical potential change. The increase in chemical potential as a result of the collision, generates an excess osmotic pressure, arising from continuous phase flow to the area of high concentration. This pressure is manifested as an energy of repulsion. The repulsive force may not always be enthalpic in origin, as a loss of conformational freedom can lead to a negative enthalpy change. By each chain losing some of it's conformational freedom, the contribution to the free energy of the system increases, which leads to repulsion(Florence, A.T, *et al.*, 1981).

It is undoubtedly the imwitor 308 and crillet 3 combination microemulsion system that proved most successful, incorporating 25 to 30% miglyol 812, an intermediate quantity of surfactant and maintaining homogeneity on dilution. The effect of the more lipophilic imwitor 742 relative to imwitor 308 was demonstrated by the reduced ability of the imwitor 742 system to produce microemulsion regions incorporating sufficient lipid to act as a lipophilic drug carrier, that are also dilutable. The usefulness of lecithins as surfactants in microemulsification has been demonstrated here, forming a microemulsion over a range of low to high surfactant and lipid content, however, dilutability proved to be a problem. Another negative feature is the use of ethanol as a cosurfactant, with limitations placed on it's use in oral pharmaceutical preparations restricting the application of this lecithin system(Engström, S., 1995). Lecithin, being a surfactant of natural origin, is unstable itself, with oxidation of the acyl chains and hydrolysis of the polar head a problem from a formulation stability point of view(Trotta, M., *et al.*, 1996).

The microemulsion used in the formulation in chapter 7 comprises imwitor 308 (27.63%), crillet 3 (27.63%), miglyol 812 (23.68%) and water (21.06%). The high surfactant content permits the dilution of this o/w microemulsion, although at high water content, on dilution, the system probably reverts to a mixed micellar solution. The lipid and water content of this formulation permits the incorporation of the drugs used. Future work could possibly look at the use of polyhydric alcohols such as sorbitol or glycerol as a substitute for ethanol as cosurfactant in the lecithin systems.

Chapter 6

Drug Stability in Formulation Components

6.1 Introduction

In the preceding chapter, work was done on the mapping of ternary and pseudo-ternary phase diagrams, to determine the areas of existence of microemulsion systems. The systems mapped were numerous, employing various surfactants and lipids. In order to narrow down the field for the microemulsions that were to be selected for testing in chapter 7, where possible, stability studies were conducted on RIF, INH and PZA in individual formulation components. The International Congress on Harmonisation (ICH) Harmonised Tripartite Guidelines for the stability testing of a new drug substance or product were used as a model for the work conducted in chapters 6 and 7 and are detailed in sections **6.2** and **6.5**. A literature search on the liquid formulation stability of RIF, INH and PZA only produced results on the stability of RIF in suspension and syrup form. Nahata *et al.* reported on the stability and effect of preparation method on RIF concentration and stability. RIF preparations were found to adhere to the theoretical concentration to be formulated, being more stable at room temperature the more finely divided the RIF powder incorporated into the syrup(Nahata, M.C.,*et al.*, 1994 a & b). The injectable solution incorporated into syrup BP produced concentrations closest to the required 10mg/ml, with stability also found to be greater than in suspension.

6.2 Drug Stability Theory

The ICH guidelines, as the name implies, serves as a guide of the essential stability data required for a new drug substance or product, not being absolute, with alternative approaches allowed if scientifically justifiable reasons are given(Carstensen, J.T., 1995).

This testing is done to provide an indication of how the drug or product varies with time under the influence of temperature, light and humidity. The design of the stability program is based on the behaviour and properties of the drug substance, accounting for the drug stability data and possible changes that may take place on storage, if available. The test criteria assessed in chapters 6 and 7 are the chemical stability of RIF, INH and PZA and the physical stability of the formulation and components, with the analytical procedures employed, validated in chapters 3 and 4.

No standards or literature on the degradation products of RIF, INH and PZA was available, making the identification and quantitative assessment of these degradation products not possible. The concentrations of the drugs incorporated into formulation were assessed as the quantity remaining at specific stages of stressing, with the limit of drug degradation at the end of the accelerated degradation study stated to not be greater than 5% of the starting drug concentration by the ICH(Carstensen, J.T., 1995). The conditions and criteria set out by the ICH for the accelerated testing of a drug product are exposure to a temperature of $40^0C \pm 2^0C$ and a relative humidity (RH) of 75% ± 5% for 6 months.

For acceptance, the product should also adhere to the pH limits, appearance and physical property specifications and dissolution criteria with degradants not exceeding stated specification limits, in addition to the loss of potency limit of 5%. Significant change in any of these general criteria as they apply to the specific drug or product, requires the registration application of the product to be accompanied by at least 6 months of data from an ongoing one year study at an intermediate condition(Carstensen, J.T., 1995). In the case of this study $25^0C \pm 2^0C$ at 60%RH ± 5% and $40^0C \pm 2^0C$ at 75%RH were the conditions set. The frequency of testing needs to be sufficient to establish the stability of the product, with testing to be carried out in the final packaging proposed for marketing.

Accelerated testing is designed to increase the rate of physical change in the formulation or

chemical change of the active drug or product by exaggerating the storage conditions that the product will ultimately be exposed to, in so doing, providing information for the stability enhancement of the product if need.

6.3 Instrumentation and Equipment

The chromatographic system used for the HPLC analysis of all aqueous fractions was the same as that developed and validated in Chapter 3.

The equipment listed in section **4.2.1** for the lipid-aqueous partitioning method developed and validated in chapter 4 was used in the analysis of RIF, INH and PZA in the lipids investigated.

6.4 Solvents and Reagents

Rifampicin(RIF), isoniazid(INH) and pyrazinamide(PZA) were donated by the Druggists Group Reasearch unit(Pharmacare-Lennon, RSA), as was sorbitol(70%m/v) and cremophor RH. Polyoxyethylene 20 sorbitan monooleate(Crillet 3) was donated by Croda, Southern Africa.

Methanol and acetonitrile were purchased from BDH®, England(purity >99.9%). All aqueous fractions were prepared using double distilled deionised water, obtained from a Milli-RO 15 water purification system, manufactured by Millipore®(Massachusetts, USA). Miglyol 812 and Imwitor 308 were supplied by Hüls, Southern Africa and corn oil purchased from Sigma(St. Louis, USA).

6.5 Methodology

For each solvent and surfactant tested in this chapter, saturated solutions of RIF, INH, PZA, RIF with INH, INH with PZA and RIF with PZA, were prepared using the method, volume of solvent and equipment outlined in section **2.4.4**.

The saturated solutions were filtered by vacuum filtration and 50ml of each filtrate solution drawn and purged with air for one hour. 0.3ml fractions of aerated solution were pipetted into 0.5ml amber glass ampoules(126 ampoules per solvent investigated) and sealed by a four way converging flame bunsen burner. This procedure was repeated for the original nitrogenated solutions, with the ampoules filled with nitrogen prior to sealing to displace air above the solution. Based on the solubility tables constructed, 0.2ml fractions of the saturated solutions were drawn and appropriately diluted to within the validated region of the calibration curve, for HPLC analysis. HPLC analysis of the stock solutions give the zero time starting drug concentrations, from which all subsequent determinations are calculated as a percentage of the starting concentration.

42 aerated and 42 nitrogenated ampoules of drug solution were then placed in thermostatically controlled convection ovens at 25, 40 and 60^0C. 3 aerated and 3 nitrogenated solution samples were drawn at 24 hour intervals from each oven, diluted to within the validated concentration range and determined by HPLC **System 1** detailed and validated in chapter 3. An average of the 3 determinations for each set of conditions was taken as the mean percentage drug remaining.

Where miglyol 812 and corn oil, were used as solvents, the lipid-aqueous extraction procedure developed in chapter 4, was used prior to HPLC analysis. The UV-VIS spectrophotometric technique developed in chapter 4 served as a daily system check, prior to HPLC analysis, to ensure that the aqueous fractions from the extraction procedure did

not contain an appreciable quantity of lipid. Aqueous solutions of 10% m/m of cremophor, 10%m/v of crillet 3, 5% m/v of imwitor 308 and 70% m/v of sorbitol were used for the drug stability investigation in these solubility enhancers/surfactants. HPLC analysis of the drug solutions give the degradation profiles of RIF, INH, PZA, RIF with INH, INH with PZA and RIF with PZA, under air and nitrogen, at 25, 40 and 60^0C over a period of 7 days. The degradation study was conducted in duplicate for each solvent and the mean values obtained are reported in this chapter.

6.6 Results and Discussion

The stability of RIF, INH and PZA was investigated in aqueous solution, miglyol 812, corn oil, 10%m/v cremophor RH, 5%m/v imwitor 308, 10%m/v crillet 3 and 70%m/v sorbitol solution. The microemulsion selected for assessment in chapter 7 was comprised of water, miglyol 812, imwitor 308 and crillet 3, hence it is these drug stability assessments that are reported in detail in this chapter.

6.6.1 Aqueous Stability

The results for the stability assessment of RIF, INH and PZA in water are graphically represented in figures **6.1** to **6.9**. The stability of RIF, INH and PZA were assessed in solution, individually and in combination. The graphs constructed, illustrate the stability of the individual drugs at the stated temperatures, under air and nitrogen, as well as the influence of the drugs on the stability of each other. Figures **6.1**, **6.2** and **6.3** illustrate RIF stability, **6.4**, **6.5** and **6.6** INH stability and **6.7**, **6.8** and **6.9** PZA stability.

Figure 6.1 RIF stability in aqueous solution

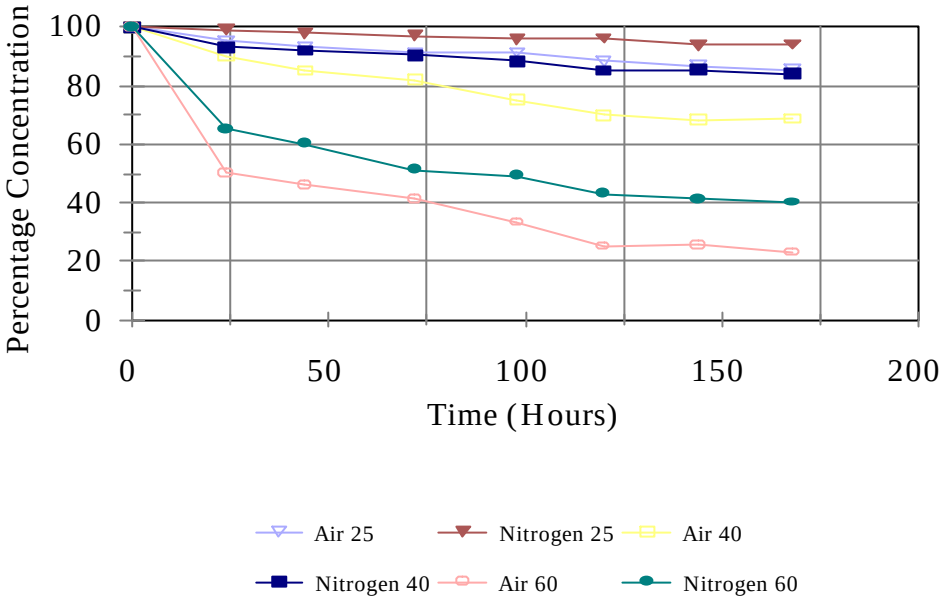


Figure 6.2 RIF stability in the presence of INH in aqueous solution

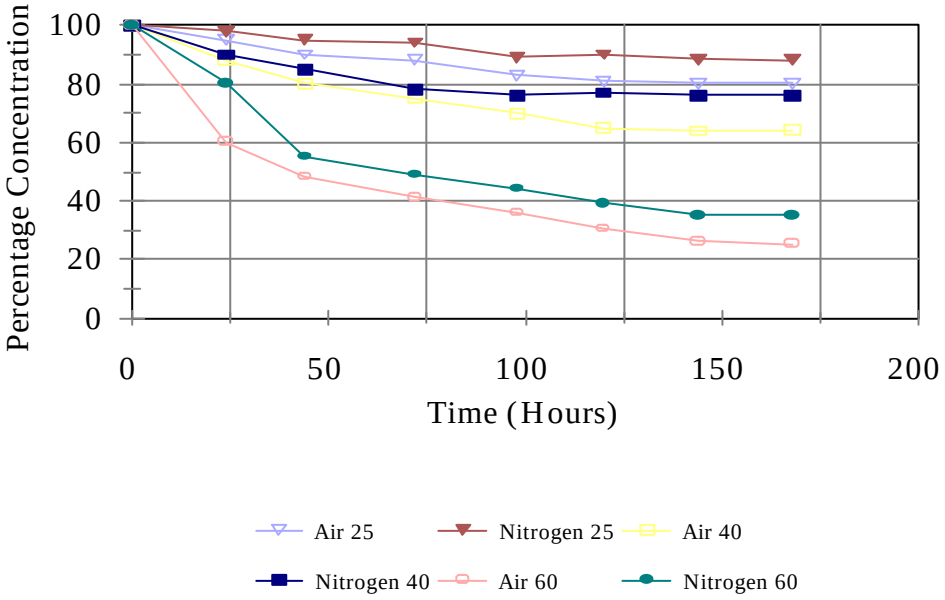


Figure 6.3 RIF stability in the presence of PZA in aqueous solution

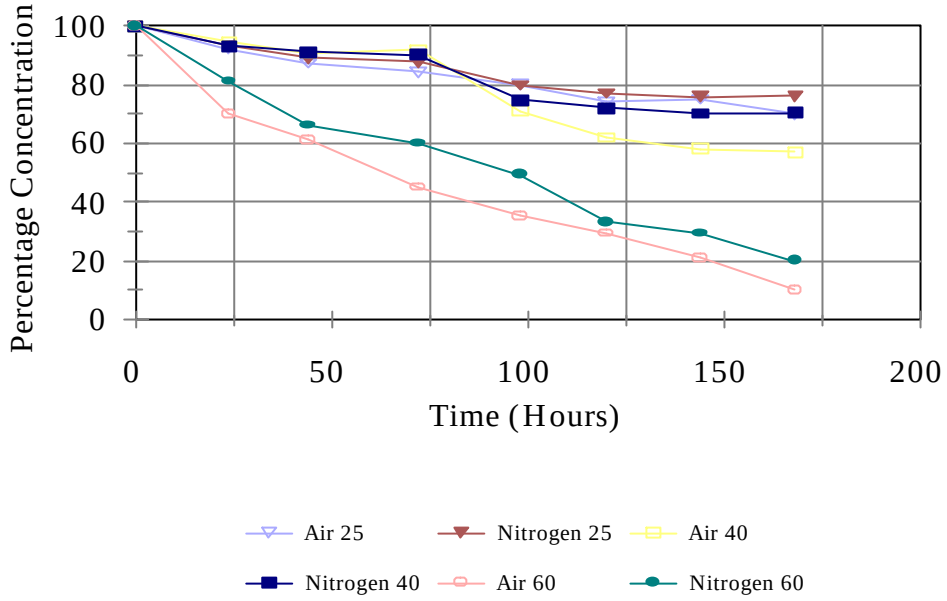


Table 6.1 Percentage RIF remaining after 7 days - AQUEOUS

Aqueous Solution				
Temperature ^{0}C	**Gas**	**RIF**	**RIF with INH**	**RIF with PZA**
25	**Air**	86	80	70
	Nitrogen	94	88	76
40	**Air**	69	64	57
	Nitrogen	84	76	70
60	**Air**	23	25	10
	Nitrogen	40	35	20

In figures **6.1**, **6.2** and **6.3**, whether alone, or in the presence of INH or PZA, the temperature and oxidation dependent degradation of RIF is evident. The percentage RIF remaining at the end of the 7 day study is displayed in table **6.1**, which helps illustrate the oxidative degradation of RIF and the effect of INH and PZA on it's stability, with nitrogenated samples degrading to a lesser degree.

The temperature dependent degradation is clearly displayed in the graphs constructed with an initial rapid rate over the first 48 hours at 60 ^{0}C which subsequently tapers off with time. The accelerated degradation effect of INH on RIF is displayed by figure **6.2** relative to figure **6.1**, with PZA having no observable enhancing effect on RIF degradation or stability in figure **6.3**.

Figure 6.4 INH stability in aqueous solution

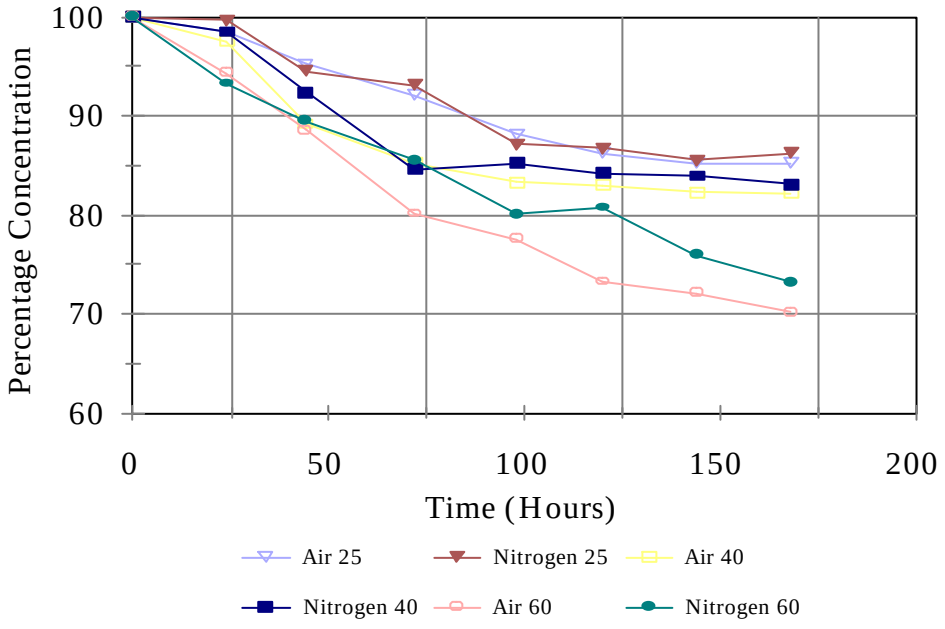


Figure 6.5 INH stability in the presence of RIF in aqueous solution

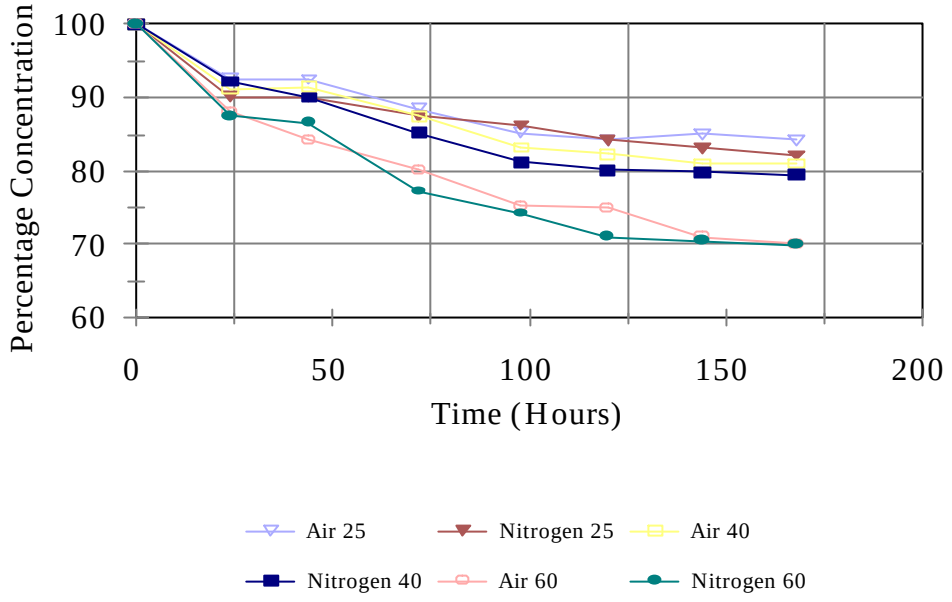
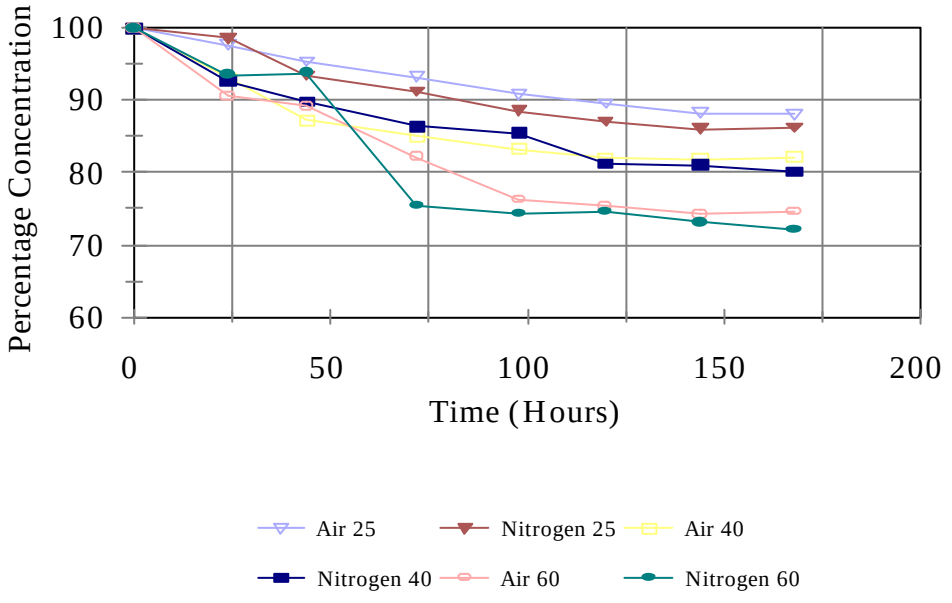


Figure 6.6 INH stability in the presence of PZA in aqueous solution



As depicted in figures **6.4**, **6.5** and **6.6**, INH stability is less affected by temperature than in the case of RIF.

Table 6.2 Percentage INH remaining after 7 days - AQUEOUS

Aqueous Solution				
Temperature ^{0}C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	85	84	88
	Nitrogen	86	82	86
40	**Air**	82	81	82
	Nitrogen	83	79	80
60	**Air**	70	70	74
	Nitrogen	73	69	72

Even at elevated temperature, INH concentrations do not drop below 69%, as indicated in table **6.2**. The independent nature of INH degradation on oxidation is demonstrated by the nitrogenated samples producing stability that is not significantly greater than those stored under air, with a slight increased degradation under nitrogen actually indicated. The INH dependent reduction in RIF concentration is not reflected here either, with RIF having no significant effect on INH stability, as seen in table **6.2**. INH and PZA have no effect on the stability of the other, with the structural similarity and hence lack of interaction of these substances probably being responsible for this.

As can be seen in figures **6.7**, **6.8** and **6.9** the degradation of PZA appears somewhat haphazard. Degradation is shown to be independent of oxygenation or nitrogenation prior to sealing, with the effect of heat being of little significance, with drug concentration not dropping below 86% at 60^{0}C, as shown in table **6.3**. The effect of INH on the drug is shown to be negligible, with a slight decrease in PZA concentration, when combined with RIF. This decrease in PZA although only slight, due to it's structural similarity to INH, was expected to be unaffected by RIF, especially as PZA seems to have no effect on RIF

stability.

Figure 6.7 PZA stability in aqueous solution

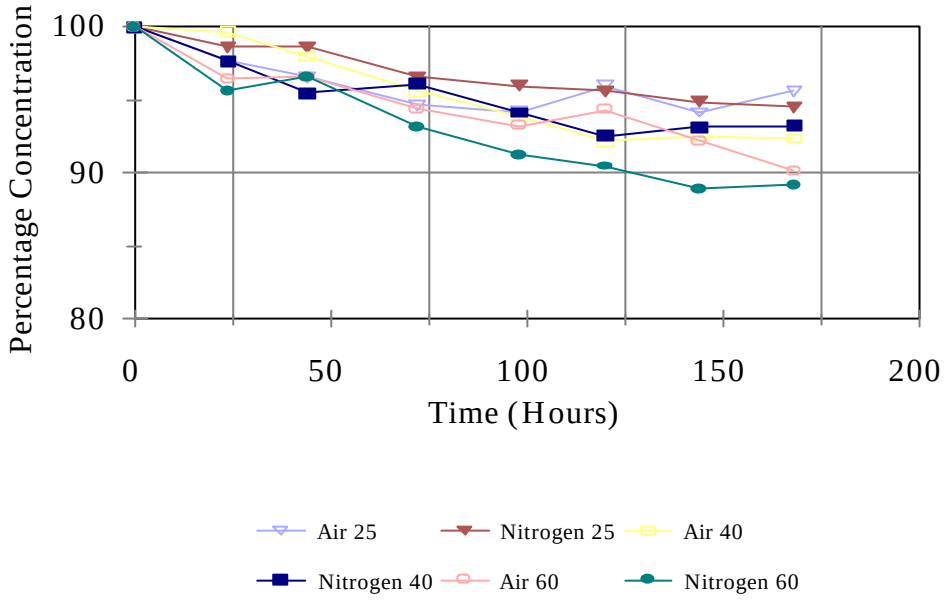


Figure 6.8 PZA stability in the presence of RIF in aqueous solution

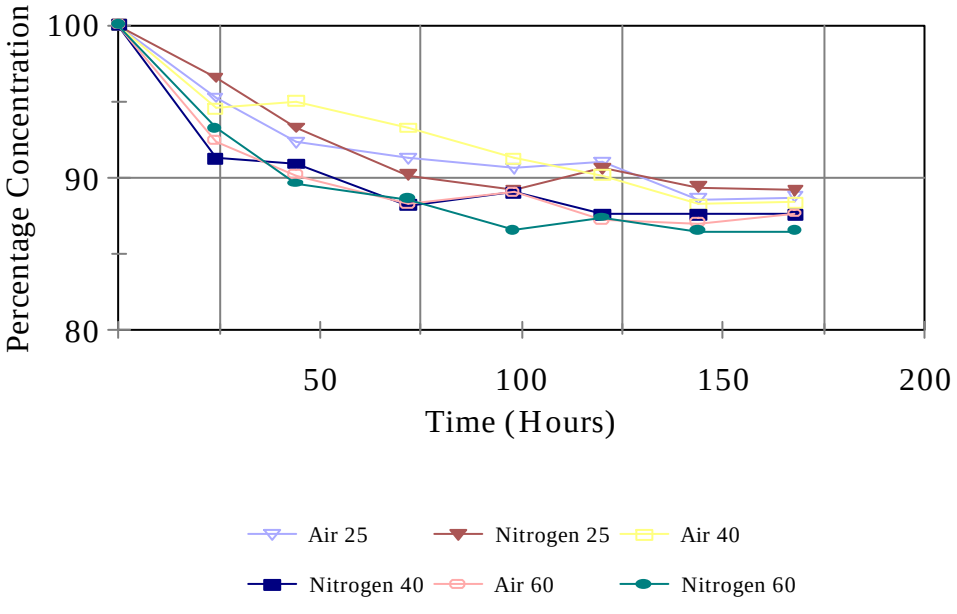


Figure 6.9 PZA stability in the presence of INH in aqueous solution

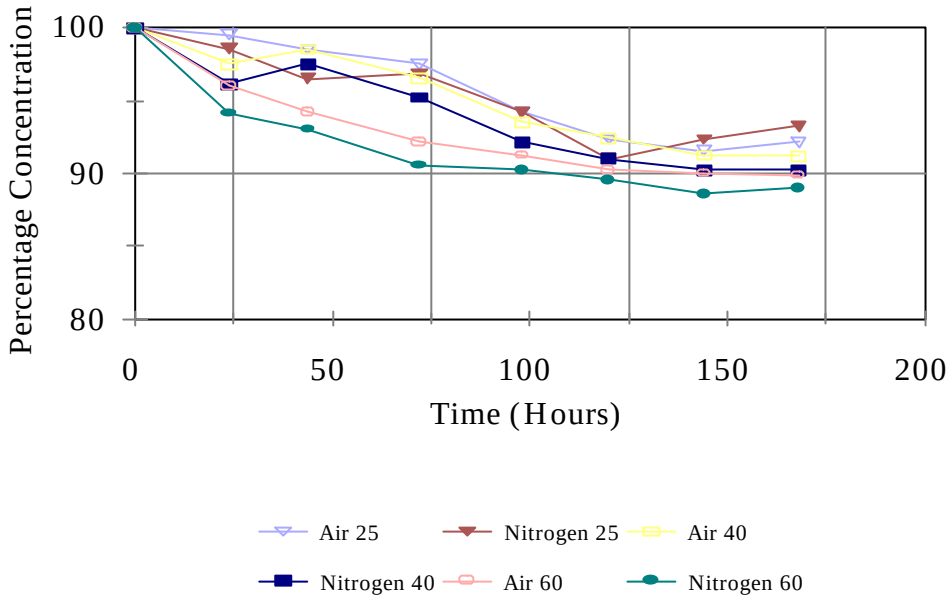


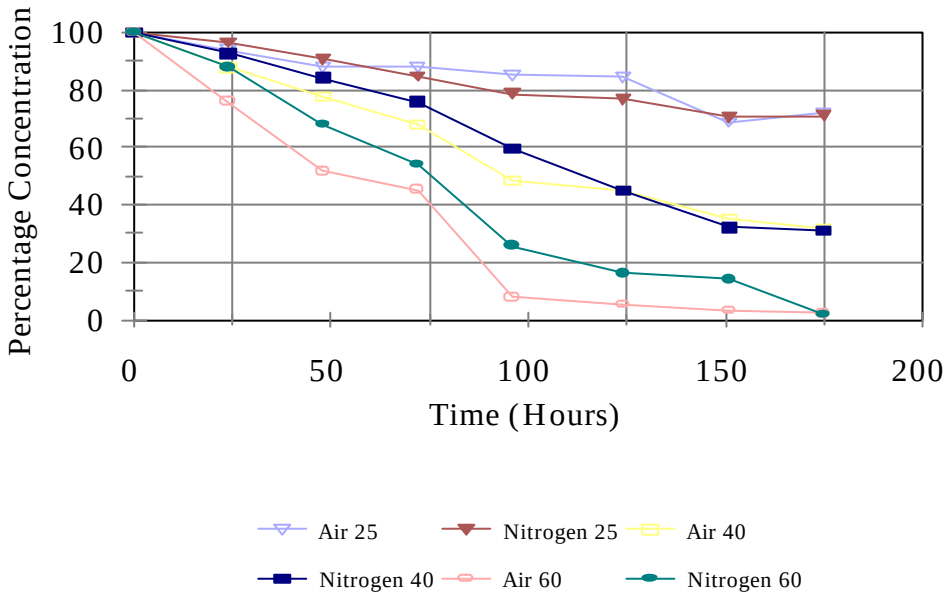
Table 6.3 Percentage PZA remaining after 7 days - AQUEOUS

Aqueous Solution				
Temperature ^{0}C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	96	88	92
	Nitrogen	94	89	93
40	**Air**	92	88	91
	Nitrogen	93	87	90
60	**Air**	90	87	89
	Nitrogen	89	86	88

6.6.2 Miglyol 812 Stability

Miglyol 812 is a semi-synthetic lipid composed of a mixture of C8 and C10 triglycerides. The stability results for RIF are depicted in figures **6.10**, **6.11** and **6.12**, INH in figures **6.13**, **6.14** and **6.15** with PZA in figures **6.16**, **6.17** and **6.18**. Tables **6.4, 6.5** and **6.6** report the percentage drug remaining after 7 days.

Figure 6.10 RIF stability in miglyol 812 solution



RIF stability in miglyol 812 displays the temperature dependency illustrated by the drug in aqueous solution. Oxidative degradation of the drug is more pronounced for RIF in miglyol 812 alone, than combined with INH and PZA, with the solutions stored under nitrogen indicating greater stability over the course of the experiment. At the end of the study, the percentage drug remaining is very similar at all temperatures. RIF stability is affected by INH as it was in section **6.6.1** with PZA affecting it only slightly, as displayed in table **6.4**. At 60^0C complete degradation of RIF in miglyol 812 occurs after 7 days, this degree of degradation of RIF relative to that in aqueous solution is believed to be due to it's increased solubility in the lipid. RIF stability is believed to be concentration dependent,

with RIF catalysing it's own degradation being another proposed explanation of it's instability. The low solubility of INH in miglyol 812 results in INH only being a small contributor to the degradation of RIF in the lipid.

Figure 6.11 RIF stability in the presence of INH in miglyol 812 solution

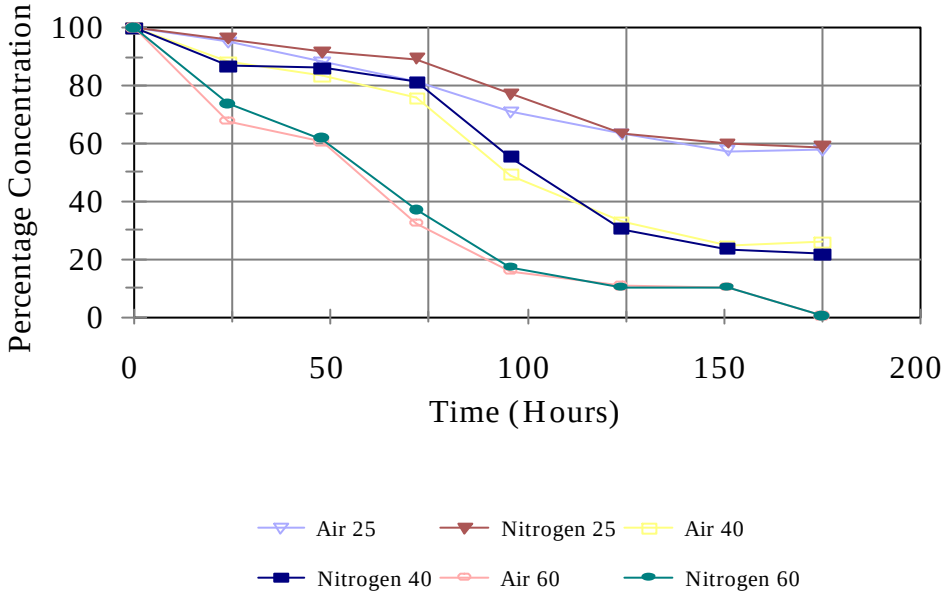


Figure 6.12 RIF stability in the presence of PZA in miglyol 812 solution

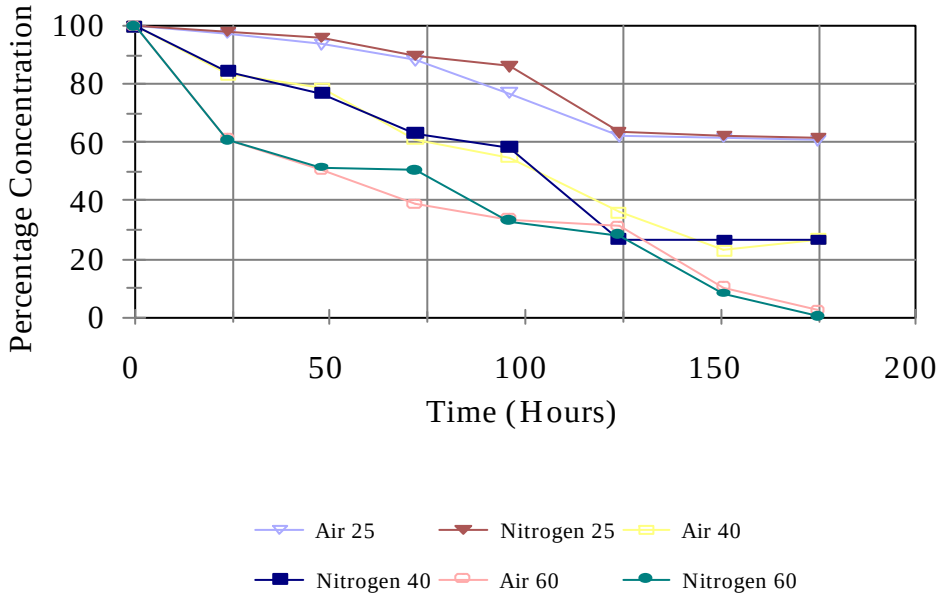


Table 6.4 Percentage RIF remaining after 7 days - MIGLYOL

Miglyol 812 Solution				
Temperature ^{0}C	**Gas**	**RIF**	**RIF with INH**	**RIF with PZA**
25	**Air**	72	58	61
	Nitrogen	71	59	62
40	**Air**	32	26	27
	Nitrogen	31	22	27
60	**Air**	2	0.3	2
	Nitrogen	2	0.3	0.3

INH stability is unaffected by the presence of air, with storage under nitrogen providing no added stability as is evident in figures **6.13**, **6.14** and **6.15** and in table **6.5**. Temperature dependence is demonstrated in solution on it's own, but not in combination with RIF and PZA where degradation suggests a slight oxidation dependency.

Figure 6.13 INH stability in miglyol 812 solution

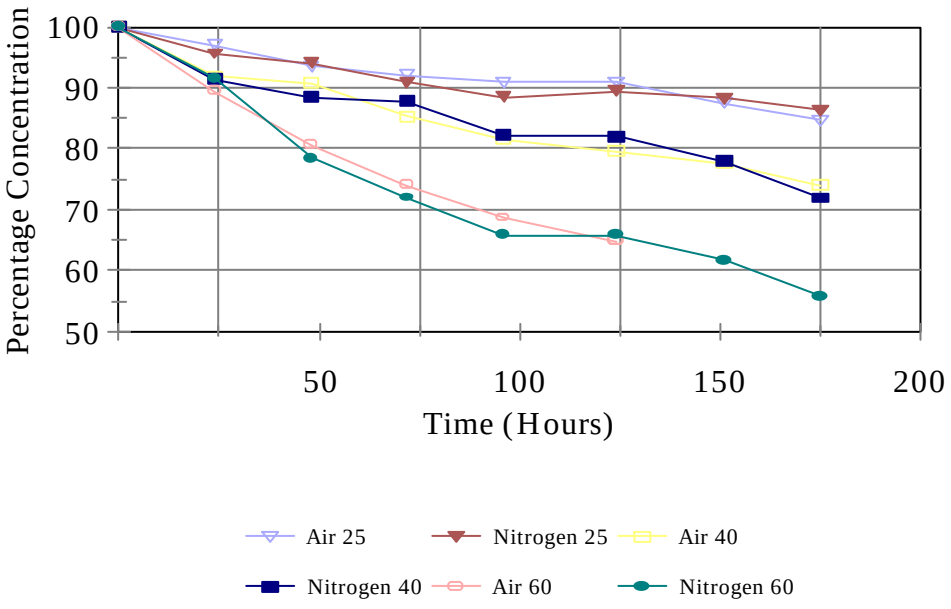


Figure 6.14 INH stability in the presence of RIF in miglyol 812 solution

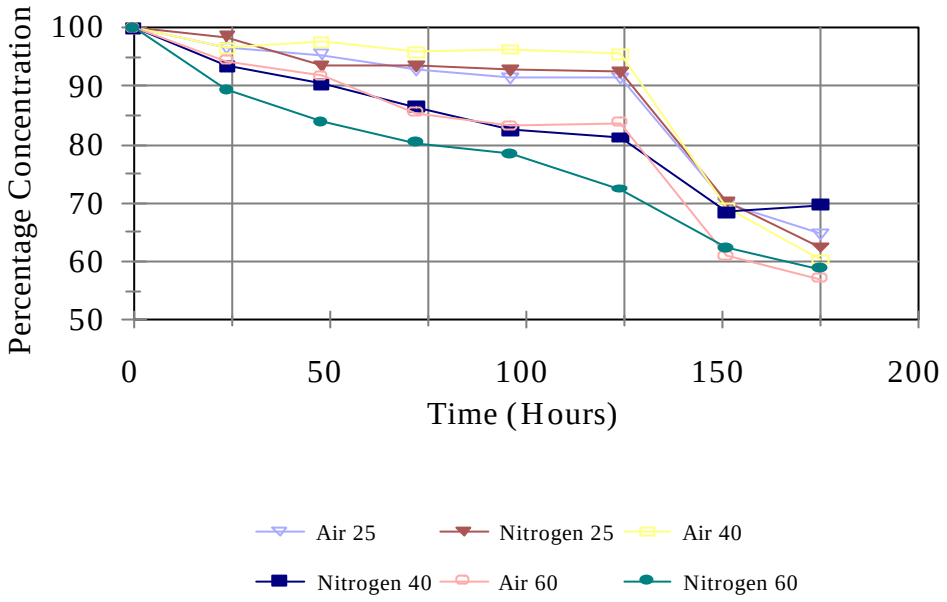


Figure 6.15 INH stability in the presence of PZA in miglyol 812 solution

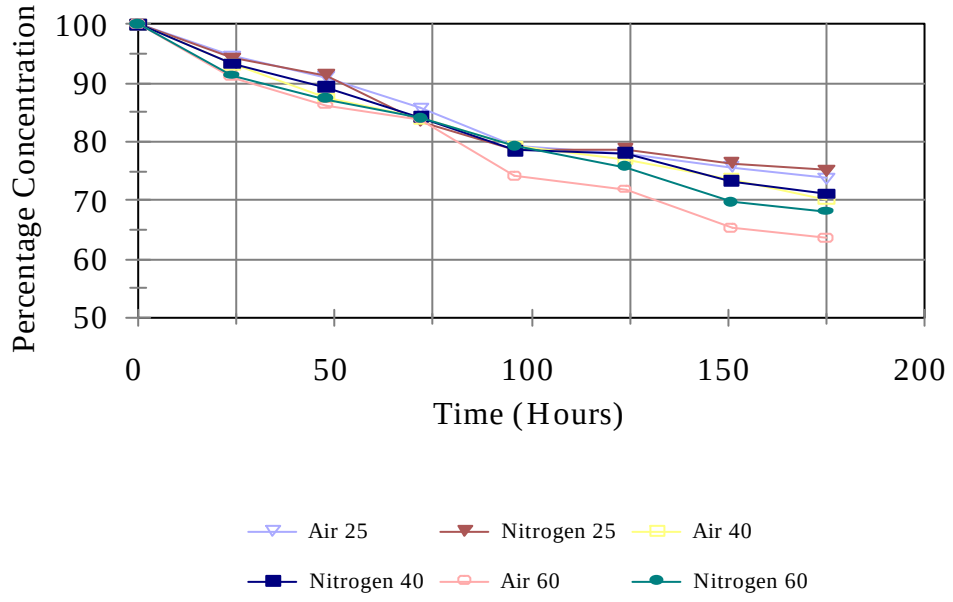


Table 6.5 Percentage INH remaining after 7 days - MIGLYOL

Miglyol 812 Solution				
Temperature ^{0}C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	85	65	74
	Nitrogen	86	62	75
40	**Air**	74	60	70
	Nitrogen	71	70	71
60	**Air**	58	57	64
	Nitrogen	55	59	68

For INH in combination with RIF in figure **6.14**, degradation is constant for the first 125 hours, after which there is a rapid increase to the point where the drug remaining after 7 days, is significantly less than that for INH alone or with PZA.

Figure 6.16 PZA stability in miglyol 812 solution

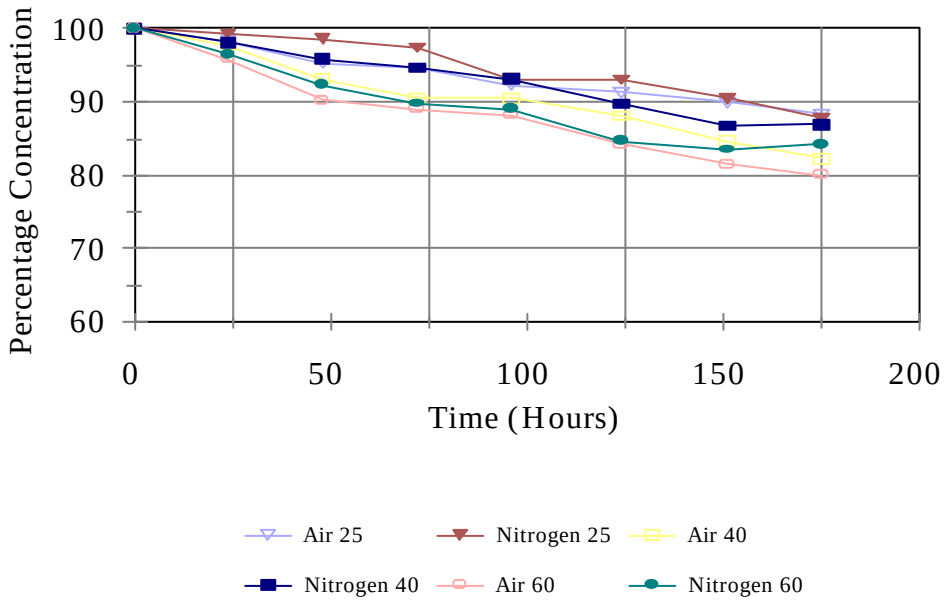


Figure 6.17 PZA stability in the presence of RIF in miglyol 812 solution

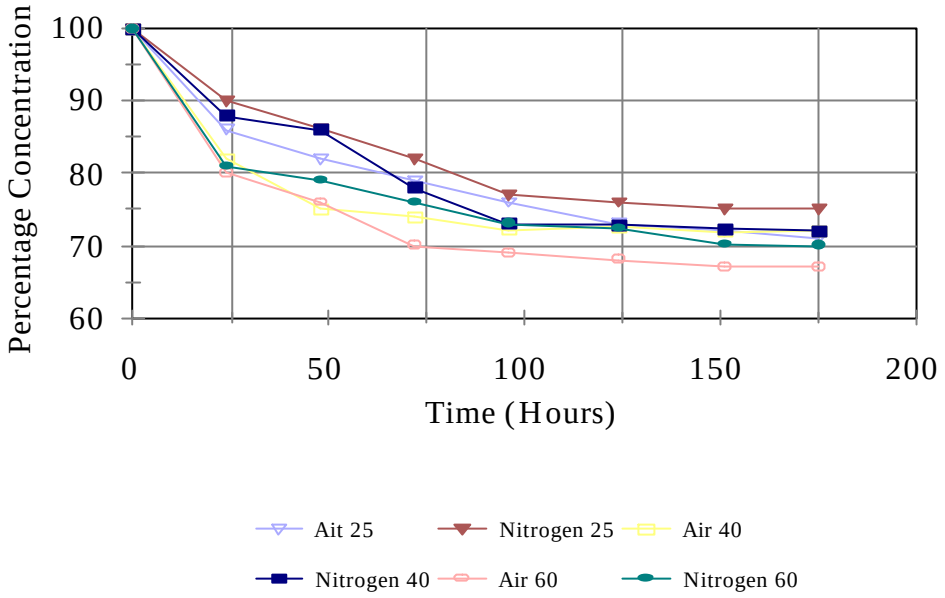


Figure 6.18 PZA stability in the presence of INH in miglyol 812 solution

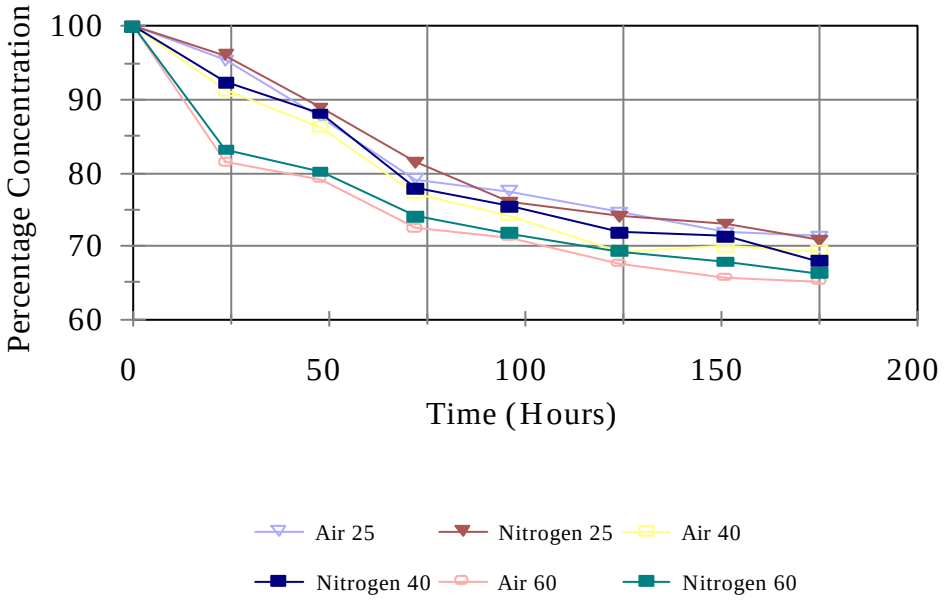


Table 6.6 Percentage PZA remaining after 7 days - MIGLYOL

Miglyol 812 Solution				
Temperature ^{0}C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	88	71	71
	Nitrogen	88	75	71
40	**Air**	82	72	69
	Nitrogen	87	72	68
60	**Air**	80	67	65
	Nitrogen	84	70	66

PZA in miglyol 812 reflects slightly greater stability when stored under nitrogen rather than air. This difference is more pronounced at elevated temperature, with the effect of varying temperature having minimal effect on the stability of PZA. PZA stability was greatest when alone in miglyol 812, with degradation of the drug slightly greater in the presence of RIF and INH as demonstrated in table **6.6**.

6.6.3 Imwitor 308 Stability

The stability of RIF, INH and PZA alone and in combination was assessed in 5%m/v aqueous imwitor 308 solutions due to this surfactant's poor water solubility. Imwitor 308 consists of a mixture of 80-90% monoglycerides of stearic and palmitic acids and variable quantities of di- and tri- glycerides, more commonly known as glyceryl monostearate(Martindale, 1989). It is a nonionic surfactant with an HLB value of approximately 6, forming a cloudy but homogeneous solution with no sedimentation on standing, having no apparent effect on the solubility of RIF, INH or PZA. The results of the stability study are depicted in figures **6.19** to **6.27** and in tables **6.7** to **6.9**.

Figure 6.19 RIF stability in aqueous imwitor 308 solution

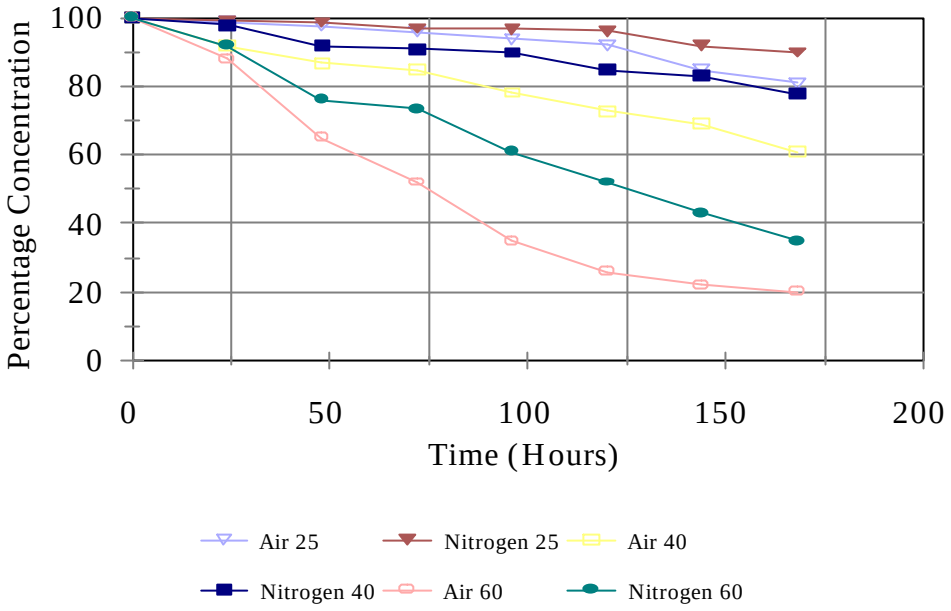


Figure 6.20 RIF stability in the presence of INH in aqueous imwitor 308 solution

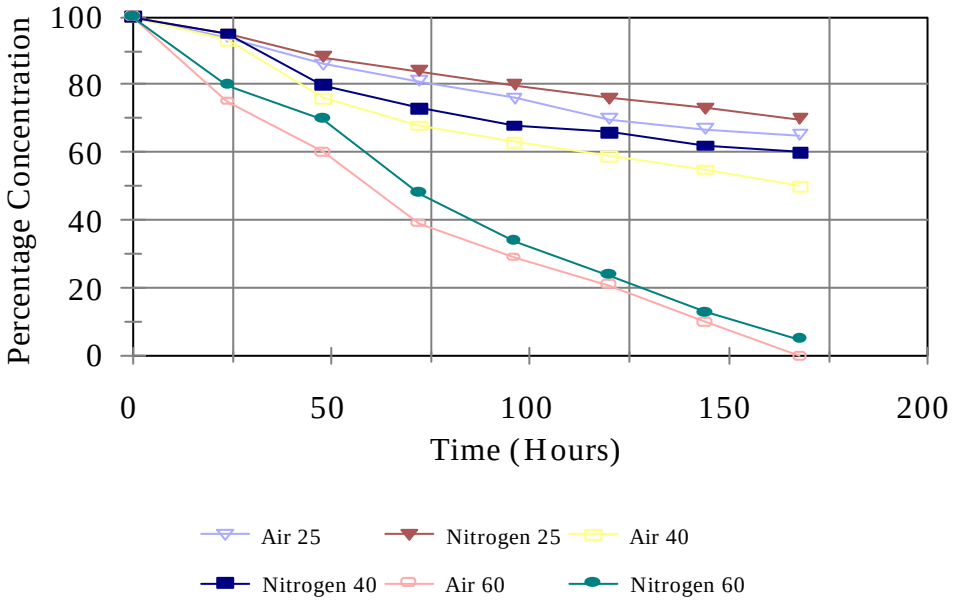


Figure 6.21 RIF stability in the presence of PZA in aqueous imwitor 308 solution

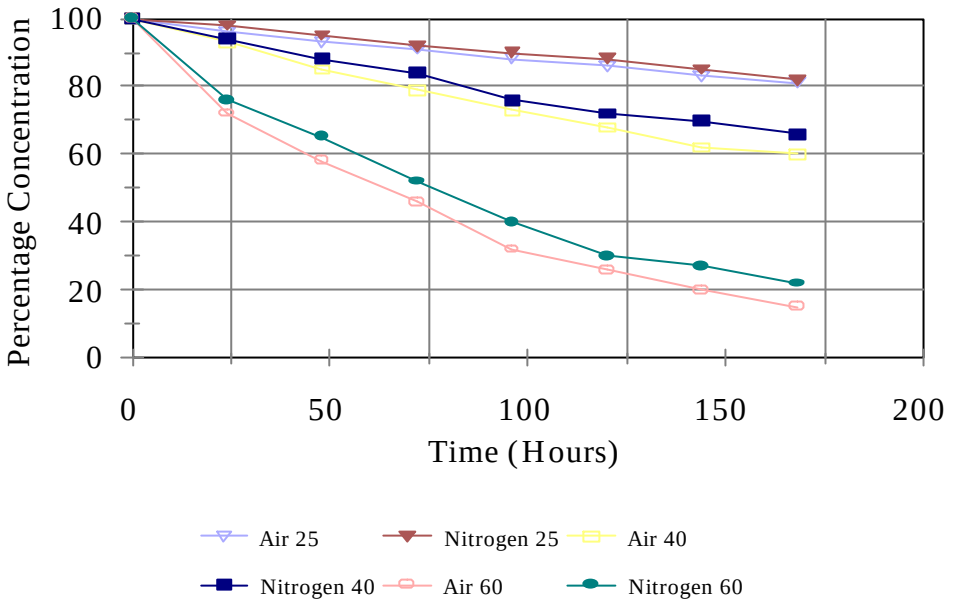


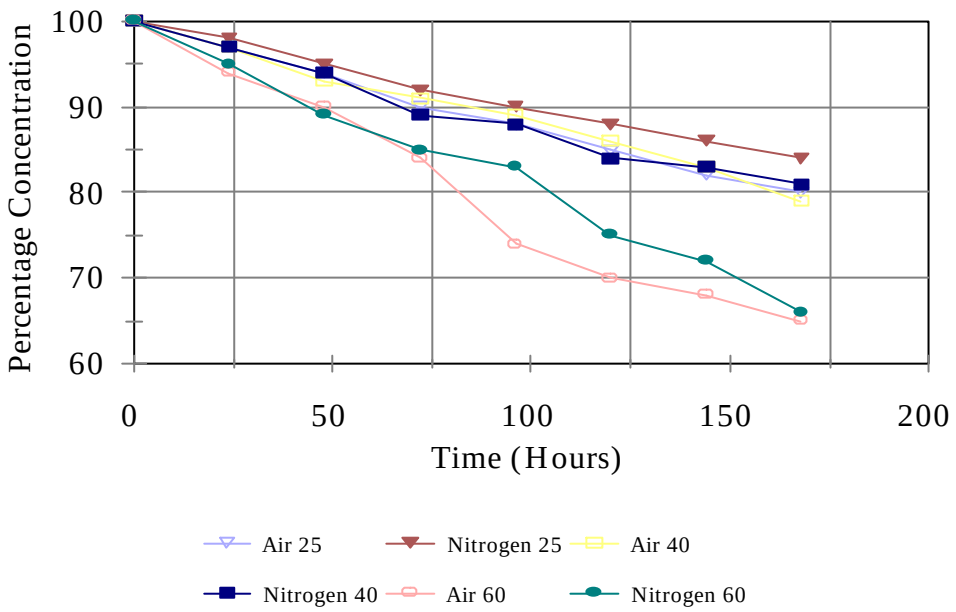
Table 6.7 Percentage RIF remaining after 7 days - IMWITOR 308

Imwitor 308 5%m/v Solution				
Temperature ^{0}C	**Gas**	**RIF**	**RIF with INH**	**RIF with PZA**
25	**Air**	81	65	81
	Nitrogen	90	70	82
40	**Air**	61	50	60
	Nitrogen	78	60	66
60	**Air**	20	0	15
	Nitrogen	35	5	22

As shown by the profiles of RIF in aqueous solution, both temperature and oxidation dependent degradation are demonstrated in figures **6.19**, **6.20** and **6.21**. The accelerated degradation of RIF in the presence of INH is illustrated in figure **6.20** , degrading to a

greater extent in imwitor 308 than in aqueous solution. This enhanced effect is postulated to be due to the presence of the surfactant imwitor 308, in part by increasing the solubility of RIF. Degradation in the presence of PZA is also slightly greater than in solution alone, but this does not occur to the extent observed in the presence of INH.

Figure 6.22 INH stability in aqueous imwitor 308 solution



The results obtained for INH in aqueous imwitor 308 solution indicate no significant benefit of storing the drug solutions under nitrogen. Temperature dependence is clearly indicated here, with INH in the presence of RIF degrading to a greater extent, which is in keeping with the results obtained for RIF in the presence of INH in sections **6.6.1** and **6.6.2**.

Figure 6.23 INH stability in the presence of RIF in aqueous imwitor 308 solution

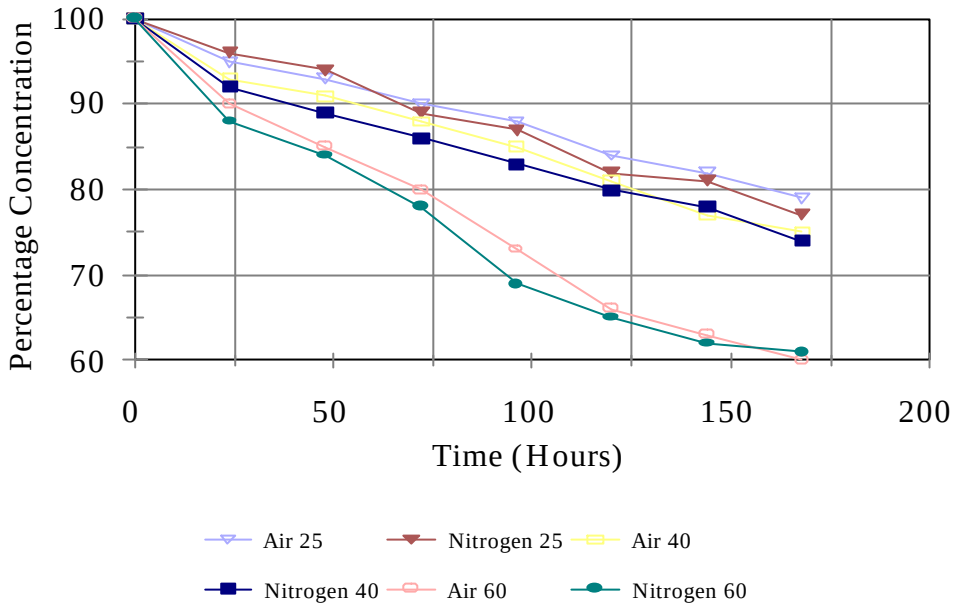


Figure 6.24 INH stability in the presence of PZA in aqueous imwitor 308 solution

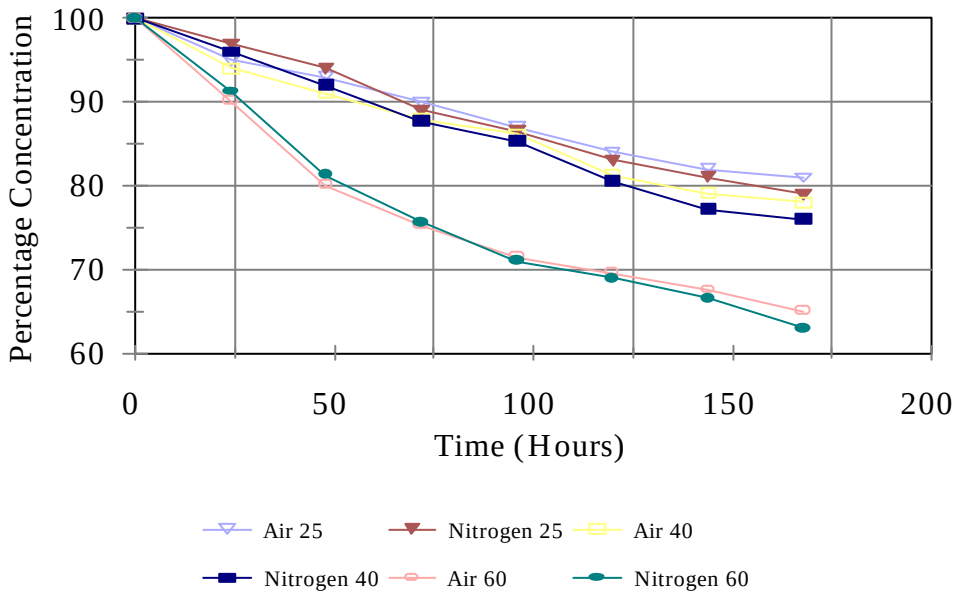


Table 6.8 Percentage INH remaining after 7 days - IMWITOR 308

Imwitor 308 5%m/v Solution				
Temperature ^{0}C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	80	79	81
	Nitrogen	84	77	79
40	**Air**	79	75	78
	Nitrogen	81	74	76
60	**Air**	65	60	65
	Nitrogen	66	61	63

Figure 6.25 PZA stability in aqueous imwitor 308 solution

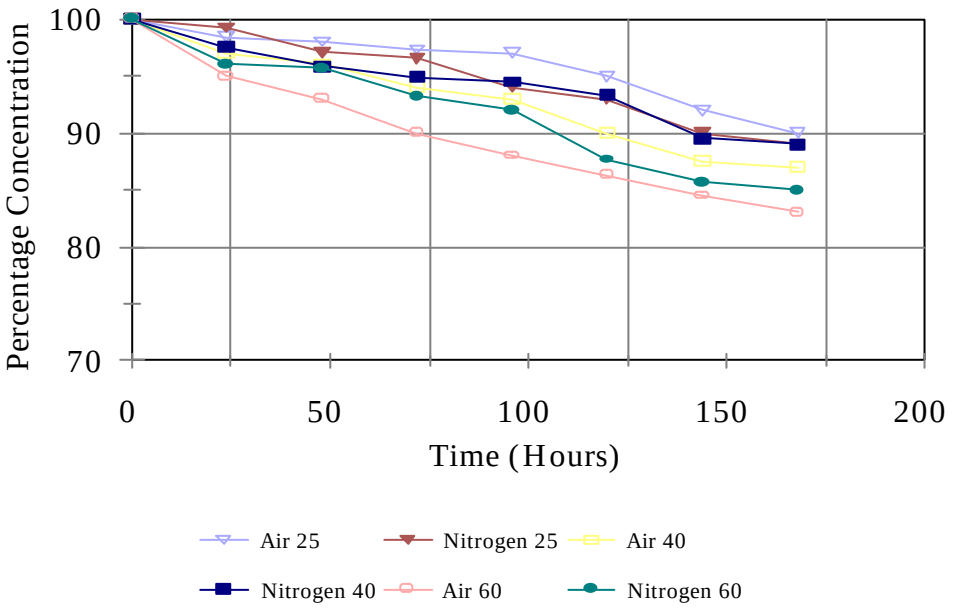


Figure 6.26 PZA stability in the presence of RIF in aqueous imwitor 308 solution

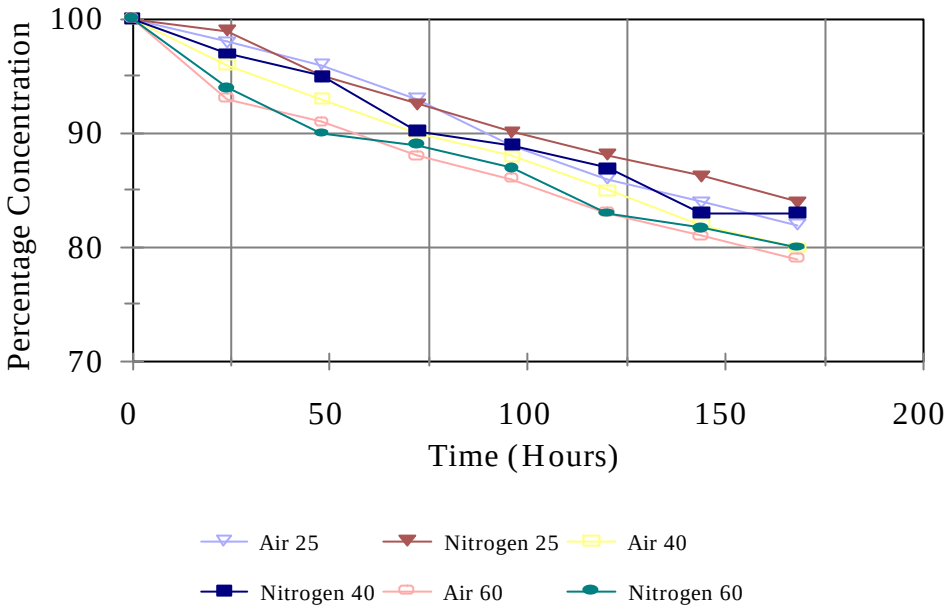


Figure 6.27 PZA stability in the presence of INH in aqueous imwitor 308 solution

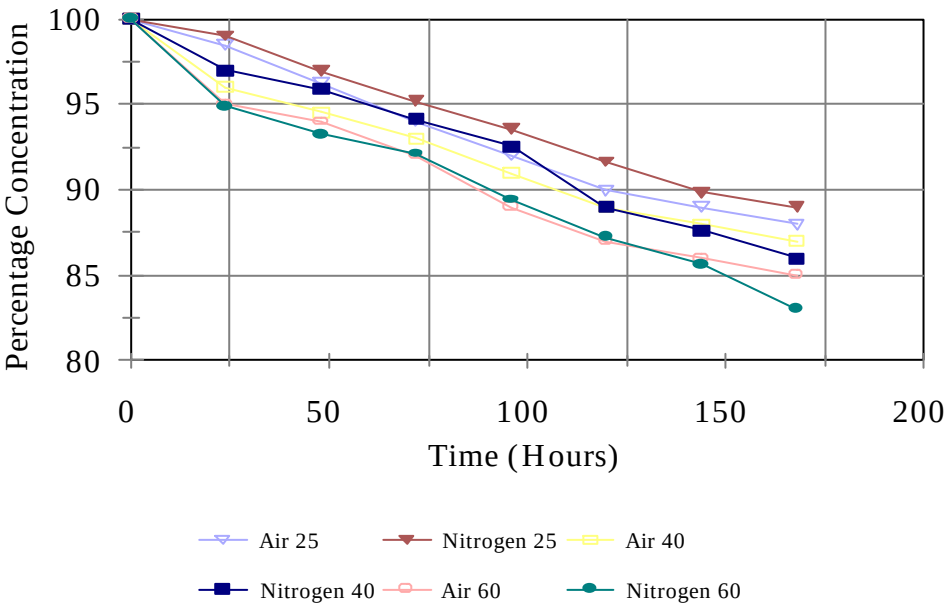


Table 6.9 Percentage PZA remaining after 7 days - IMWITOR 308

Imwitor 308 5%m/v Solution				
Temperature ^0C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	90	82	88
	Nitrogen	89	84	89
40	**Air**	87	80	87
	Nitrogen	89	83	86
60	**Air**	83	79	85
	Nitrogen	85	80	83

The previous stability studies for PZA have indicated it's greater stability at elevated temperature and resistance to oxidation relative to INH and RIF. This is demonstrated in imwitor 308, as can be seen in figures **6.25**, **6.26** and **6.27** and table **6.9**. Samples stored under nitrogen show only a small improved stability over those stored under oxygen.

6.6.4 Crillet 3 Stability

Crillet 3 (Tween 80) consists of a mixture of partial oleic esters of sorbitol and it's mono and di anhydrides copolymerised with approximately 20 moles of ethylene oxide for each mole of sorbitol and it's anhydrides. It is a hydrophilic nonionic surfactant with an HLB value of 15, and is commonly used in the production of stable oil-in-water emulsions and as a solubility enhancing agent(Martindale, 1989). The 10%m/v solution prepared resulted in an increase in the solubility of RIF and PZA to 3.5 and 27.9 mg/ml respectively, with INH solubility apparently unaffected. The results of the crillet 3 stability study are displayed in figures **6.28** to **6.36** and tables **6.10** , **6.11** and **6.12**.

Figure 6.28 RIF stability in aqueous crillet 3 solution

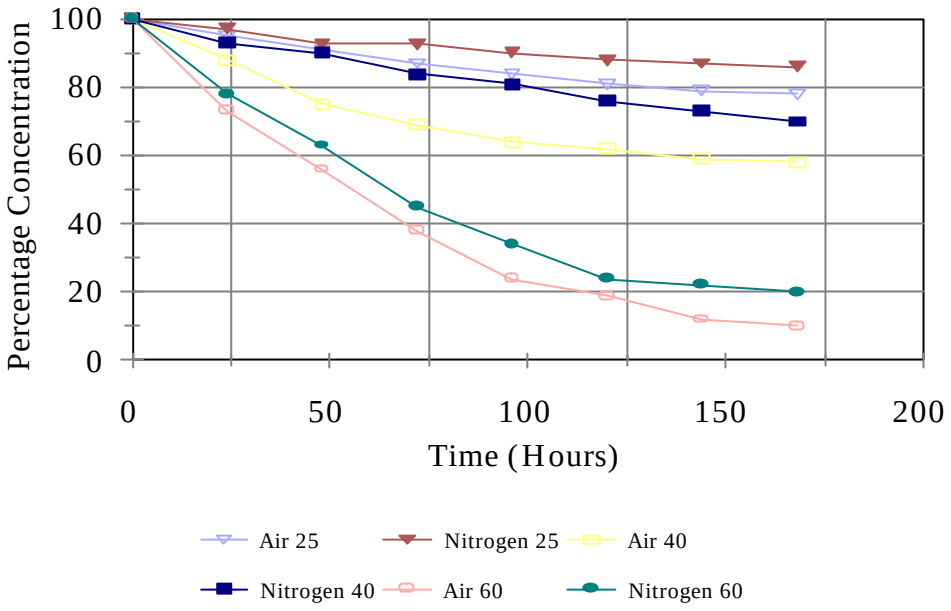
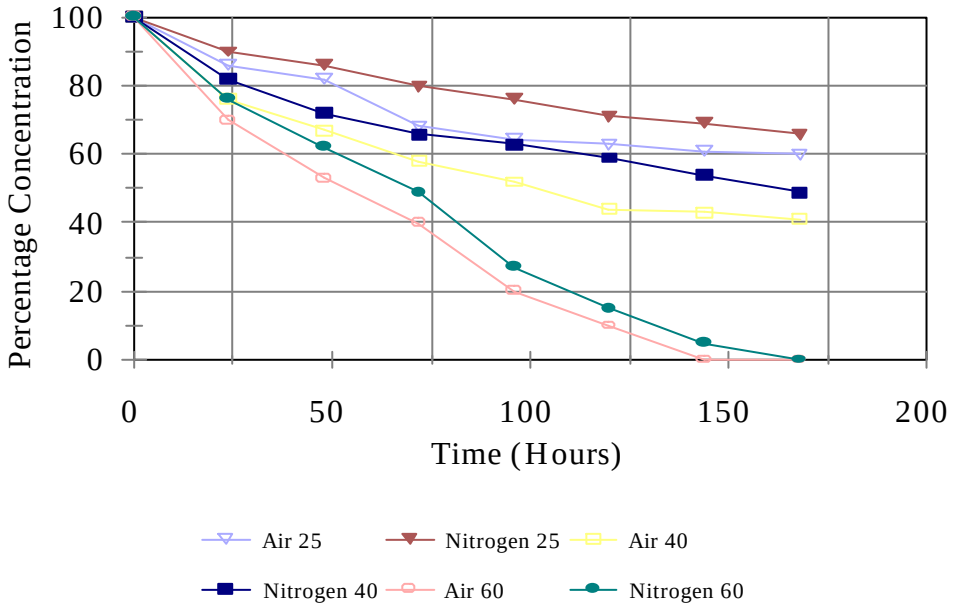


Figure 6.29 RIF stability in the presence of INH in aqueous crillet 3 solution



Oxidation and temperature dependent degradation of RIF has been a feature of all the

stability studies conducted, portrayed once again in figures **6.28**,**6.29**, **6.30** and table **6.10**.

Figure 6.30 RIF stability in the presence of PZA in aqueous crillet 3 solution

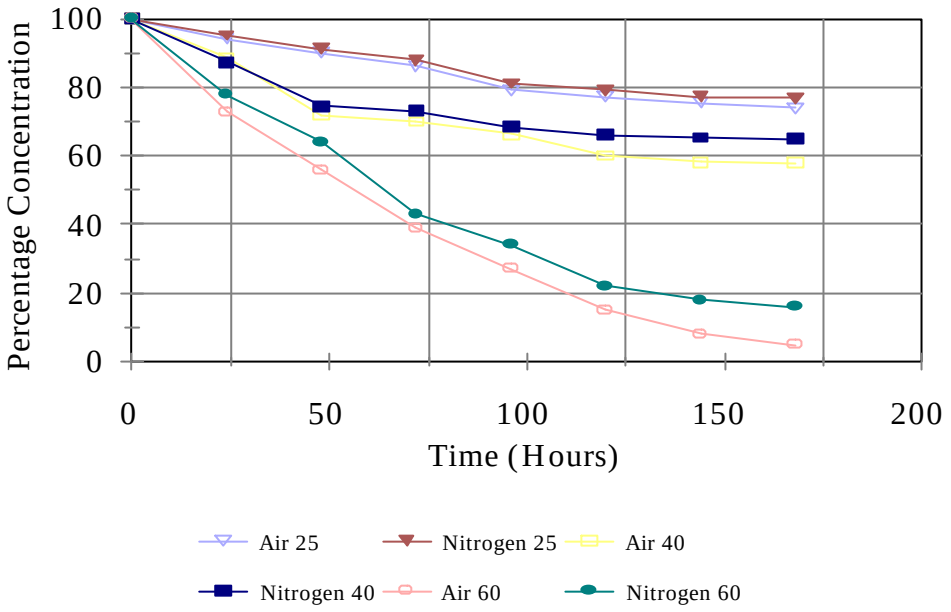


Table 6.10 Percentage RIF remaining after 7 days - CRILLET 3

Crillet 3 10%m/v Solution				
Temperature ^{0}C	**Gas**	**RIF**	**RIF with INH**	**RIF with PZA**
25	**Air**	78	60	74
	Nitrogen	86	66	77
40	**Air**	58	41	58
	Nitrogen	70	49	65
60	**Air**	10	0	5
	Nitrogen	20	0	16

Figure **6.28** shows a greater degree of degradation for RIF compared to that in aqueous solution, this being due to the surfactant/solubility enhancing nature of crillet 3, with figure **6.29** displaying an even greater degree of degradation of RIF in the presence of INH, with complete degradation after 6 days at 60 ^{0}C.

Figure 6.31 INH stability in aqueous crillet 3 solution

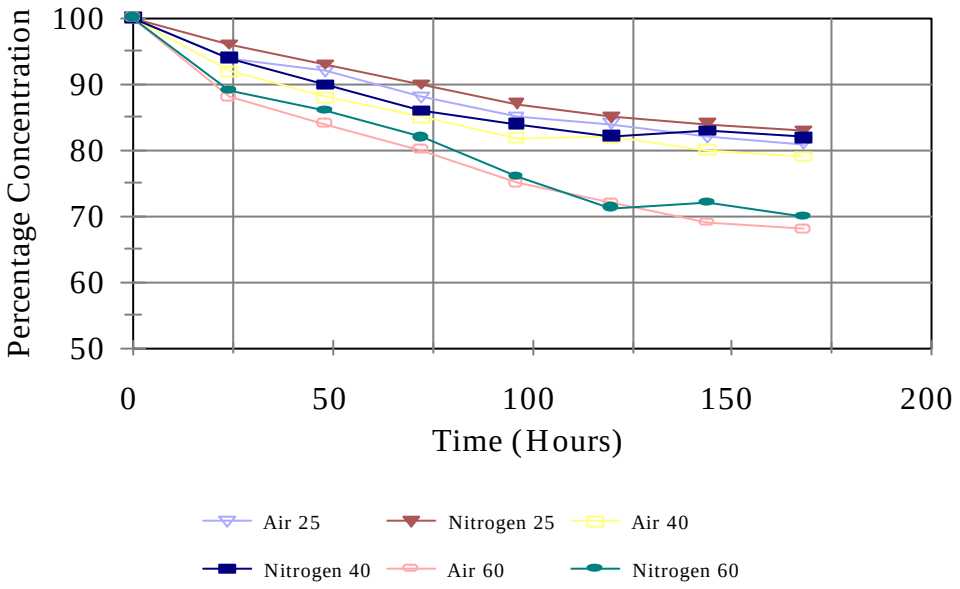
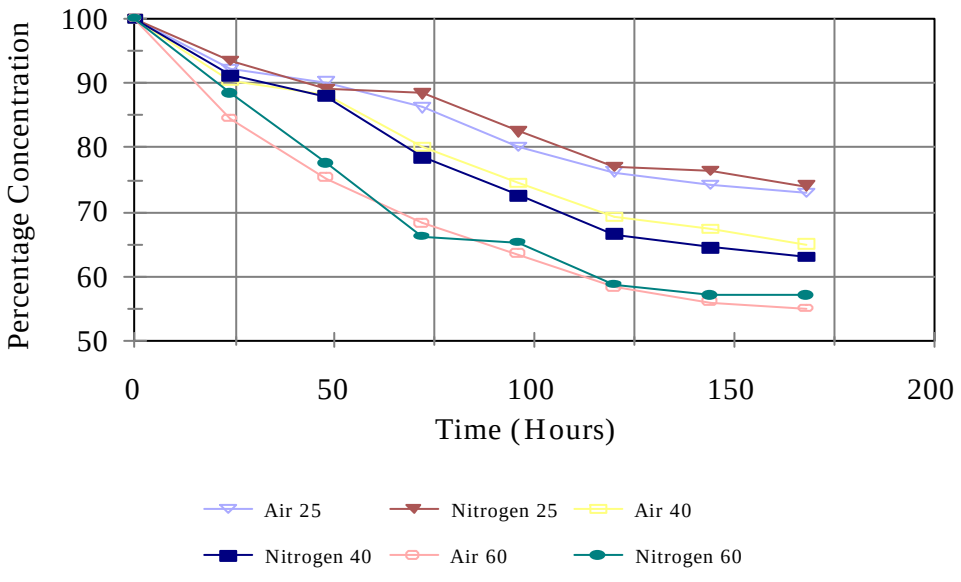


Figure 6.32 INH stability in the presence of RIF in aqueous crillet 3 solution



INH in crillet 3 aqueous solution is slightly more stable when stored under nitrogen, however, this is not significant enough to be of much benefit.

Figure 6.33 INH stability in the presence of PZA in aqueous crillet 3 solution

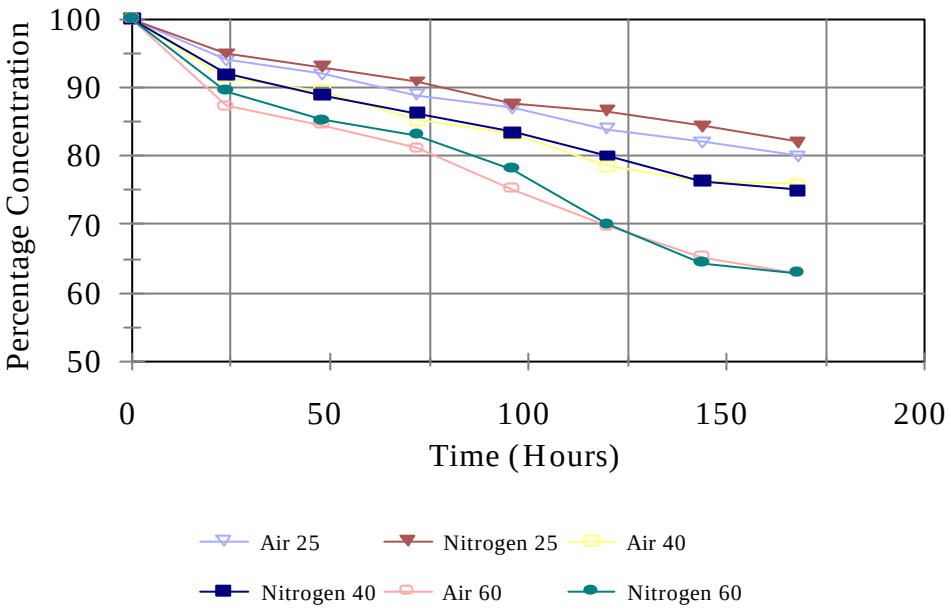


Table 6.11 Percentage INH remaining after 7 days - CRILLET 3

Crillet 3 10%m/v Solution				
Temperature ^{0}C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	81	73	80
	Nitrogen	83	74	82
40	**Air**	79	65	76
	Nitrogen	82	63	75
60	**Air**	68	55	63
	Nitrogen	70	57	63

Degradation was greater when in the presence of RIF, with PZA having minimal effect on the stability of INH. Temperature dependent degradation was demonstrated in figures **6.30**, **6.31** and **6.32**, being more defined when in combination with INH and PZA.

Figure 6.34 PZA stability in aqueous crillet 3 solution

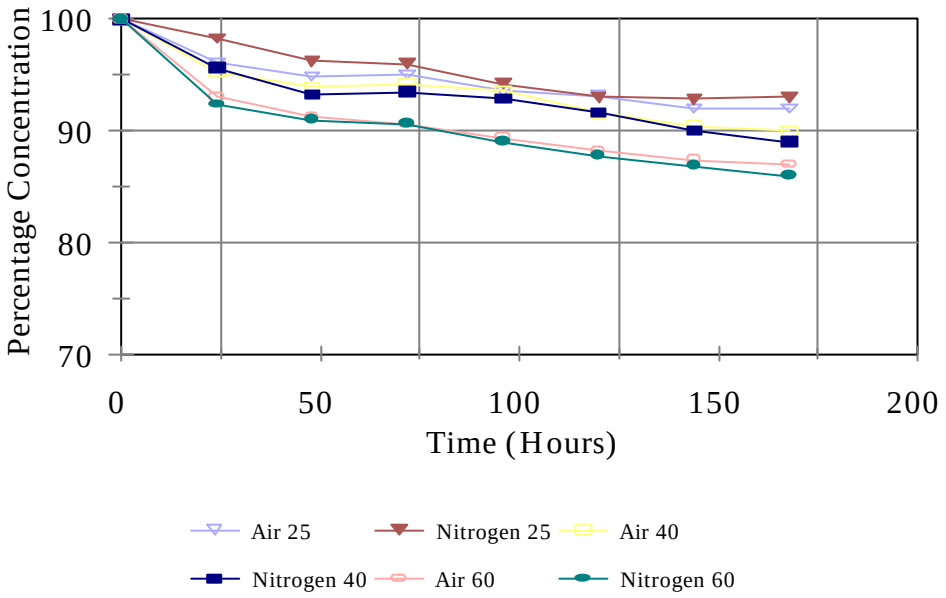


Figure 6.35 PZA stability in the presence of RIF in aqueous crillet 3 solution

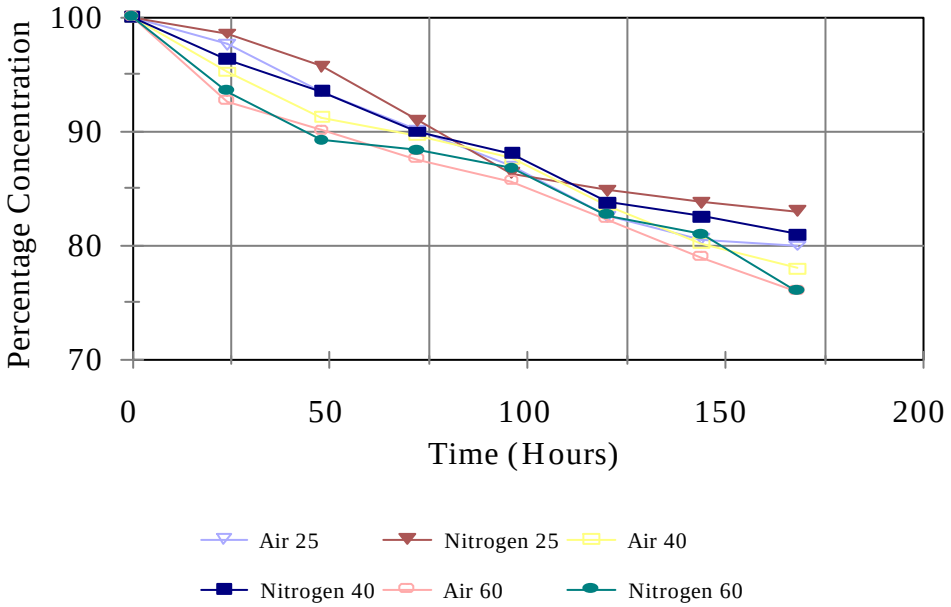


Figure 6.36 PZA stability in the presence of INH in aqueous crillet 3 solution

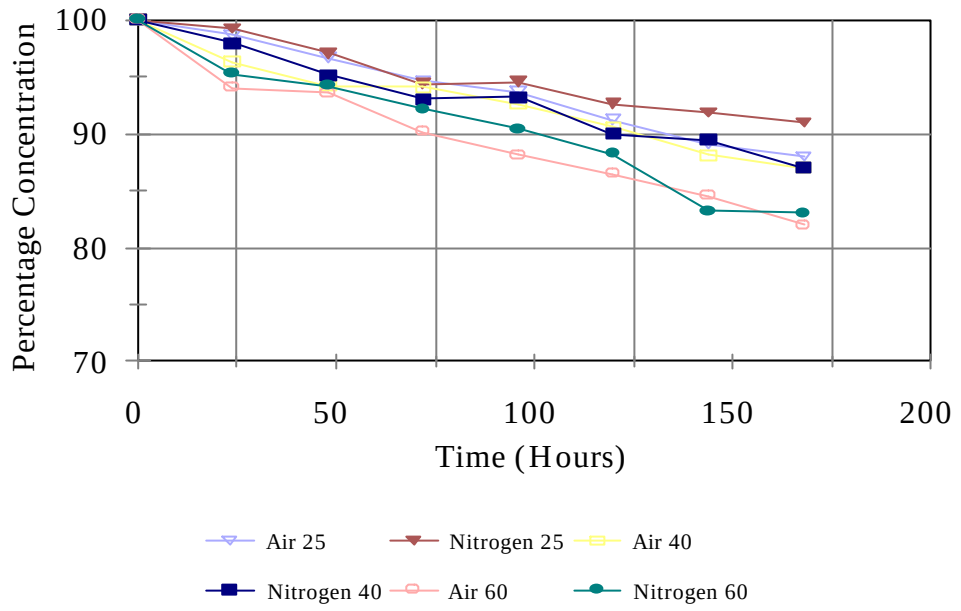


Table 6.12 Percentage PZA remaining after 7 days - CRILLET 3

Crillet 3 10%m/v Solution				
Temperature ^{0}C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	**92**	80	88
	Nitrogen	**93**	83	91
40	**Air**	**90**	78	87
	Nitrogen	**89**	81	87
60	**Air**	87	76	82
	Nitrogen	86	76	83

PZA stability relative to RIF and INH is demonstrated with greater than 86% of the drug

remaining after 7 days. A decrease in the amount of drug remaining after 7 days to 76 % is shown for PZA in combination with RIF, with a small decrease to 82% with INH. Temperature dependent degradation is not significant, with the profiles for 25, 40 and 60^{0}C almost overlapping. Storage under nitrogen also has little effect on PZA stability.

6.6.5 Corn oil Stability

The solubility of RIF, INH and PZA proved to be a limiting factor in the stability study of these drugs in corn oil. INH and PZA, from the solubility tables listed in sections **2.5.2.8** and **2.5.3.8**, have solubilities in corn oil that are too low to permit analysis in an accelerated degradation study over a 7 day period. RIF proved to be rather unstable in corn oil, with complete degradation of the drug occuring after 6 days at 40^{0}C and 2 days at 60^{0}C. At 25^{0}C, under air and nitrogen, only 48 and 67% of the RIF remained after 7 days. The stability profile indicated significant oxygen dependent degradation at 25^{0}C.

6.6.6 Cremophor RH Stability

Cremophor RH (Polyoxyl 40 Hydrogenated Castor Oil) is a mixture of the trihydroxystearate ester of ethoxylated glycerol, with smaller amounts of macrogol trihydroxystearate and the corresponding free glycols. Cremophor RH is a nonionic surfactant known for it's emulsifying and solubilising ability, commonly used in the production of pharmaceutical preparations(Martindale, 1993). It is very soluble in water, and for the purpose of this study was used to prepare a 10%m/v aqueous solution. The stability results obtained for RIF, INH and PZA, are reported in tables **6.13**, **6.14** and **6.15** respectively.

Table 6.13 Percentage RIF remaining after 7 days - CREMOPHOR

Cremophor RH 10%m/v Solution				
Temperature ^0C	**Gas**	**RIF**	**RIF with INH**	**RIF with PZA**
25	**Air**	79	**69**	**75**
	Nitrogen	89	74	**81**
40	**Air**	62	53	**58**
	Nitrogen	76	66	**70**
60	**Air**	20	5	**20**
	Nitrogen	33	15	**30**

The stability profiles constructed for RIF illustrate a clear oxidation and temperature dependent influence on degradation, over the entire 7 day study. The results listed in table **6.13** confirm this with the stability of RIF being enhanced when stored under nitrogen, although the presence of INH still produces greater RIF degradation, than when in it's absence. This occurrence is consistent, independent of the type of vehicle used.

Table 6.14 Percentage INH remaining after 7 days - CREMOPHOR

Cremophor RH 10%m/v Solution				
Temperature ^0C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	**89**	**80**	**82**
	Nitrogen	**82**	**83**	**85**
40	**Air**	**77**	**70**	**78**
	Nitrogen	**72**	**71**	**78**
60	**Air**	**81**	**67**	**66**
	Nitrogen	**79**	**69**	**68**

No oxidation or temperature dependence was evident for INH stability on it's own, with temperature dependence becoming a feature when in the presence of RIF and PZA. Here greater degradation seems to occur at 60^0C after 7 days, implying a temperature induced interaction between INH and RIF or PZA. The benefit of nitrogenation is not justified, with no significant increase in stability noted.

Table 6.15 Percentage PZA remaining after 7 days - CREMOPHOR

Cremophor RH 10%m/v Solution				
Temperature ^{0}C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	**85**	**85**	**84**
	Nitrogen	**89**	**90**	**86**
40	**Air**	**83**	**85**	**81**
	Nitrogen	**86**	**88**	**86**
60	**Air**	**81**	**86**	**80**
	Nitrogen	**84**	**82**	**84**

PZA stability remains consistently unaffected by temperature, air or the presence of RIF or INH. After 7 days, in the presence of RIF, INH or alone, independent of the temperature or the presence of air, at no point does the percentage PZA remaining drop below 80%.

6.6.7 Sorbitol Stability

Sorbitol is a polyhydric alcohol, commonly employed in the preparation of numerous pharmaceutical preparations. A 70%m/v solution was used in this stability study, and in microemulsion preparation. It is not a reducing sugar, and is therefore not expected to affect the stability of INH, which is a concern. The solubility of RIF in 70%m/m sorbitol

solution was found to be negligible as indicated in table 2.5.1.8, thus not making the stability assessment of RIF alone and in combination with INH and PZA in solution, possible. The results of INH and PZA stability in the sorbitol solution are reported in tables 6.16 and 6.17.

Table 6.16 Percentage INH remaing after 7 days - SORBITOL

Sorbitol 70%m/v				
Temperature ^{0}C	**Gas**	**INH**	**INH with RIF**	**INH with PZA**
25	**Air**	**91**	**-**	**93**
	Nitrogen	**94**	**-**	**94**
40	**Air**	**86**	**-**	**88**
	Nitrogen	**88**	**-**	**89**
60	**Air**	**79**	**-**	**78**
	Nitrogen	**80**	**-**	**79**

INH stability in sorbitol solution proved to be greater than in any of the other formulation components tested, with all concentrations recorded in table **6.16**, being greater than 79% and as high as 94% at 25^{0}C. The presence of PZA had no effect on the drug stability, with the benefit of storage under nitrogen being minimal.

PZA, although not as stable as INH at 25^{0}C, possesses greater temperature stability up to 60^{0}C in sorbitol solution, even when combined with INH. These results are displayed in table **6.17**.

Table 6.17 Percentage PZA remaining after 7 days - SORBITOL

Sorbitol 70%m/v				
Temperature ^0C	**Gas**	**PZA**	**PZA with RIF**	**PZA with INH**
25	**Air**	**85**	**-**	**80**
	Nitrogen	**85**	**-**	**86**
40	**Air**	**81**	**-**	**81**
	Nitrogen	**83**	**-**	**83**
60	**Air**	**82**	**-**	**79**
	Nitrogen	**83**	**-**	**80**

6.7 Conclusion

The first four formulation components assessed in this chapter were used in the production of the final microemulsion selected for the formulation stability assessment of RIF, INH and PZA. The ideal situation that this work is striving for is one where RIF is soluble and stable in a lipid formulation component, with minimal interaction with INH or PZA, and the opposite situation where INH and PZA are soluble and stable in an aqueous formulation component with minimal interaction. The overriding limit set on this project was the selection of formulation components, that can deliver the doses of RIF, INH and PZA required in the small quantities of formulation components comprising the final unit dose.

Trends in the stability assessments conducted on RIF, INH and PZA were noted, with slight variation depending on the formulation component being evaluated. RIF when investigated in solution on it's own, invariably demonstrated temperature and oxidation dependent degradation in all vehicles. Definite distinction could be made between samples

stored at 25, 40 and 60^{0}C over the 7 day trial period. A definite advantage of storing RIF solutions under nitrogen was also observed, with these solutions showing less degradation over the trial period, than those stored under air. The final drug concentrations remaining after 7 days, reported in the tables of each study illustrate this oxidation and temperature dependence with the graphs constructed, showing the drug degradation with time. The temperature dependent degradation of RIF has been proposed to be via the formation of a highly reactive carbene intermediate which degrades rapidly. Air oxidation of the *p*-phenolic groups in the naphthalene ring is responsible for the instability of the drug in the presence of air, with the corresponding *p*-quinones formed(Connors, K.A., *et al.*, 1986; Foye, W.O., *et al.*, 1995; Lund, W., 1994). Acid catalyse hydrolysis of RIF is another route of degradation, with RIF concentration dependent degradation also reported by Connors *et al.*(Connors, K.A., *et al.*, 1986). As can be seen in the degradation studies of RIF in combination with INH and PZA, these drugs affect RIF stability differently, despite the structural similarity of the two compounds. INH produces a pronounced increase in the degree of degradation of RIF, whereas PZA has a negligible effect on it's stability. The mechanism for the INH enhanced degradation of RIF is postulated to be due to an INH degradation product interacting with 3-formylrifamycin. 3-formylrifamycin exists in equilibrium with RIF, the binding of an INH degradation product with this molecule, effectively reduces the concentration of RIF in solution accounting for the lower concentrations of RIF found when combined with INH. This relationship appears to be INH concentration dependent and occurs by a mechanism not yet elucidated, trapping the drug in the 3-formyl rifamycin form, effectively reducing the RIF available and enhancing the degradation of the drug. From the results reported in this chapter, the effect of the vehicle on RIF stability was unexpected, with the stability in aqueous solution generally being better than in lipid. Variations between stability results in aqueous media vary. The presence of a surfactant promoting aqueous solubility of RIF appears to have as a result a larger degree of degradation of the drug, this is particularly noticable in the presence of INH. This effect is believed to be due to a concept relied upon in this work for the

emulsification process to be effective. The presence of a surfactant acts to reduce the degree of electrostatic repulsion between molecules in solution, enabling INH and RIF molecules to approach each other more closely, thus potentiating the degradation enhancing effect of INH. This effect is demonstrated by the results reported in order of greater HLB value (degree of hydrophilicity), with crillet 3(15)producing greatest RIF degradation followed by cremophor(13.5) and then imwitor 308(6), in order of decreasing hydrophilicity(HLB value). Of the two lipids investigated RIF is more stable in miglyol 812 than in corn oil, this believed to be due to miglyol being a semi-synthetic lipid, over which a greater degree of control can be exerted when concerned with it's synthesis. Corn oil is obtained from a natural more variable source, where interaction with the mixture of fatty acid esters comprising it is more likely. A positive point of miglyol 812 as a prospective vehicle is the poor solubility of INH and PZA in it, while RIF solubility is significantly better.

INH proved to be most stable in the 70%m/v sorbitol solution with no significant oxidation or temperature dependent degradation indicated. Oxidation is not a pathway for degradation of INH, based on the structure of the molecule. INH is next most stable in aqueous solution, crillet 3, imwitor 308, cremophor and is least stable in miglyol 812, being affected by the insufficient solubility of INH in this lipid. Temperature dependent degradation is small, with this only being noticable when INH is in combination with RIF, most significantly in crillet 3 solution.

PZA is the most stable of the three drugs, but is unfortunately the one that is most expendable and neither lipophilic or hydrophilic enough to be incorporated into the formulation selected. Stability is relatively unaffected by temperature and the presence of air, and is very stable compared to RIF and INH, independent of the vehicle employed, although the drug remaining does decrease slightly in the presence of RIF.

Due to drug dose specifications and solubility limitations, the final formulation assessed in

chapter 7, only contained RIF and INH, despite INH and PZA having no significant effect on the stability of each other. This cannot be said for RIF in the presence of INH and PZA, where the presence of either of these two drugs, produced a greater degree of degradation of RIF, INH exhibiting this effect to a greater degree than PZA. The solubility of PZA in the lipid and aqueous components of the microemulsion was not significant enough to achieve the required 500 mg/10ml dose, while RIF and INH could achieve the respective 150mg/10ml and 100mg/10ml dose.

Chapter Seven

Microemulsion Stability Study

7.1 Introduction

The concept of a microemulsion is very appealing for the increased bioavailability and delivery of several drugs of differing properties and compatabilities in combination. Historically, microemulsions have proven to be problematic when identifying surfactant combinations to produce a thermodynamically stable formulation, easily overcome with adequate preformulation work, with the benefit of a stable microemulsion being more desirable for the delivery of certain drugs than other liquid dosage forms. The only example of a microemulsion as a preconcentrate available on the market at present, is Sandimmune Neoral®, a cyclosporine formulation designed to optimise the oral absorption of this drug. Following oral administration, this formulation immediately forms a microemulsion in aqueous fluids, resembling a mixed micellar phase(Friman, S., *et al.*, 1996; Kovarik, J.M., *et al.*, 1994; Meinzer, A., 1995). The result is the rapid availability of the drug for absorption, enabling the utilisation of the entire length of the absorption window. The microemulsion is not dependent on the lipid dispersion step of digestion, which results in absorption being less affected by bile flow and pancreatic lipase, with the effect on absorption anticipated, and determined to affect the pharmacokinetics of the drug. The microemulsion demonstrated reduced inter and intraindividual variability, relative to other marketed cyclosporine formulations, yielding a more consistent and predictable concentration-time profile (Meinzer, A., 1995; Kovarik, J.M., *et al.*, 1994). Microemulsions were targeted as a desirable method for formulating RIF in particular, due to the success with cyclosporine, also being a high molecular mass lipophilic molecule (Kim, C-K, *et al.*, 1997; Meinzer, A., 1995). The Sandimmune Neoral® results show promise in the areas of greater therapeutic efficacy and the reduction of drug doses required due to improved

bioavailability.

This chapter encompasses an accelerated stability study of a selected microemulsion developed in chapter 5, which incorporates RIF and INH as the active drug substances. The desired drug doses required per dosing unit are 150mg of RIF and 100mg of INH. The stability of the drugs and the formulation were assessed over a 10 week period.

The theory for the stability assessment of a new drug substance or product as outlined by the ICH was used as a model for this formulation study, as used for the assessment of RIF, INH and PZA in formulation components in chapter 6. The ICH guidelines for this stability assessment are detailed in section **6.2**.

7.2 Instrumentation and Equipment

The chromatographic system used for the HPLC analysis of all aqueous fractions was the same as that developed and validated in Chapter 3.

The equipment listed in section **4.2.1** for the lipid-aqueous partitioning method developed and validated in chapter 4 was used in the analysis of RIF and INH in the microemulsion formulation investigated. Forced circulation ovens, type FSOH, made by Labcon(USA) were used for the heating of samples.

7.3 Solvents and Reagents

Rifampicin(RIF) and isoniazid(INH) were donated by the Druggists Group Reasearch unit(Pharmacare-Lennon, RSA). Methanol and acetonitrile were purchased from BDH®, England(purity >99.9%). All aqueous fractions were prepared using double distilled deionised water, obtained from a Milli-RO 15 water purification system, manufactured by

Millipore®(Massachusetts, USA). Miglyol 812 and Imwitor 308 were donated by Hüls, Southern Africa and Crillet 3 by Croda, Southern Afica.

7.4 Preliminary Formulation Stability Methodology

Ternary and pseudo-ternary phase diagrams were constructed in chapter 5 for numerous systems, with successful w/o and o/w macro and microemulsions then selected for a preliminary formulation stability study. The preliminary stability study was conducted to determine possible formulations that would remain stable over the length of time of this chapter's end formulation study. Four 10ml samples of each prospective formulation were prepared and placed in amber glass ampoules, two sealed under air and two under nitrogen. One air and one nitrogen sample was placed in a convection oven at 25^0C and the same done for the remaining samples at 60^0C. The physical stability of the samples was visually assessed daily and recorded over a two week period. The samples were prepared in the form most closely resembling the final proposed packaging in production. The method of drug delivery in practice was intended to be in single sealed 10ml unit doses made of an opaque gas impermeable polymer. This situation is most closely resembled by sealed amber 10ml glass ampoules.

7.5 Drug Stability Methodology

Following on from the microemulsion selected after the preliminary formulation stability study, drug stability of RIF and INH in formulation was assessed. The selected formulation of composition stated in section **7.6.1** was prepared by the method detailed here. 315.9g of water was measured and 15g of INH dissolved in it, using a magnetic stirrer, at room temperature. 414.5g of crillet 3 was weighed and added to the aqueous solution of INH. 355.2g of miglyol 812 was measured and 22.5g of RIF dissolved in it using a magnetic stirrer at room temperature. 414.5g of imwitor 308 was weighed and mixed with the lipid

solution of RIF. Half of the lipid solution was decanted and 750mg of vitamin A acetate powder dissolved in it, vitamin A being a lipid soluble oxygen radical scavenger. Half of the aqueous fraction was in turn, decanted, and the resultant aqueous and lipid fractions mixed together to spontaneously produce two clear pourable dark red/orange homogeneous o/w microemulsions. The one microemulsion contained no antioxidant(**M1**) while the other contained a 0.1%m/v concentration of vitamin A, theoretically, entirely dissolved in the lipid phase(**M2**). Partitioning of a small quantity of the vitamin A into the aqueous phase could not be ruled out, however, this would be minimal due to it's poor aqueous solubility. 10ml aliquots of the formulations produced were placed in 10ml amber glass ampoules, half of these ampoules were filled with nitrogen prior to sealing and half were sealed under air. 24 ampoules of each microemulsion **M1** and **M2**, half of which were nitrogen filled and half not, were then placed in the convection ovens at 25, 40 and 60^0C. Samples were drawn at weekly intervals and after undergoing the lipid-aqueous extraction procedure, the aqueous fraction was injected onto the HPLC in triplicate. These results are reported in the figures and tables in section **7.6.2**.

7.6 Results and Discussion

7.6.1 Preliminary Formulation Stability Assessment

The visual assessment of the stability of the prospective formulations for use, was done primarily by observing homogeneity. Any sign of phase separation or cracking would not allow for the formulation to maintain integrity over the length of the stability study. The formulations were observed and the results recorded in table **7.1** as being stable or not, at 25 and 60^0C over a two week period. The surfactant:cosurfactant (SAA:Co-SA) combinations are listed in the table. The subsequent SAA:Co-SA (SA) combination proportions relative to lipid are listed as well(SA:Lipid). Miglyol 812 was used as the lipid in all the formulations assessed.

Table 7.1 Preliminary formulation stability after two weeks

Formulation Stability				
			Temperature (^{0}C)	
Composition	**SAA: Co-SA**	**SA:Lipid**	**25**	**60**
Cremophor:Sorbitol(pwdr)	7 : 3	9 : 1	T	W
		8 : 2	T	W
		7 : 3	T	W
		6 : 4	W	W
	5 : 5	9 : 1	T	W
		8 : 2	T	W
		7 : 3	T	W
Imwitor 308:Crillet 3	5 : 5	9 : 1	T	W
		8 : 2	T	W
		7 : 3	T	T
	3 : 7	9 : 1	T	W
		8 : 2	T	W
Brij 97 alone		9 : 1	T	W
Brij97:Sorbitol(70%)	9 : 1	1 : 9	W	W
		2 : 8	T	W
Cremophor:Sorbitol(70%)	9 : 1	9 : 1	T	W
	7 : 3	9 : 1	T	T
	5 : 5	9 : 1	W	W

From the results listed in table **7.1** almost all the formulations selected for testing were stable at room temperature, except the coarse emulsions which cracked early in the assessment. Only two formulations were stable at 60^{0}C over the two week period with only one able to incorporate the quantities of RIF and INH required. The microemulsion selected from this

table, was composed of imwitor 308: crillet 3 (5:5) in the starting point ratio of 7:3 with lipid, to which water was added. The composition of this stable formulation is water(21.06%), miglyol 812(23.68%), imwitor 308(27.63%) and crillet 3(27.63%). This formulation maintained homogeneity, with no colour or appearance changes over the period of assessment, remaining a clear liquid o/w microemulsion, and maintained integrity on excess dilution with water.

7.6.2 Drug Stability Assessment in Formulation

M1 and **M2** formulated following the method outlined in section **7.2.4**, were assessed weekly and the results graphically depicted in figures **7.1**, **7.2**, **7.3** and **7.4**.

Figure 7.1 Percentage RIF remaining after 10 weeks - no antioxidant

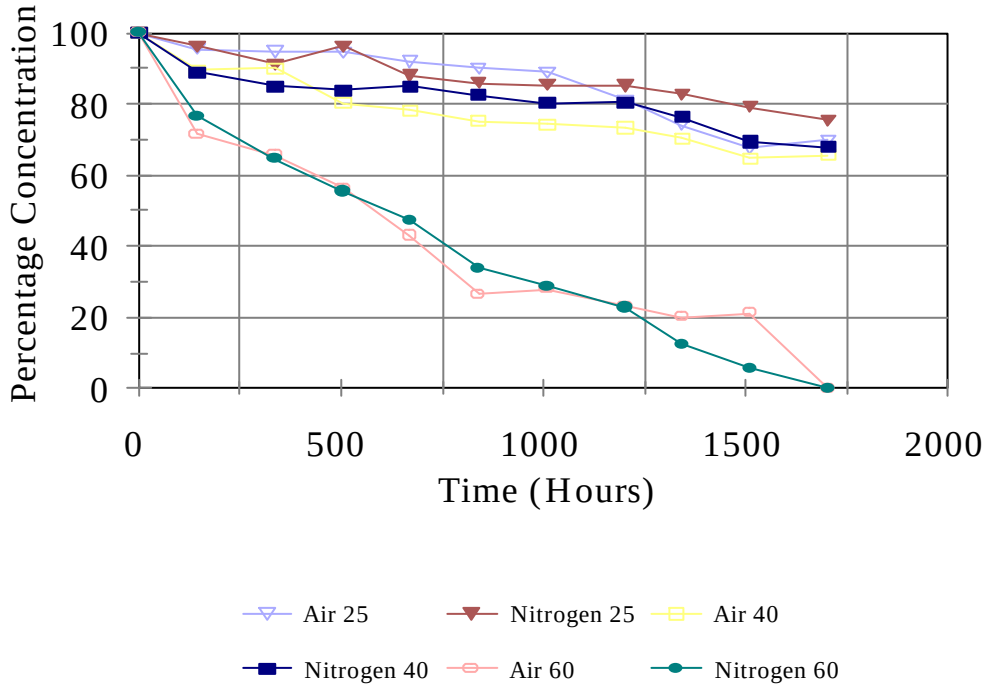
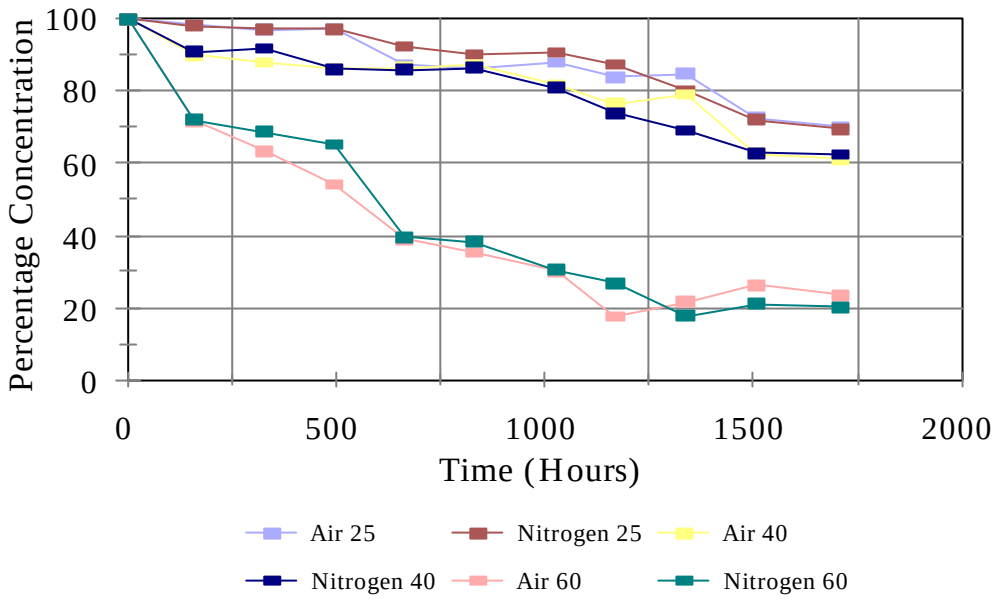


Figure 7.2 Percentage RIF remaining after 10 weeks - antioxidant



The formulations produced for this stability study remained physically stable for the entire 10 week period of the trial, maintaining homogeneity and clarity at all temperatures and conditions.

Table 7.2 Percentage RIF remaining after 10 weeks

		Microemulsion	
Temperature ^{0}C	**Gas**	**M1**	**M2**
25	**Air**	70	70
	Nitrogen	75	70
40	**Air**	66	61
	Nitrogen	68	63
60	**Air**	0	24
	Nitrogen	0	20

The 10 week time frame of this stability study falls short of the ICH minimum suggested time of 6 months at 40^0C, but the results produced, do remain of interest. In formulation, RIF stability at 25 and 40^0C proved to be superior to that in the individual formulation components. The benefit of storage under nitrogen has been shown to be negligible at these temperatures, with the presence of the antioxidant in the lipid phase being of little benefit at 25 and 40^0C. Temperature instability was demonstrated at 60^0C in **M1** and **M2**, with an accelerated rate and degree of degradation relative to the samples at 25 and 40^0C**.** Storage under nitrogen produced no benefit over storage under air at this temperature, although the benefit of the antioxidant became more apparent. At 60^0C , **M2** containing the antioxidant, proved to have greater stability over the 10 week period, with 20 to 24% RIF remaining, whereas in **M1** complete degradation of the drug had occured. Of particular interest is the result that RIF retained adequate therapeutic concentration in formulation of greater than 90% for the first six weeks, at 25^0C, stored under nitrogen and in the presence of the antioxidant. Both **M1** and **M2** maintained RIF drug concentrations greater than 80% over the first 8 weeks, whether under air or nitrogen.

Table 7.3 Percentage INH remaining after 10 weeks

		Microemulsion	
Temperature ^{0}C	**Gas**	**M1**	**M2**
25	**Air**	30	72
	Nitrogen	32	69
40	**Air**	25	50
	Nitrogen	27	51
60	**Air**	3	45
	Nitrogen	2	40

Figure 7.3 Percentage INH remaining after 10 weeks - no antioxidant

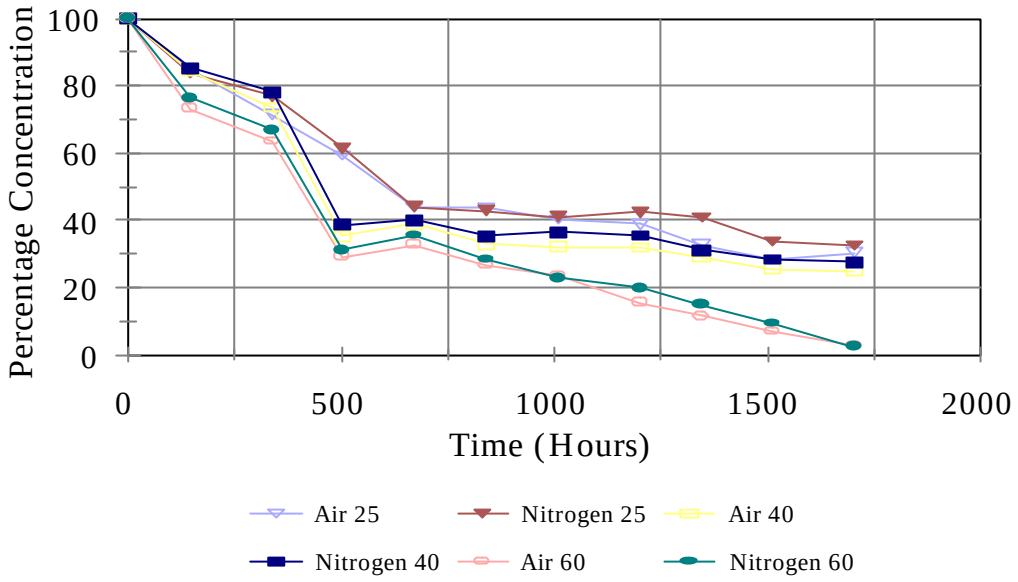
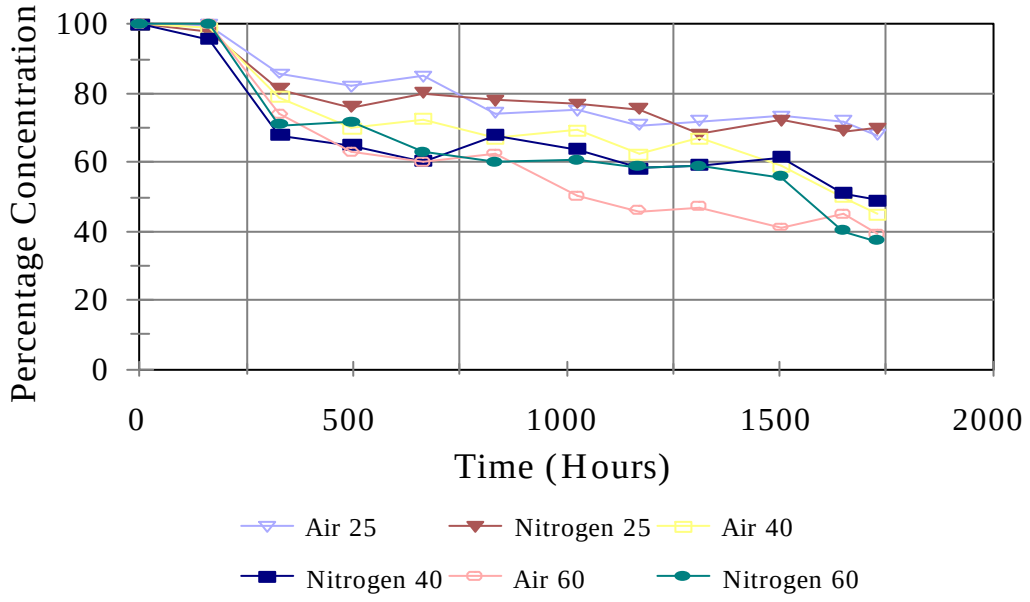


Figure 7.4 Percentage INH remaining after 10 weeks - antioxidant



The results obtained for INH deviated from those predicted. It was anticipated that INH, being separated from RIF would prove to be more stable and vice versa. This was proven for RIF, but not for INH. Despite INH being isolated in the aqueous phase and RIF in the lipid phase, INH degradation was significant, more so in the absence of antioxidant than in it's presence. The formulation containing the antioxidant in the lipid phase produced greater INH stability, which is unusual, since INH possesses poor miglyol 812 solubility of 0.32mg/ml with the antioxidant correspondingly insoluble in water but soluble in lipid. Degradation of INH at 25, 40 and 60^0C is significant in the absence of the antioxidant, as is demonstrated by the results in table **7.3**, with almost complete degradation of the drug in **M1** by the end of the trial. Degradation is significant in the first four weeks of the study in **M1**, with 60 to 70% of the starting concentration degrading, tapering off thereafter. In the presence of the antioxidant, degradation is consistent over the entire length of the study, with storage under nitrogen and temperature effects on stability not being as dramatic as in **M1**. In both **M1** and **M2** INH stability is poor, not being acceptable by ICH standards.

7.7 Conclusion

RIF stability was improved, as anticipated, being isolated in the internal lipid phase of the o/w microemulsion. Solubilisation of the RIF in the lipid droplets and lipophilic chains of the surfactants comprising the surfactant/cosurfactant interfacial film surrounding the lipid droplets increases the quantity of RIF that can be incorporated into the formulation, while protecting it from oxidative degradation to which it is sensitive. The incorporation of RIF into this internal phase also decreases it's contact with INH which has been shown to affect it's stability. The incorporation of an antioxidant into this internal phase acts as an added safety measure against oxidation of the drug.

INH which proved to be relatively stable when alone in aqueous solution in chapter 6 was

expected to exhibit the same stability in formulation, even more so, being separated from RIF in the lipid phase. RIF behaved as predicted, possessing greater stability than shown in the individual formulation components in chapter 6, however, INH behaved unexpectedly. INH was less stable in formulation in the absence of antioxidant, than in it's presence. It would be expected that the antioxidant in the lipid phase would have little effect, if any, on INH stability. INH is almost exclusively soluble in the aqueous phase of this formulation, and vitamin A in the lipid phase. The only viable explanation that could possibly be furnished for this stability enhancing effect of the vitamin A on INH is that either some undissolved vitamin A particles were suspended in the aqueous phase; solubilisation of vitamin A in the interfacial film surrounding the lipid droplet imparts a degree of protection to INH; the presence of the surfactants used, increased the solubility of this antioxidant in the aqueous phase. Although any of these explanations may be possible, none should apply, since INH in all previous studies, has proved to be relatively stable to oxidation.

Chapter Eight

Conclusion

The aim of this project has been defined as the development of a microemulsion dosage form for the oral delivery of RIF, INH and PZA in combination, addressing the preliminary assessment of the stability of such a formulation and the stability of the drugs concerned, in the formulation and it's components. The prime motivator for this work is the current lack of availability of dosage forms for the delivery of the antitubercular agents RIF, INH and PZA in the treatment of paediatric and geriatric patients as outlined in chapter 1.

Microemulsion science was chosen as an alternative solution to this problem that deserved investigation given the novel advantages associated with these systems, although not having received much attention by the pharmaceutical industry, the benefits are rapidly becoming apparent. Research indicating advantages for the oral delivery of drugs subject to poor solubility and bioavailability is making combination formulation possible. Microemulsions offer the benefits of ease of preparation, as they form spontaneously, producing clear, stable fluid formulations for the combined delivery of incompatible and lipophilic drugs(Lawrence, M.J., 1994; Aboofazeli, R., *et al.*, 1993, 1994 a & b, 1995; Malcolmson, C., *et al.*, 1993, 1995).

This project followed a systematic approach in the development and assessment of such a formulation and of methods for the routine analysis of the drugs concerned. RIF, INH and PZA were adequately characterised in chapter 2, adhering to the monograph standards referenced and were found to be sufficiently pure to be used in subsequent work. An HPLC method for the simultaneous determination of RIF, INH and PZA was developed and validated in chapter 3, as was a method for the extraction and analysis of these drugs from lipid media in chapter 4.

The chromatographic system and conditions selected and validated as being optimal for HPLC analysis of RIF, INH and PZA in combination is **System 1,** employing the ∶Bondapak™ C18 column with MeCN:tBAH(0.0002M) mobile phase in a ratio of 42.5:57.5%v/v, within a pH range of 3.10 to 3.20. The lipid-aqueous partitioning method developed and validated involves the partitioning of the drugs from 0.2ml of the lipid sample into a 60:40 (methanol:water) aqueous fraction after 2 minutes of agitation, with subsequent filtration through a 0.22∶m Cameo® hydrophilic syringe filter. This method produced optimum partitioning of RIF, INH and PZA into the aqueous fraction, while minimising the amount of lipid that appears in the final aqueous filtrate. These analytical procedures were used in the assessment of the three antitubercular agents in chapters 6 and 7.

A detailed literature review into the science of microemulsion formulation in chapters 1 and 5 provided a starting point, providing ideas on the types of lipids and surfactants required to produce a stable and usable system. HLB and CPP are aids previously used in the selection of surfactants and cosurfactants in emulsion formulation, although as stated in chapter 5, it has been proven that surfactants with similar HLB and CPP values do not always produce the same systems, they merely act as a rough guide(Ansel, H.C., 1981; Lieberman, H.A., *et al.*, 1988). The lipid to be used as the oil phase to be incorporated into these systems was chosen, based on the optimum short to middle chain length lipids for microemulsification, and the solubility of the drugs in the lipid. Greatest RIF solubility was achieved in miglyol 812 which is employed in all reported phase mapping in chapter 5. Selected pseudo-ternary and ternary systems were also mapped employing corn oil and IPM as the lipid phases, however the results obtained were not of any use in practice, with drug solubility remaining a limiting factor.

Chapter 5 deals with the labour of identifying microemulsion regions within ternary and pseudo-ternary systems for specific combinations of surfactants, cosurfactants and lipids.

The objective was the determination of systems that will prove useful for the combination delivery of RIF, INH and PZA in the doses required, given the nature and solubilities of these drugs. RIF is a large lipophilic molecule with poor aqueous solubility, with INH and PZA being the opposite. A microemulsion incorporating sufficient lipid to dissolve the RIF, maintaining integrity on dilution, and keeping RIF, INH and PZA stable to degradation, is the ideal. The final unit dose is to be a 10ml opaque gas impermeable polymer delivering RIF(150mg), INH(100mg) and PZA(500mg) (SAMF, 1997). From the phase diagrams mapped, the 5 pseudo-ternary systems illustrated in figures **5.1** to **5.5** were the most promising, each with desirable attributes. The nonionic surfactants, cremophor RH and Brij 97 produced microemulsion regions which tend to be dilutable, incorporating a large fraction of miglyol 812. When combining sorbitol with a relatively lipophilic surfactant, the result produced was a stable, flexible film forming microemulsion regions over greater compositions of lipid and water than would ordinarily be seen. In the case of cremophor RH and Brij 97, the HLB values were approximately 13, thus the addition of the hydrophilic sorbitol does not have a very significant effect when combined with these, being capable of forming microemulsion regions without the aid of a cosurfactant like sorbitol. The solubility of RIF in microemulsions formed employing these agents was insufficient, failing to incorporate the drug doses required in the 10ml unit microemulsion dose. Ovothin 200 produced dilutable microemulsion regions at low surfactant and lipid content, but it was the imwitor 308:crillet 3 system which produced a stable, dilutable microemulsion incorporating a substantial quantity of miglyol 812. The effect of the more lipophilic imwitor 742 demonstrated the reduced isotropic regions formed when using this surfactant of the same class.

Preliminary formulation stability studies conducted in chapter 7 aided in the isolation of a stable system that would prove valuable in the formulation of the three drugs. Formulation combinations that were expected to incorporate the quantities of drug required, were identified and then subjected to a formulation stability assessment. A two week trial at 25

and 60^0C identified the imwitor 308:crillet 3(5:5) and cremophor RH:sorbitol(70%m/v)(7:3) surfactant combinations as favoured formulations, being stable at these temperatures employing miglyol 812 as lipid for both.

The solubility of RIF was the prime determinator in the selection of the microemulsion from the two proven stable at elevated temperature.

The composition of the successful candidate microemulsion is:

Imwitor 308	27.63%(m/m)
Crillet 3	27.63%(m/m)
Miglyol 812	23.68%(m/m)
Water	21.06%(m/m)

Stability studies of RIF, INH and PZA alone and in combination in the components of this microemulsion followed. The effects of oxidation and temperature dependence on the degradation of the three drugs was assessed over a 7 day period and reported in chapter 6.

PZA indicated no oxidation dependent degradation, with temperature not affecting the stability of this drug to any great extent. Stability of PZA in the presence of RIF and INH was demonstrated, with solvent dependent degradation only indicating miglyol 812 as having an influence, this believed to be due to the poor solubility and resultant low starting drug concentration of PZA. RIF stability was found to be greater in the aqueous solutions investigated than in the lipid(miglyol 812). Temperature accelerated degradation was demonstrated for all solutions of RIF as was oxidation dependence, with solutions stored under nitrogen possessing greater stability. RIF exhibited enhanced degradation in all solutions when in the presence of INH and PZA, with INH enhanced degradation of RIF being the most significant. INH degradation proved to be unaffected by the presence of PZA or by oxidation, with storage under nitrogen providing no significant benefit. INH

degradation was shown to be enhanced by the presence of RIF and at elevated temperature, with solvent dependent degradation shown to be greater in miglyol 812 and in the surfactant solutions than in aqueous solution.

The stability results produced, illustrated the need for the separation of RIF from INH and PZA in formulation, with the solubility of the three drugs favouring this situation. Despite INH and PZA stability in the presence of the other, the combination of the two drugs in the selected formulation will not be possible as PZA cannot achieve the 500mg dose required due to it's aqueous solubility being inadequate. RIF achieved the drug dose of 150mg required, as did INH, incorporating the required 100mg of drug into the 10ml volume of formulation.

The resultant formulation containing RIF and INH was subjected to a 10 week stability trial reported in chapter 7, with weekly sampling providing an indication of drug stability in formulation. The formulation maintained integrity over the entire period of the trial at all temperatures and conditions evaluated. RIF stability was found to be better in formulation than in individual components, being isolated in the dispersed droplets of the internal lipid phase. This stability was enhanced by being separated from the INH in the aqueous phase, with the presence of an antioxidant in the lipid enhancing stability to oxidation. Temperature dependent degradation was exhibited for RIF and INH. INH reacted unexpectedly in formulation, being isolated in the aqueous phase. In aqueous solution in chapter 6, INH proved to be relatively stable, this being the opposite in formulation. Several theories are proposed for this anomaly in chapter 7, however, none have been conclusively proven. Despite INH instability in formulation, RIF nevertheless maintained a drug concentration of greater than 90% for the first 6 weeks at 25^0C under nitrogen.
In conclusion, this project succeeded in producing a novel microemulsion formulation capable of delivering the incompatible RIF and INH in combination, with the drugs showing no destabilising effect on the microemulsion produced. Numerous oral

pharmaceutically acceptable microemulsion systems were mapped, with the capacity of being used for the delivery of other lipophilic drugs and drug combinations. The stability of RIF, INH and PZA in selected formulation components was assessed in addition to the development of successful extraction and analytical procedures for the determination of these drugs. Although the final formulation did not produce the desired stability of RIF and INH over the length of the formulation trial, it provided valuable information into possible future improvements of the microemulsion. Factors that may be investigated, are the effect of the pH of the aqueous phase on INH or the possibility of the surfactant and cosurfactant producing a combined destabilising effect on INH. The incorporation of an antioxidant into the aqueous continuous phase and optimising the concentration of that in the lipid are other aspects that may warrant attention with the objective of stabilising these drugs in formulation.

References

Aboofazeli, R., Lawrence, M.J., The phase behaviour of quarternary systems containing lecithin. *J. Pharm. Pharmacol.*, 43 (Suppl.) (**1991**) 87P.

Aboofazeli, R., Lawrence, M.J., Investigations into the formation and characterization of phospholipid microemulsions. I. Pseudo-ternary phase diagrams of systems containing water-lecithin-alcohol-isopropyl myristate. *Int. J. Pharm.*, 93 (**1993**) 161-175.

Aboofazeli, R., Lawrence, M.J., Investigations into the formation and characterization of phospholipid microemulsions. II. Pseudo-ternary phase diagrams of systems containing water-lecithin-isopropyl myristate and alcohol: influence of purity of lecithin. *Int. J. Pharm.*, 106 (**1994**) (a) 51-61.

Aboofazeli, R., Lawrence, C.B., Wicks, S.R. and Lawrence, M.J., Investigations into the formation and characterization of phospholipid microemulsions. III. Pseudo-ternary phase diagrams of systems containing water-lecithin-isopropyl myristate and either an alkanoic acid, amine, alkanediol, polyethylene glycol alkyl ether or alcohol as cosurfactant. *Int. J. Pharm.*, 111 (**1994**) (b) 63-72.

Aboofazeli, R., Patel, N., Thomas, M. and Lawrence, M.J., Investigations into the formation and characterization of phospholipid microemulsions. IV. Pseudo-ternary phase diagrams of systems containing water-lecithin-alcohol and oil; the influence of oil. *Int. J. Pharm.*, 125 (**1995**) 107-116.

Ansel, H.C., Introduction to Pharmaceutical Dosage Forms. Lea & Febiger, Philadelphia, 1981, Third Edition, pg 154-162.

Arkegar**t**, A.P., Kunjir, S.S. and Purandare, K.S., Simultaneous determination of rifampicin, isoniazid and pyrazinamide by high performance thin layer chromatography. *J. Pharm. & Biomed. Anal.*, 14 (**1996**) 1645-1650.

Attwood, D., Mallon, C. and Taylor C.J., Phase studies on oil-in-water phospholipid microemulsions. *Int. J. Pharm.*, 84 (**1992**) R5-R8.

Bhargava, H.N., Narurkar, A. and Lieb, L.M., Using microemulsions for drug delivery. *Pharm. Tech.*, 11 (**1987**) 46-54.

Brewer, G.A., Isoniazid. Analytical Profiles of Drug Substances., 6 (**1977**) 183-258.

British Pharmacopoeia, HMS Office, London, volumes 1 & 2, **1993**, pg 363-364, 526, 564, 580-581, 972, 1085, 1089-1090.

Carrigan, P.J. and Bates T.R., Biopharmaceutics of Drugs Administered in Lipid-Containing Dosage Forms I: GI Absorption of Griseofulvin from an Oil-in-Water Emulsion in the Rat. *J. Pharm. Sci.*, 62 (9) (**1973**) 1476-1479.

Carstensen, J.T., Drug Stability: Principles and Practices, Marcel Dekker Inc., New York, Second Edition, **1995**, pg539-579.

Charman, W.N., Lipids, lymph and lipidic formulation. *Bull. Tech. Gattefossé*, (**1997**) 28-29.

Clarke, G.S., The validation of analytical methods for drug substances and drug products in UK pharmaceutical laboratories. *J. Pharm. & Biomed. Anal.*, 12 (**1994**) 643-652.

Connors, K.A., Amidon, G.L. and Stella, V.J., Chemical Stability of Pharmaceuticals. Wiley-Interscience Publications, Second Edition, **1986**, pg 728-732.

Craig, D.M.Q., Barker, S.A., Banning, D. and Booth, S.W., An investigation into the mechanism of self-emulsification using particle size analysis and low frequency dielectric spectroscopy. *Int. J. Pharm.*, 114 (**1995**) 103-110.

Constantinides, P.P. and Yiv, S.H., Particle size determination of phase-inverted water-in oil microemulsions under different dilution and storage conditions. *Int. J. Pharm.*, 115 (**1995**) 225-234.

Dadgar, D., Burnett, P.E., Choc, M.G., Gallicano, K. and Hooper J.W., Application issues in bioanalytical method validation, sample analysis and data reporting. *J. Pharm. & Biomed. Anal.*, 13 (2) (**1995**) 89-97.

De Gennes, P.G. and Taupin, C., Microemulsions and the flexibility of oil/water interfaces. *J. Phys. Chem.*, 86 (**1982**) 2294-2304.

Edwardson, P.A.D., Bhaskar, G. and Fairbrother J.E., Method validation in pharmaceutical analysis. *J. Pharm. & Biomed. Anal.*, 8 , 8-12 (**1990**) 929-933.

Engström, S., Law and Order in Lipid-Water Systems - From Drug Delivery to the Barrier Properties of the Skin. *Bull. Tech. Gattefossé*, 88 (**1995**) 27-35.

Felder, E. and Pitre, D., Pyrazinamide. Analytical Profiles of Drug Substances., 12 (**1983**) 433-462.

Florence, A.T. and Attwood, D., Physicochemical Principles of Pharmacy. Macmillan Press, First Edition, **1981**, pg 214-345.

Florence, A.T. and Whitehill, D., The formulation and stability of multiple emulsions. *Int. J. Pharm.*, 11 (**1982**) 277-308.

Foye, W.O., Lemke, T.L. and Williams, D.A., Principles of Medicinal Chemistry. Williams & Wilkins, Fourth Edition, **1995**, pg 747-758.

Friman, S. and Bäckman, L., A New Microemulsion Formulation of Cyclosporin - Pharmacokinetics and Clinical Features. *Clin. Pharmacokinet.*, 30 (3) (**1996**) 181-193.

Fubini, B., Gasco, M.R. and Gallarate, M., Microcalorimetric study of microemulsions as potential drug delivery systems. I. Evaluation of enthalpy in the absence of any drug. *Int. J. Pharm.*, 42 (**1988**) 19-26.

Furniss[t], B.S., Hannaford, A.J., Rogers, V., Smith, P.W.G. and Tatchell, A.R., Vogel's Textbook of Practical Organic Chemistry including Qualitative Organic Chemistry, Longman, London, Fourth Edition, **1978**, pg 940-1062.

Gaitonde[t], C.D. and Pathak P.V., Rapid and sensitive estimation of Isoniazid, Pyrazinamide and Rifampicin in combined dosage form by reverse-phase liquid chromatography. *Drug Dev. Ind. Pharm.*, 17 (9) (**1991**) 1201-1214.

Gallo, G.G. and Radaelli, P., Rifampicin. Analytical Profiles of Drug Substances., 5 (**1976**) 467-513.

Gharbo, S.A., Cognion, M.M. and Williamson, M.J., Modified Dissolution Method for Rifampicin. *Drug Development and Industrial Pharmacy*, 15 (2) (**1989**) 331-335.

Gautier, J.C., Microemulsions something of interest at research laboratory scale as well as for industrial applications. *Bull. Tech. Gattefossé*, 88 (**1995**) 61-64.

Herfindal, E.T., Gourley, D.R. and Lloyd hart, L., Clinical Pharmacy and Therapeutics. Williams & Wilkins, Fifth edition, **1991**, pg 1092-1109.

Ho, H-O., Hsiao, C-C. And Sheu, M-T., Preparation of Microemulsions Using Polyglycerol Fatty Acid Esters as Surfactant for the delivery of Protein Drugs. *J. Pharm. Sci.*, 85 (2) (**1996**) 138-143.

International Committee on Harmonisation, Harmonised Tripartite Guideline, Validation of Analytical Procedures, ICH Steering Committee, **1996**, pg 1-9.

Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W., Theory of self assembly of hydrocarbon amphiphiles into micelles and bilayers. *J. Chem. Soc. Faraday Trans. II*, 72 (**1976**) 1525-1568.

Jenke, D.R., Chromatography Method Validation: A Revision of Current Practices and Procedures. II. Guidelines for Primary Validation Parameters. *J. Liq. Chrom. Rel. Tech.*, 19 (5) (**1996**) 737-757.

Jindal, K.C., Chaudhary, R.S., Singla, A.K., Gangwal, S.S. and Khanna S., Dissolution test method for rifampicin-isoniazid fixed dose formulations. *J. Pharm. & Biomed. Anal.*, 12 (4) (**1994**) 493-497.

Kale, N.J. and Allen, L.V., Studies on microemulsions using Brij 96 as surfactant and glycerin, ethylene glycol and propylene glycol as cosurfactants. *Int. J. Pharm.*, 57 (**1989**) 87-93.

Kim, Chong-Kook., Ryuu, Sang-A., Park, Kyung-Mi., Lim, Soo-Jeong. and Hwang, Sung-Joo., Preparation and physicochemical characterization of phase inverted water/oil microemulsion containing cyclosporin A. *Int. J. Pharm.*, 147 (**1997**) 131-134.

Kovarik, J.M., Mueller, E.A., Van Bree, J.B., Tetzloff, W. and Kutz, K., Reduced Inter and Intraindividual Variability in Cyclosporine Pharmacokinetics from a Microemulsion Formulation. *J. Pharm. Sci.*, 83 (3) (**1994**) 444-446.

Krukenberg, C.C., Mischler, P.G., Massad, E.N., Moore, L.A. and Chandler, A.D., Stability of 1% rifampicin suspensions prepared in five syrups. *Am. J. Hosp. Pharm.*, 43 (**1986**) 2225-2228.

Lang, J.R. and Bolton, S., A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry - 1. Experimental considerations., *J. Pharm. & Biomed. Anal.*, 9 (55) (**1991**) 357-361.

Lawrence, M.J., Surfactant Systems: Microemulsions and Vescicles as Vehicles for Drug Delivery. *Eur. J. of Drug Met. And Phamacokinet.*, 3 (**1994**) 257-269.

Lieberman, H.A., Rieger, M.M. and Banker, G.S., Pharmaceutical Dosage Forms - Disperse Systems, volume 1, Marcel Dekker Inc., New York, First Edition, **1988**, pg 49-91, 199-425.

Lund, W., The Pharmaceutical Codex - Principles and Practice of Pharmaceutics. The Pharmaceutical Press, London, Twelfth Edition, **1994**, pg 82-197, 928-930, 1039-1041.

Magdassi, S., Frenkel, M. and Garti, N., Correlation between nature of emulsifier and multiple emulsion stability. *Drug Dev. & Ind. Pharm.*, 11 (4) (**1985**) 791-798.

Malcolmson, C. and Lawrence, M.J., A comparison of the incorporation of model steroids into nonionic micellar and microemulsion systems. *J. Pharm. Pharmacol.*, 45 (**1993**) 141-143.

Malcolmson, C. and Lawrence, M.J., Three-component non-ionic oil-in-water microemulsions using polyoxyethylene ether surfactants. *Colloids & Surfaces B: Biointerfaces*, 4 (**1995**) 97-109.

Mallon, C., Attwood, G., Ktistis, G. and Taylor, C.J., The influence of formulation on the particle size of oil-in-water microemulsions. *J. Pharm. Pharmacol.*, 43 (Suppl.) (**1991**) 33P.

Martindale, The Extra Pharmacopoeia, Royal Pharmaceutical Society of Great Britain, The Pharmaceutical Press, London, England, Twenty Nineth Edition, **1989**, pg 546-579, 1243-1293, 1583.

McMurry, J., Organic Chemistry, Brooks/Cole Publishing, California, Second Edition, **1988**, pg 380-451.

Meinzer, A., Mueller, E. and Vonderscher, J., Microemulsion - A Suitable Galenical Approach for the Absorption Enhancement of Low Soluble Compounds? *Bull. Tech. Gattefosse*!, 88 (**1995**) 21-26.

MIMS, Medical Specialities, Times Media, Pretoria, 39 (10), October, **1999**, pg 289-293.

Merck Index, The, Merck & Co. Inc., Rahway, NJ, USA, Tenth Edition, **1983**, pg 361, 745-746, 749, 1148, 1187, 1248-1249.

Merck Manual of Diagnosis and Therapy, The Merck Research Laboratories, Rahway, NJ, 1992, Sixteenth Edition.

Muranishi, S., Lipid-micellar and lipid-conjugated delivery systems for peptide drugs. *Bull. Tech. Gattefossé*, (1997) pg 35-41.

Nahata, M.C., Morosco, R.S. and Hipple, T.F., Stability of rifampicin in two suspensions at room temperature. *J. Clinical Pharm. and Therapeutics*, no.19 (1994) (a) pg 263-265.

Nahata, M.C., Morosco, R.S. and Hipple, T.F., Effect of preparation method and storage on Rifampicin concentration in suspensions. *Annals of Pharmacotherapy*, vol.28 (1994) (b) pg 182-185.

Prankerd, R.J., Walters, J.M. and Parnes, J.H., Kinetics for degradation of rifampicin, an azomethine-containing drug which exhibits reversible hydrolysis in acidic solutions. *Int. J. Pharm.*, vol.78 (1992) pg 59-67.

Ricker[t], R.D., Sandoval, L.A., Permar, B.J. and Boyes, B.E., Improved reversed-phase high performance liquid chromatography columns for biopharmaceutical analysis.[t] *J. Pharm. & Biomed. Anal.*, vol.14 (1995) pg 93-105.

Rushforth, D.S., Sanchez-Rubio, M., Santos-Vidals, L.M., Wormuth, K.R., Kaler, E.W., Cuevas, R. and Puig, J.E., Structural Studies of One-Phase Microemulsions. *J. Phys. Chem.*, vol.90 (1986) pg 6668-6673.

Saint Ruth, H., Attwood, D., Ktistis, G. and Taylor, C.J., Phase studies and particle size analysis of oil-in-water phospholipid microemulsions. *Int. J. Pharm.*, vol.116 (1995) pg 253-261.

Salo, J-P. and Salomies, H., High performance thin layer chromatographic analysis of hydrolysed tinidazole solutions - 1. Development and validation method. *J. Pharm. & Biomed. Anal.*, no.14 (1996) pg 1261-1266.

Sarciaux, J.M., Hilgers, A.R., Cooper, A.M., Cook, K.J., Burton, P.S. andPatel, M.V., Oral absorption potential of peptidic drugs from microemulsion formulations: a case study of U-71038. *Bull. Tech. Gattefossé*, no.88 (1995) (a) pg 11-19.

Sarciaux, J.M., Acar, L. and Sado, P.A., Using microemulsions for oral drug delivery of therapeutic peptides. *Int. J. Pharm.*, vol.120 (1995) (b) pg 127-136.

Saxena, S.J., Stewart, J.T., Honigberg[t], I.L., Washington, J.G. and Keene, G.R., Liquid Chromatography in Pharmaceutical Analysis VIII: Determination of Isoniazid and Acetyl Derivatives in Plasma and Urine Samples. *J. Pharm. Sci.*, vol.66, no.6 (1977) pg813-816.

Shah, Y., Khanna, S., Jindal, K.C. and Dighe, V.S., Determination of Rifampicin and Isoniazid in pharmaceutical formulations by HPLC. *Drug Development & Industrial Pharmacy*, 18 (14) (**1992**) 1589-1596.

Shinoda, K., Araki, M., Sadaghiani, A., Khan, A. and Lindman, B., Lecithin-based Microemulsions: Phase Behaviour and Microstructure. *J. Phys. Chem.*, 95 (**1991**) 989-993.

South African Medicines Formulary. Dept. of Pharmacology, UCT, Pioneer Press, Fourth edition, **1997**, pg 260-270.

Tarr, B.D. and Yalkowsky, S.H., Enhanced Intestinal Absorption of Cyclosporine in Rats Through the Reduction of Emulsion Droplet Size. *Pharm. Res.*, 6 (1) (**1989**) 40-43.

Trotta, M., Ugazio, E. and Gasco, M.R., Pseudo-ternary Phase Diagrams of Lecithin-based Microemulsions: Influence of Monoalkylphosphates. *J. Pharm. Pharmacol.*, 47 (**1991**) 451-454.

Trotta, M., Cavalli, R., Ugazio, E. and Gasco, M.R., Phase behaviour of microemulsion systems containing lecithin and lysolecithin as surfactants. *Int. J. Pharm.*, 143 (**1996**) 67-73.

United States Pharmacopoeia 1995 National Formulary. USP Convention Inc.©, Rockville, MD, **1994**, pg 311-321, 389-397, 728-729, 845-846, 1344-1345, 1381-1383, 1768-1844.

Wahlich, J.C. and Carr, G.P., Chromatographic system suitability tests - what should we be using?[t] *J. Pharm. & Biomed. Anal.*, 8 (8-12) (**1990**) 619-623.

Wechsler, J. The Paediatric Drug Development Challenge, *Pharm. Tech.*, August, (**1998**) 12-16 and 70.

World Health Organisation TB Report 1998, World Health Organisation Press Office, Geneva, **1998**, pg 1-198.