

**THE SOLUBILITY ENHANCEMENT
AND THE STABILITY ASSESSMENT
OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE
IN AQUEOUS MEDIA**

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LIST OF ABBREVIATIONS

"	Alpha
AUFS	Absorbance unit full scale
\$	Beta
Conc.	Concentration
CV	Coefficient of variance
COM _{min}	Complexation model with the minimum potential energy
CyD	Cyclodextrin
CyD _{min}	Cyclodextrin model with the minimum potential energy
D _{min}	Drug model with the minimum potential energy
DSC	Differential scanning calorimetry
E _{CyD}	The potential energy of the cyclodextrin in that particular arrangement
E _{Drug}	The potential energy of the drug in that particular arrangement
E _{Drug-CyD}	The potential energy of the drug-cyclodextrin complex in that particular arrangement
E _{Water-CyD}	The potential energy of the cyclodextrin (with the water molecule included within) in that particular arrangement
(Gamma
Hcl	Hydrochloric acid
HETP	Height equivalent theoretical plate
HPLC	High performance liquid chromatography
HP---CyD	Hydroxypropylated cyclodextrin
I	Isoniazid (for figure illustration)
INH	Isoniazid
IR	Infrared
K	Solubility constant
LOD	Limit of detection
LOQ	Limit of quantitation
MeCN	Acetonitrile
MeOH	Methanol
m. pt.	Melting point
MS	Mass spectroscopy
N	Number of theoretical plate
MaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
<i>P.D.</i> value	Total potential energy differences
P	Pyrazinamide (for figure illustration)
PZA	Pyrazinamide
R	Rifampicin (for figure illustration)
R.A.	Relative abundance
RIF	Rifampicin
Rt	Retention time
S _o	Solubility of the initial component

TB	Tuberculosis
tBAH	Tetrabutyl ammonia hydroxide
TLC	Thin layer chromatography
UV-vis	Ultraviolet-visible
V	Distance between two HPLC peaks during HETP experiments
v/v	Volume to volume ratio
W	Peak width at 4.4% peak height during HETP experiments

CHAPTER ONE

INTRODUCTION

1.1. TUBERCULOSIS

1.1.1. A GLOBAL EMERGENCY

Tuberculosis is an infectious disease caused by the *Mycobacterium tuberculosis*. Although the primary infection site always involves the lungs, other organ systems are also susceptible. Tuberculosis is usually chronic and may be almost lifelong in duration. Despite the advancement of the therapeutic methods in both the prevention and treatments of this infection, tuberculosis still remains as a major cause of mortality and morbidity throughout the world particularly in many underdeveloped countries of Latin America, Asia and Africa [Herfindal et al., 1992].

After the very first discovery of the tuberculosis bacillus by Dr. Robert Koch on the 24th of March 1882, a hundred and fifteen years later, in the report presented by the National Institutes of Health of United States on March 1997, it is stated that about 17 billion people, one-third of the world's population, are infected with the predominant tuberculosis organism annually. Among those, 8 million people develop active tuberculosis and there are about 3 million deaths in the world. Furthermore, a 9-14% case increase would be expected each year. There are several reasons for the increasing incidence of tuberculosis with the current increase in cases of human immunodeficiency virus (HIV) as part of the reason, while the development of the multi-drug-resistant tuberculosis (MDR-TB) mutants and patient non-compliance are also the major factors. As the bacilli are able to survive in the host body for years, about 92 percent of the new cases of clinical tuberculosis arise in individuals who have been infected by tuberculosis previously. Males are twice likely to have clinical tuberculosis compared to females with age-specific risk lowest in the 5-14-year-old group [Klaudt, 1998; Nyström et al., 1998].

Each and every year, the World Health Organization (WHO) spend billions of U.S. dollars on the issue of tuberculosis control. The Global Tuberculosis Programme (GTB) of WHO is vigorously

promoting DOTS (Directly Observed Treatment, Short-Course), one of the most clinically successful and cost-effective systems available. It comprises of a proper short-course chemotherapy (six-eight months), regular progress monitoring and programmatically ensuring patient compliance [Owen et al., 1998].

Despite the excellent health infrastructure provided by the South African Department of Health to the people in the rural areas, South Africa is one of the 16 nations lagging in controlling tuberculosis [Klaudt, 1998; Owen et al., 1998]. The country is said to have one of the highest averages of new cases tuberculosis and an estimation of 10,000 South Africans are killed by the disease every year (equivalent to every 33 minutes someone dies from tuberculosis in South Africa). The problem is clearly noticeable in most of the rural areas in the Western Cape Province where an annual case rate of 682 per 100,000 people has been reported [Weyer et al., 1995]. According to the South African National Tuberculosis Association (SANTA), of the 102,000 patients treated in 1996, only 70 percent of the patients completed their DOTS treatment. Among those patients who completed their treatment, for the compliant patient, a cure rate of 85-95 percent is expected. The cure rate dropped to 55 percent for those non-compliant patients. However, if without treatment, most people will die within two to three years. DOTS spent 130 million U.S. dollars in South Africa on curing tuberculosis in 1996. With the proper use of DOTS, an approximation of 500 million U.S. dollars will be saved in South Africa annually. However, for South Africa's tuberculosis management to be effective, it should be readily accessible at the basic level - in the crowded squatter camps, townships and the rural areas [Isacson, et al., 1998; Kumareson et al., 1996].

1.1.2. MICROBIOLOGY

Mycobacteria are acid-fast, non-spore forming, non-motile and non-encapsulated bacilli. They are obligate slow growing aerobes (requiring 2-6 weeks for growth on solid media) which grow best at human body temperature. The genus *Mycobacterium* contains many species, some pathogenic and some saprophytic. *Mycobacterium leprae*, the causative organism for leprosy, and *M. africanum*, *M. avium* and *M. bovis*, are responsible for a small fraction of the tuberculosis infections, but the most important strict pathogen is the tubercle bacilli called *Mycobacterium tuberculosis*. Three major histological and functional populations are identified and these populations can potentially coexist during the infection, at different locations [Henry et al., 1993].

The first population of bacilli is the rapidly multiplying bacilli which seek out a neutral pH environment such as the lungs. The second population is the slow metabolizing organisms that tend to cultivate themselves in the acidic solid-caseous material. The remaining population of organisms can generally be found inside the active macrophage, which ordinarily degrades pathogens with enzymes. All of the above target sites have different properties and therefore the bacilli need to be treated with different antitubercular drugs [Herfindal et al., 1992].

M. tuberculosis has an unusual waxy cell wall comprised of fatty molecules, whose structure and function are unknown. This waxy coat allows *M. tuberculosis* to survive in its preferred environment and although being inactivated by the immune cell, the organisms can remain alive for years. The bacilli can be spread throughout the body *via* the lymphatic system, as well as through the blood stream. The reactivation of the *M. tuberculosis* will generally occur when the immune system of the host body is low (patients with acquired immunodeficiency syndrome are generally very susceptible to tuberculosis) and the fact that its waxy coat is impermeable to many common drugs, also contributes towards the survival of *M. tuberculosis*. Only the few specific antitubercular agents which are listed in section 1.1.9. showed unique abilities in destroying *M. tuberculosis* by preventing its cell multiplication [Mannella, 1997].

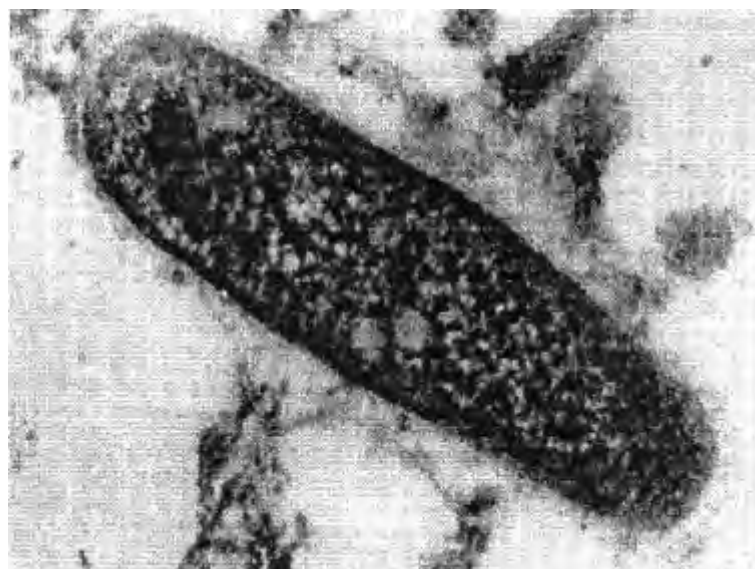


Figure 1.1. Electron Micrograph of *Mycobacterium Tuberculosis* [Mannella, 1997]

1.1.3. TRANSMISSION

Tuberculosis infection which can be classified as either a primary (childhood) infection or reinfection (adult) tuberculosis is primarily an airborne disease. *Mycobacterium tuberculosis* is transmitted by airborne droplet nuclei of about 1-10 micrometers in diameter. They are small enough to bypass the natural physical defences of the upper respiratory passages, reaching the alveoli and ingested by the alveolar macrophages. Some of the bacilli may be killed immediately. Other bacilli may multiply within the macrophages, causing the death of the macrophage and the release of more bacilli to be ingested by other macrophages. The primary site of tuberculosis destruction is always the lungs (pulmonary tuberculosis). The high oxygen level and relatively poor lymphatic drainage (especially in the posterior apical segments of the lung) favour the bacterial multiplication. If the infection is not subsequently contained, the gradual emergence of tuberculosis may cause a destructive, progressive and even fatal aftermath [Herfindal et al., 1992].

Within 2-6 weeks after inhalation, these infected macrophages may spread to the nearby lymph nodes (hilar and mediastina) and further spread all over the host body. This process is called bacillemia. Central nervous system (especially the brain parenchyma), metaphyseal end of the bones, choroid layer of retina, reproductive system, adrenal glands, kidneys and urinary system are the few other ideal sites of extrapulmonary tuberculosis infection. Both the infection and bacillemia are asymptomatic or only accompanied by mild self-limited systemic illness [Herfindal et al., 1992].

Within 2-10 weeks, the infected macrophage present pieces of bacilli which display on their cell membrane surface. This triggers the T-cells to response causing hypersensitivity. The chemical signals produce the inflammatory reaction as well as activating other specialized immune cells to kill the bacteria-macrophage complex. Although the immune cells may effectively kill many bacilli, the lesion of themselves and the nearby tissues would generally be caused by the bacilli. The lesion of the caseous material produces excellent nutrients and leads to more intensive multiplication of the bacilli (approximately $10^9 - 10^{11}$ bacilli/gm) and the cycle repeats itself [Herfindal et al., 1992].

1.1.4. SIGNS AND SYMPTOMS

There are no obvious signs and symptoms for an individual who is infected by *M. tuberculosis*. If the immune system of an individual is able to control the infection, the person is asymptomatic and the only evidence may be a positive skin test or possibly changes in the chest roentagenogram. If the active tuberculosis develops, the signs depend on the location of the infection, but the symptoms tend to be at late onset and non-specific. These non-specific symptoms include anorexia, weight loss, fatigue, fever, and night sweats. Acute onset of fevers, chills, malaise and an influenza-like illness may be also expected from some patients. However, occasionally patients may have the active disease and yet be totally asymptomatic. Unless proper diagnosis is made, one tends to be unaware of the disease and therefore endanger one's own life [Coetzee et al., 1996; Kochi et al., 1997; Mqoqi et al., 1997].

Insidious cough which progresses over weeks and muco-purulent sputum production usually occurs with pulmonary infection. Hemoptysis may gradually appear in the advance stages. Other symptoms such as chest pain or tightness and acute or recurrent pleuritic pain are also related to pulmonary infection. If the patient has very extensive lung disease or large pleural effusion, shortness of breath may be expected [Herfindal et al., 1992].

Various signs and symptoms would be found in extrapulmonary tuberculosis. Skeletal tuberculosis usually offset together with arthritis and osteomyelitis and the Pott's disease (compression on the granulation tissue or abscess formation may cause paralysis of the lower extremities) is associated when the infection targets the spine. Gross hematuria, dysuria, frequency, and flake pain are all part of the extrapulmonary tuberculosis symptoms. When the bacilli targets the central nervous system (CNS), meningitis is presented as a mild headache or slight change in mentation. Fulminant meningitis may also manifest which causes a low-grade fever in patients. The peripheral white blood count is usually normal, but tends to be lower than average [Herfindal et al., 1992]. Other potential infection target sites include the reproductive system, spine, liver, urinary system and sometimes even the muscle.

1.1.5. DIAGNOSIS AND CLINICAL FINDINGS

Tuberculin skin testing also known as the Mantoux test helps to identify most people infected with tubercle bacilli six to eight weeks after initial exposure. However, the Mantoux test is not specific for the diagnosis of active tuberculosis. A positive result may be the indication of an individual's

history of being exposed to *M. tuberculosis* or some mycobacteria. Therefore it is necessary for an individual who is Mantoux-test-positive to take a further examination [Herfindal et al., 1992; Henry et al., 1993].

An appropriate chest roentagenogram can be a strong presumptive diagnosis of tuberculosis diseases, although other diseases such as histoplasmosis can also cause a similar clinical radiograph. The most definitive ways of diagnosis of tuberculosis requires isolating the *M. tuberculosis* in the laboratory. Only with the additional information obtained from the laboratory analysis can a physician make a conclusive diagnosis. These analyses include staining and culturing the early-morning sputum specimen for a positive smear of acid-fast bacteria, fiberoptic bronchoscopy and analysis of early-morning gastric aspirates [Henry et al., 1993; Herfindal et al., 1992].

1.1.6. USE OF VACCINE

The only available vaccine for prevention of tuberculosis is the bacillus Calmette-Guérin (BCG). BCG is a vaccine composed of live, attenuated *M. bovis* from cows. It is used in those parts of the world where the disease is common and is given to infants as part of the recommended immunization program. BCG vaccine is usually given as an intracutaneous inoculation, most commonly in the deltoid region. A post vaccinal scar about 8 millimeter in diameter indicates having received the vaccine. BCG only prevents the spread of *M. tuberculosis* within infants but does not prevent initial infection. Although the vaccine is cheap, safe and normally requires only single inoculation, it is far from being ideal, due to the unpredictable immunity for different populations and sometimes complications follow the inoculation. Local ulceration and regional suppurative lymphadenitis are the common side-effects. In addition, people who had BCG vaccination would result in a positive Mantoux skin test reaction, thus limiting the effectiveness of the test. [Henry et al., 1993; Herfindal et al., 1992].

1.1.7. TREATMENT REGIMENS AND STRATEGIES

An adequate drug administration is the backbone of tuberculosis treatment and the selection of an effective chemotherapeutic strategy is essential. Rifampicin, isoniazid, pyrazinamide, streptomycin

and ethambutol are the few common antitubercular agents. Many drugs can be used in the treatment and several principles have been used in the selection process. The most important requirement is using a dosage form of combining at least two or more drugs in order to prevent the emergence of the multi-drug resistance tubercular bacilli (MDR-TB) which is caused by inconsistent and partial treatment. There is no affordable cure for some underdeveloped countries concerning MDR-TB strains. From the public health perspective, no tuberculosis treatment is better than uncompleted tuberculosis treatment. In these countries, tuberculosis treatment costs about 2000 U.S. dollars per patient, but may cost as much as 250,000 U.S. dollars per patient with MDR-TB [Herfindal et al., 1992; Peto et al., 1996].

Since most drugs have different physical/chemical properties, combining different drugs ensures the multi-targeting of all three different populations (refer to section 1.1.3.) of *M. tuberculosis*. The population comprising the rapid multiplying extracellular bacteria is best treated with isoniazid, streptomycin, and to an extent rifampicin. Rifampicin demonstrates a unique ability to destroy the slow metabolizing, solid caseous acid environment-seeking organism. Due to the acidity within the macrophage, therapeutic activities of most antitubercular agents are inhibited. Pyrazinamide has shown greatest activity against these intracellular bacilli, whereas rifampicin has only a marginal activity against this population [Henry et al., 1993; Herfindal et al., 1992].

The emergence of MDR-TB while under the proper treatment is not very common. The rates of bacterial mutation have been calculated as rifampicin, 1 in 10^8 ; isoniazid, 1 in 10^6 ; pyrazinamide, 1 in 10^5 and ethambutol, 1 in 10^6 . Therefore the probability of the emergence of MDR-TB is less than 1 in 10^{19} if a patient is taking the proper combination of treatment of rifampicin, isoniazid and pyrazinamide. Since the spontaneous formation of MDR-TB can only arise at a rate of 1 in 10^6 to 1 in 10^8 [Herfindal et al., 1992; de Villers et al., 1996], the multiple antitubercular drugs administration would thus prevent the emergence of MDR-TB.

1.1.8. DOSAGE RECOMMENDATIONS

Various dosages have been recommended by the South Africa Antituberculosis Program Guidelines (1996) to suit various categories of patients. These regimens need to be administered daily or five

times a week [Rey, 1998; SAHD, 1996; UCT DPMS, 1995]. The requirements are listed in the tables below (tables 1.1. to 1.4.).

Table 1.1. Dosage Recommendation For Adult Patients Never Treated Before or Previously Completed A Set of Treatment

Adult Patients		
	Under 50 Kg	Over 50 Kg
<i>Intensive Phase (12 weeks)</i>		
Rifampicin/isoniazid/pyrazinamide	480/320/1000 mg	600 /400/1250mg
Ethambutol	800mg	1200mg
<i>Continuation Phase (16-24 weeks)</i>		
Rifampicin / isoniazid	450/300mg	600/300mg

Table 1.2. Dosage Recommendation For Adult Patients With Interrupted Treatments

Adult Patients		
	Under 50 Kg	Over 50 Kg
<i>Intensive Phase (12 weeks)</i>		
Rifampicin/isoniazid/pyrazinamide	480/225/1000 mg	600 /300/1500mg
Ethambutol/streptomycin	900/750mg	1200/1000mg
<i>Transition Phase(4 weeks)</i>		
Rifampicin/isoniazid/pyrazinamide	480/225/1000mg	600/300/1500mg
Ethambutol	900mg	1200mg
<i>Continuation Phase (16-24 weeks)</i>		
Rifampicin/isoniazid	450/300mg	600/300mg

Table 1.3. Dosage Recommendation for Paediatric Patients Who have Primary TB And Effusion

Paediatric Patients			
	5-10Kg	11-20Kg	20-30Kg
<i>Intensive Phase (8 weeks)</i>			
Rifampicin/isoniazid/pyrazinamide	75/50/250mg	150/100/500mg	300/200/1000mg
<i>Continuation Phase (8 weeks)</i>			
Rifampicin/isoniazid	75/50mg	150/100mg	300/200mg

Table 1.4. Dosage Recommendation For Paediatric Patients With Primary TB and Non-pulmonary TB

Paediatric Patients			
	5-10Kg	11-20Kg	20-30Kg
<i>Intensive Phase (8 weeks)</i>			
Rifampicin/isoniazid/pyrazinamide	75/50/250mg	150/100/500mg	300/200/1000mg
<i>Continuation Phase (16 weeks)</i>			
Rifampicin/isoniazid	75/50mg	150/100mg	300/200mg

1.2. PROJECT PROPOSAL

The development of a dosage form that is suitable for each age group and user friendly to the physicians, parents and patients is often quite challenging. The ideal medication for paediatric use should be relatively simple to administer, of acceptable taste, low toxicity, good stability and not requiring refrigeration. Shortcomings in any of above mentioned conditions will lead to patient non-compliance and unfortunate consequences. Administration of tablets or capsules is difficult for the infant patients and studies have shown that many children to the age of twelve, even some adults, have difficulties with these solid dosage forms. Therefore suspensions and liquid formulations are desired. However, in the respect of formulation development, masking the unpleasant taste in a liquid formulation is not a simple task and colouring and favoring agents could cause allergic reactions in children. The stabilities and the bio-availabilities of the drugs may not be satisfactory and sometimes the excipients could exacerbate the above mentioned conditions. Another challenge is when and how to conduct toxicology studies for neonatal and juvenile populations. Although the pharmacology and the toxicology of the drug may already been known for adults, the basis for extrapolating this to juveniles may not guarantee the safety.

Pharmaceutical companies have not invested more in the paediatric drugs mainly due to the fact that the paediatric pharmaceutical market is quite small. The United States pharmaceutical market spend about 70 billion U.S. dollars each year for the adults, but, only 3.5 billion were accounted for children. Therefore the Industries are only willing to cover less expensive drugs, but are uninterested in the development of the slightly more expensive treatments even when that particular therapy offers far more benefits and less risk. The limitation of demand for paediatric products further causes the lower budget of sponsorship. This becomes an obstacle for the research groups to enroll in extensive drug development and continues to be the real hurdle for designing paediatric clinical trials [Wechsler et al., 1998].

Rifampicin, isoniazid and pyrazinamide are the essential first-line antitubercular agents and the combination of these drugs is recommended by most of the health organizations around the world. However, on the current South African market, the common formulations are in the form of capsules, tablets or injectables: Rimactane[®] (capsules, tablets or injectable), Rifcin[®] (capsules or tablets), Rifadin[®] (syrup), Rifata[®] (syrup, tablets) and Rifambutin[®] (capsules) contain only

rifampicin and isoniazid. Pyrazinamide is often found as tablets under the name of Isopas[®], Pyrazide[®] and Rozide[®]. This implies that in order for an individual to take one's recommended medication, not only is there is an inconvenient method of administration but also multiple inconvenient formulations. This may cause intense patient non-compliance among infants and children, sometimes, even for older persons [FDC, 1994].

This project was initiated in response to a request from Pharmacare-Lennon[®] (Korsten, RSA), a leading generic pharmaceutical manufacturer in South Africa and is intended to investigate the solubilities and the stabilities of rifampicin, isoniazid and pyrazinamide at the pre-formulation stage. Moreover, the project will attempt to overcome the problems which could arise during the pre-formulation stage by incorporating the drugs with various solubilizing and stabilizing agents. These agents can be categorized into three different groups: the surfactant, the suspending agents and the complexing agents. The solubility and stability profiles of the drugs in the presence of these agents are able to be evaluated after a suitable high performance liquid chromatography (HPLC) method is developed and validated.

Pre-formulation solubility studies will involve the HPLC evaluation of the phase solubility of rifampicin, isoniazid and pyrazinamide in the presence of three different surfactants (poloxamer 188, poloxamer 407 and sorbitols) and three different cyclodextrins (α -cyclodextrin, hydroxypropyl- β -cyclodextrin and γ -cyclodextrin). The solubility constants will be calculated. Pre-formulation stability studies will also involve the simultaneous exposure of rifampicin, isoniazid and pyrazinamide in the presence of various stabilizing agents and storage conditions. The stabilizing agents include the above mentioned agents as well as carbopol 934 and carbopol 947P.

Enhanced aqueous stability complemented by an improved solubility would be the criteria that need to be met in order to recommend the extension of this pre-formulation investigation into a formulation development stage. The final goal is dedicated to produce a paediatric and geriatric medication which combine rifampicin, isoniazid and pyrazinamide in a liquid formulation of approximately 10-15ml quantity. Since South Africa is one of the 16 countries with the worst tuberculosis infection incidence but still being accused by WHO as "lacking progress in controlling tuberculosis" [Klaudt et al., 1998], such a formulation would considerably benefit South Africa in every possible way.

1.3. CONCLUSION

Tuberculosis is a global public health threat. The antitubercular regimen containing rifampicin, isoniazid and pyrazinamide is the cornerstone of effective treatment, and should be initiated once conclusive evidence of active tuberculosis is found. Successful strategy against tuberculosis should include not only the adequate chemotherapy but also management of patients, constant monitoring for adverse effects, evaluating bacteria eradication and, most important of all, ensuring patient compliance since patient compliance contributes a significantly factor to successful cure rate, as well as cost-effectiveness. Vice versa, the consequences of patient non-compliance results in treatment failure, additional treatment, additional expenses, development of the MDR-TB, and death. Although the major determinant of the outcome of tuberculosis treatment is the patient compliance, the current administration of antitubercular drugs is generally inconvenient and further increases the tendency of patient non-compliance. Therefore, a “dosage-friendly’ liquid formulation containing rifampicin, isoniazid and pyrazinamide is the key factor of improving tuberculosis treatment in many ways, hence necessitating the in depth studies solubility and stability of rifampicin, isoniazid and pyrazinamide.

CHAPTER TWO

DRUG CHARACTERISATION

2.1 INTRODUCTION

Three independent batches of sample, rifampicin, isoniazid and pyrazinamide with corresponding batch numbers of 97/0185, 97/0021 and 98/0054 respectively were kindly donated by the Druggist Group Research of Pharmacare-Lennon[®] (P. O. Box 4002, Korsten 6014, South Africa) for this project. It is important to characterise all three drug molecules and determine the purity before targeting the solubility and/or stability problems of the drug substances (see chapters 4 to 7). The characterisation studies enable the researchers to confirm the structure of these drug substances and as well as investigate the purity of the sample [Carstensen, 1995].

Identifying and characterising the structure of any particular organic compound generally relies on spectroscopy. Four fundamental types of spectroscopy are often used in qualitative organic chemistry: ultraviolet-visible (UV-Vis) spectroscopy, (2) infrared (IR) spectroscopy, (3) nuclear magnetic resonance (NMR) and (4) mass spectroscopy (MS). When correlating these studies, this allows the researchers to comprehend the chemical description of the sample analytes with a high degree of confidence. Additionally, thermal analyses (i.e. melting range and differential scanning calorimetry) and a simple purity test (thin layer chromatography) were also performed [McMurry, 1992] to confirm the identity and purity of each of the three drug molecules.

2.1.1 ULTRAVIOLET SPECTROSCOPY (UV)

UV-Vis spectroscopy is widely available and commonly used by laboratory workers to perform both qualitative and quantitative experiments. The λ_{max} (in nanometers) and the relative intensity calculated as the molar absorptivity are reported [Skoog et al., 1993]. All the UV-Vis spectra were recorded on a GBC (UK) UV-Vis 916 double-beam spectrophotometer.

Each individual drug molecule (rifampicin, isoniazid and pyrazinamide) was carefully weighed to obtain 0.1g and dissolved in 100ml of methanol-water (2 : 8, v/v) solvent. The stock solution was further diluted with the same solvent to give the concentration of 100 $\mu\text{g}/\text{ml}$ of sample solution and the spectrum recorded from 200 to 400nm.

2.1.2 INFRARED SPECTROSCOPY (IR)

IR spectroscopy is a good method of identifying carbon-carbon double bonds and other functional groups and thus provides a good qualitative characterisation of the drug samples. A Spectrum 2000 FT-IR spectrophotometer (Perkin Elmer, UK) was used to conduct all the IR spectroscopy experiments [McMurry, 1992 ; Vogel 1978]. The infrared spectra of drugs were obtained by using the nujol method scanning from 600.0 to 4000.0 cm^{-1} .

2.1.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

NMR spectroscopy provides the most detailed information in respect of the construction of a molecule and has become a powerful analytical tool when combining both proton and carbon NMR spectroscopy, resulting in high precision when attempting to understand the constitutional structure of the drug samples [McMurry, 1992; Vogel, 1978]. All of the NMR spectra were accomplished by using a 400MHz AMX NMR spectrometer (Bruker, Germany), dimethylsulfoxide- d_6 (DMSO) as the solvent during the analyses of isoniazid and pyrazinamide, while chloroform- d_1 (CDCl_3) was used to dissolve rifampicin.

2.1.4 MASS SPECTROSCOPY (MS)

A GCQ-MS spectrometer (Finnigan, USA) is used to determine the molecular weight of each drug sample. A direct electronic impact method was employed and while the molecular ion (M^+) peak suggests the molecular weight of the compound, each peak shown in the MS spectrum indicates the weight of a particular fragment. Moreover, by examining the relative abundance of the particular fragmentation peak, the stability of each fragment can be appreciated. The implication of the tendency of degradation occurring at that particular site can therefore be suggested [McMurry, 1992; Volgel, 1978]. The relative abundance of each fragment peak was calculated by the amount of the ion count of a particular peak dividing the total amount of ions obtained from the experiment. The results were interpreted and reported as the relative abundance (R.A.) of fragments.

2.1.5 MELTING POINT (MP)

The melting point of a pure substance is a characteristic of the substance being studied and any pure sample of the same substance shall have the same melting point. Therefore, the melting point (*mp*) is a physical constant which can be used as a measure of purity and identity. Melting point range was studied by using a Gallenkamp (UK) 50 Hz 220/240volt melting point apparatus and a open capillary tube method [McMurry, 1992; Skoog et al., 1993].

2.1.6 THERMAL ANALYSES (DSC)

Thermal analyses can also play a fundamental role in sample characterisation, since each chemical has its unique thermal properties and may further provide an indication of purity. However, this method does not indicate the amount of impurities, nor does it separate the contaminants [Atkins, 1990]. The heat curve was obtained by using a DSC 7 (Perkin Elmer, UK). Samples were accurately weighed (6-9mg) using a Sartorius MCS electronic microbalance (Goettingen, Germany) into 25 μ l aluminium pans and heated at a rate of 10 C per minute under nitrogen purge over 50-250 C temperature range.

2.1.7 THIN LAYER CHROMATOGRAPHY (TLC)

Chromatography is generally employed (chapter 3) in the sample characterisation. Relying on silica as the absorbent/stationary phase and a simple solvent as the mobile phase, thin layer chromatography is an uncomplicated, but reliable chromatography technique which most organic chemists prefer for testing the sample purity. Detection of impurities is done by visualising the silica gel 60 F₂₅₄ TLC (Merck, Germany) plate and the relative retardation factor (R_f) value can easily be calculated [Volgel, 1978]. The conditions used for analysing each drug will be specified in the sections 2.2.1.7, 2.2.2.7 and 2.2.3.7.

2.2 EXPERIMENTAL RESULTS AND DISCUSSION

2.2.1 RIFAMPICIN

Rifampicin, the international nonproprietary name of rifampin, is normally described as a molecule consisting of a naphthohydroquinone chromophoric part which is attached by an aliphatic chain with a piperazine side chain attachment. Rifampicin is designated by IUPAC rules as 2,7-(epoxypentadeca[1,11,13]trienimino)naphtho[2,1-b]-furan-1,11(2H)-dione,5,6,9,17,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-1-8-[N-(4-methyl-1-piperazinyl)-formimidoyl-21-acetate. According to the original nomenclature of rifamycins, sometimes rifampicin is also known as 2-[[[(4-methyl-1-piperazinyl)imino]methyl]-rifamycin SV. Rifamycin AMP and rifaldazine are the other two trivial names which are generally used [Gallo et al., 1974; Notvich et al., 1991; Reynold et al., 1992].

Rifampicin contains 9 asymmetric carbon atoms and 3 double bonds. However, due to the fact that rifampicin is a semi-synthetic antibiotic and all of the isomeric centers belong to the parent compound were found naturally, only one isomer can be produced. This red-orange, odourless, crystalline powder has the molecular mass of 822.95g/mol and the structure is represented in figure 2.1. (numbered according to IUPAC rules) or 2.2. (numbered according to USAN). Its empirical formula is stated as C₄₃H₅₈N₄O₁₂ [Gallo et al., 1974]. Due to the fact that USAN numbering is commonly used in most references, the USAN numbering is also preferred and used in this thesis.

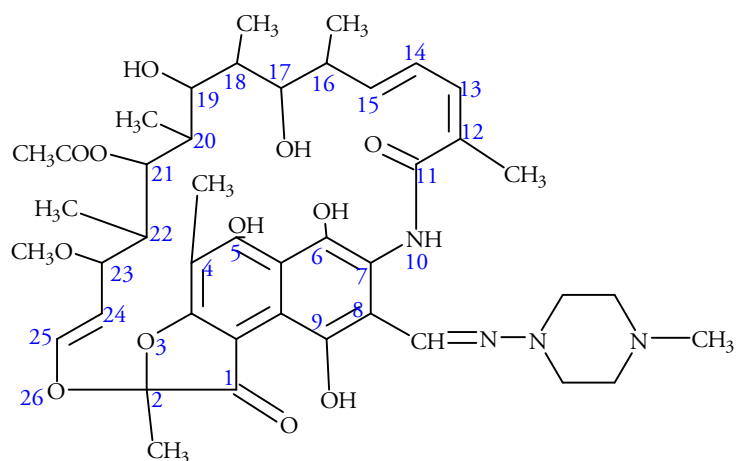


Figure 2.1 Rifampicin with IUPAC numbering [Gallo et al., 1974]

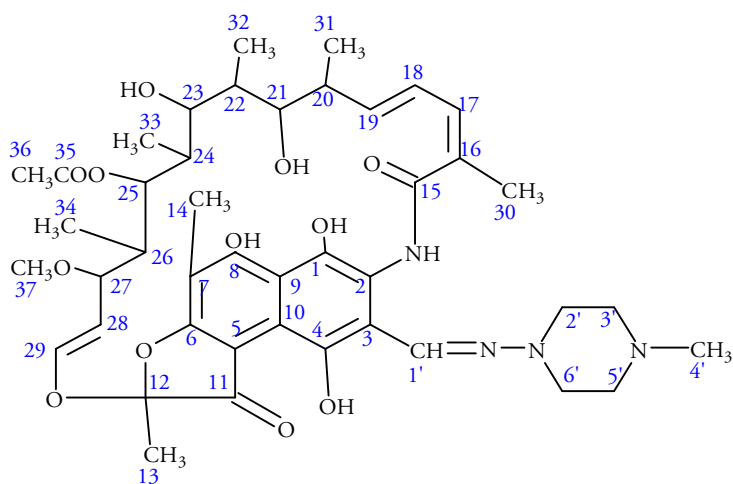


Figure 2.2 Rifampicin with USAN numbering [Gallo et al., 1974]

2.2.1.1 ULTRAVIOLET SPECTRUM OF RIFAMPICIN

The UV spectrum of rifampicin is illustrated in figure 2.3. The absorption maxima (λ_{\max}) was found at 235.3nm. The calculated ϵ value concurred with the literature given values [Gallo et al., 1974].

Table 2.1 : UV-Vis Data of Rifampicin

UV-Vis Spectrum Data			
λ_{max} (nm) (Experimental)	λ_{max} (nm) (Literature)	Abs (Experimental)	Abs (Literature)
235.3	237.0	31,554	33,200
254.1	255.0	31,082	32,100
333.4	334.0	27,895	27,000

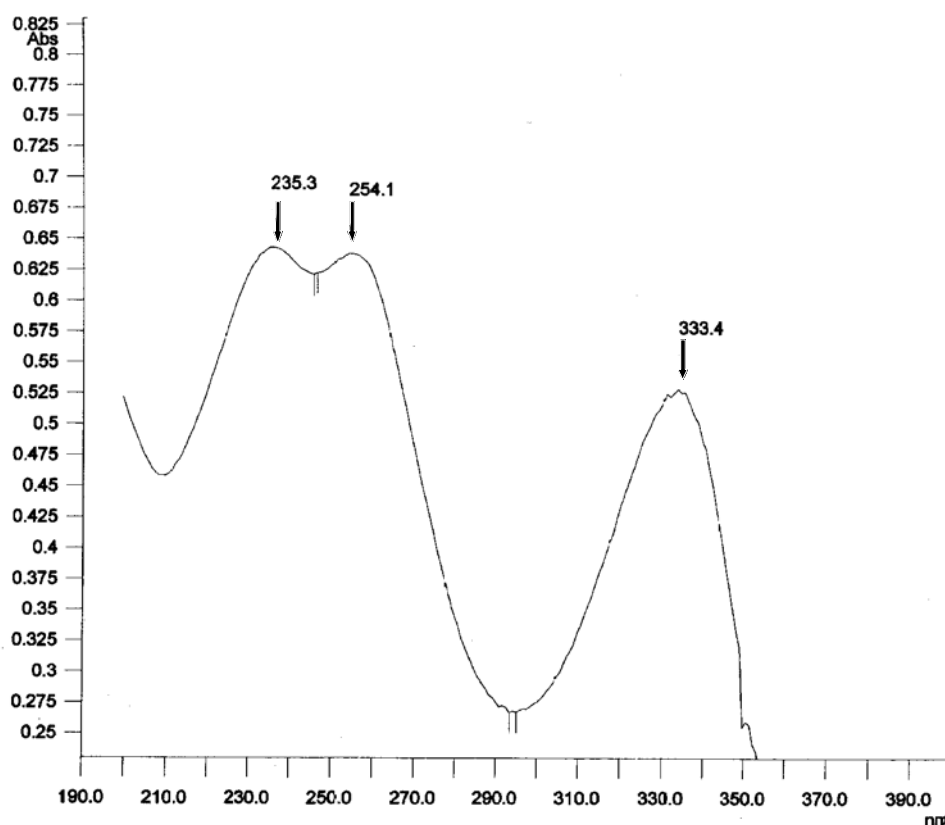


Figure 2.3 UV-vis Spectrum of Rifampicin

2.2.1.2 INFRARED SPECTRUM OF RIFAMPICIN

The infrared spectrum of rifampicin (figure 2.4) confirms the presence of the relevant functional groups (as the important peaks are listed in table 2.2) and is compared with the literature findings [Gallo et al., 1974].

Table 2.2 Infrared Result of Rifampicin

Infrared Spectrum Data		
<i>IR Absorption Band (cm⁻¹) (Experimental)</i>	<i>IR Absorption Band (cm⁻¹) (Literature)</i>	<i>Functional Groups</i>
3583.9-3449.9 broad peak	3500-3000	-OH stretching
2925.5	2930	-CH ₃ stretching
2894.3	2820	-CH ₃ O asymmetric stretching
2781.0-2682.7	2800	-CH ₃ N stretching
1712.1, 1732.5	1715, 1730	-C=O acetyl stretching
1676.6, 1655.2 and 1620.7	1670	-C=N- asymmetric bending
1567.0	1570	-C=C- stretching
1461.0-1332.6	1400	-C-N- stretching
1290.8-1021.0	1255-1020	-C-O-C- acetyl group

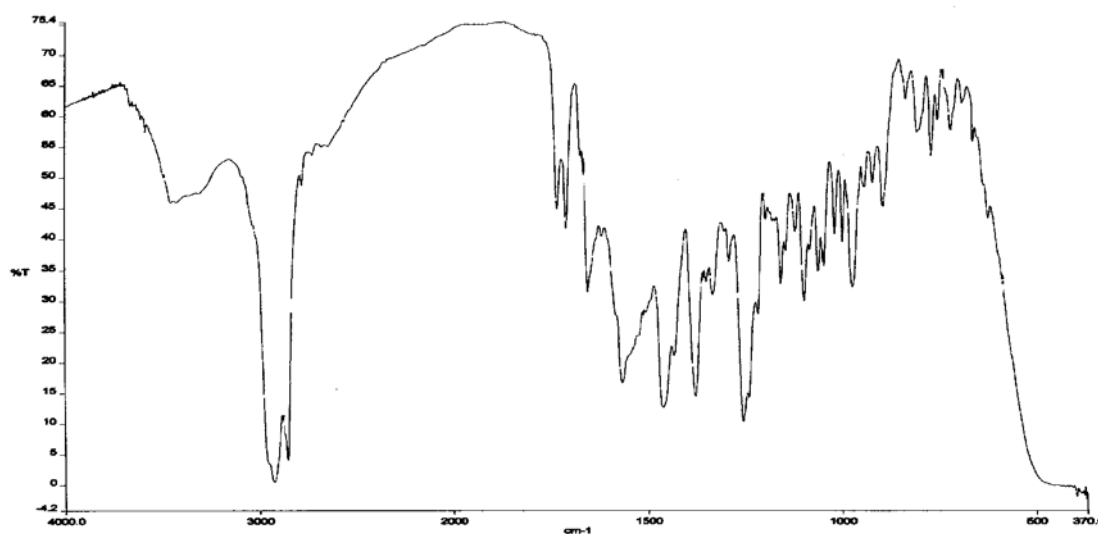


Figure 2.4 Infrared Spectrum of Rifampicin

2.2.1.3 NUCLEAR MAGNETIC RESONANCE SPECTRUM OF RIFAMPICIN

The results of the NMR spectra are interpreted according to USAN numbering and tabulated in tables 2.3. and 2.4. The experimental data were obtained by using approximately 30mg/ml of rifampicin-CDCl₃ solution and further compared with literature data reported by Gallo. *et. al.* [Gallo et al., 1974]

Table 2.3 ¹H NMR Spectrum Data of Rifampicin

¹ H NMR Spectrum Data			
<i>Proton Represented</i>	<i>Multiplicity</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>
NH	s	11.95	11.96
CH=N	s	8.23	8.22
CH ₂ (2' & 6')	m	2.9-3.3	2.9-3.3
CH ₂ (3' & 5')	m	2.5, 2.8	2.4, 2.8
N-CH ₃	s	2.35	2.34
OH (1)	s	11.4, 14.0	11.4, 14.0
OH (4 & 8)	s	13.2	13.16
CH ₃ (13)	s	1.8	1.82
CH ₃ (14)	s	2.25	2.23
H (17 & 18)	m	6.5, 6.6	6.3, 6.8
H (19)	dd	5.96	5.92
H (20)	ddq	2.22	2.26
H (21)	dd	3.73	3.78
OH (21 & 23)	bs	3.2, 4.1	3.2, 4.2
H (22)	ddq	1.72	1.70
H (23)	dd	3.05	3.04
H (24)	ddq	1.52	1.52
H (25)	dd	4.96	4.96
H (26)	ddq	1.22	1.22
H (27)	dd	3.59	3.58
H (28)	dd	5.06	5.00
H (29)	d	6.21	6.20
CH ₃ (30)	s	2.10	2.10
CH ₃ (31)	d	0.88	0.88
CH ₃ (32)	d	1.02	1.01
CH ₃ (33)	d	0.58	0.58
CH ₃ (34)	d	-0.33	-0.33
CH ₃ (36)	s	2.11	2.06
CH ₃ (37)	s	3.1	3.05

Note : s=singlet

bs= broad singlet

d=doublets

m=multiplet

dd= doublet of doublets

ddq= doublet of doublets of quartet

Table 2.4 ¹³C NMR Spectrum Data of Rifampicin

¹³ C NMR Spectrum Data					
<i>Carbon Represented</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>	<i>Carbon Represented</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>
1	138.5	138.6	23	76.9	76.7
2	105.2	105.9	24	37.6	37.6
3	111.0	111.0	25	74.1	74.4
4	147.2	147.2	26	39.5	39.5
5	112.7	112.8	27	76.7	76.7
6	174.5	174.1	28	118.7	118.7
7	120.33	120.3	29	142.6	142.6
8	169.4	169.3	30	20.7	20.7
9	104.5	104.4	31	17.8	17.8
10	117.8	117.8	32	10.9	10.9
11	159.0	195.3	33	8.5	8.5
12	108.7	108.7	34	8.8	8.8
13	21.5	21.5	35	171.9	171.9
14	7.4	7.6	36	20.7	20.7
15	169.6	169.6	37	57.1	57.0
16	129.0	129.4	1'	134.6	134.4
17	135.1	135.0	2'	50.3	50.2
18	123.1	123.2	3'	54.0	53.9
19	142.6	142.6	4'	54.0	53.9
20	38.66	38.66	5'	50.3	50.2
21	70.7	70.7	6'	46.0	45.8
22	33.4	33.4			

The interpretation of these spectra confirmed the rifampicin structure.

2.2.1.4 MASS SPECTRUM OF RIFAMPICIN

Figure 2.5 shows the fragmentation peaks where 822g/mol indicates the molecular ion (M^+) of the sample. The mass spectrum confirmed the samples to be rifampicin after comparison with the literature [Gallo et al., 1974]. The major fragments are represented in figure 2.6. The base peak m/e of 98 suggests the -N-N- bond on the piperazine side chain could be fragmented since this bond is easily cleaved. The degradant at m/e of 398 implies that the bond of -N-C2= and -O-C12- could also be weak resulting a peak with high intensity (100%). The significant peaks are m/e of 822, 424, 398, 300 and 98 with the relative abundance of 2.14, 79.83, 2.43 and 30.24% respectively. This low relative abundance of the molecular ion ($m/e = 822$) could suggest the rifampicin molecule is not stable and easily fragmented into vast amount of different fragments.

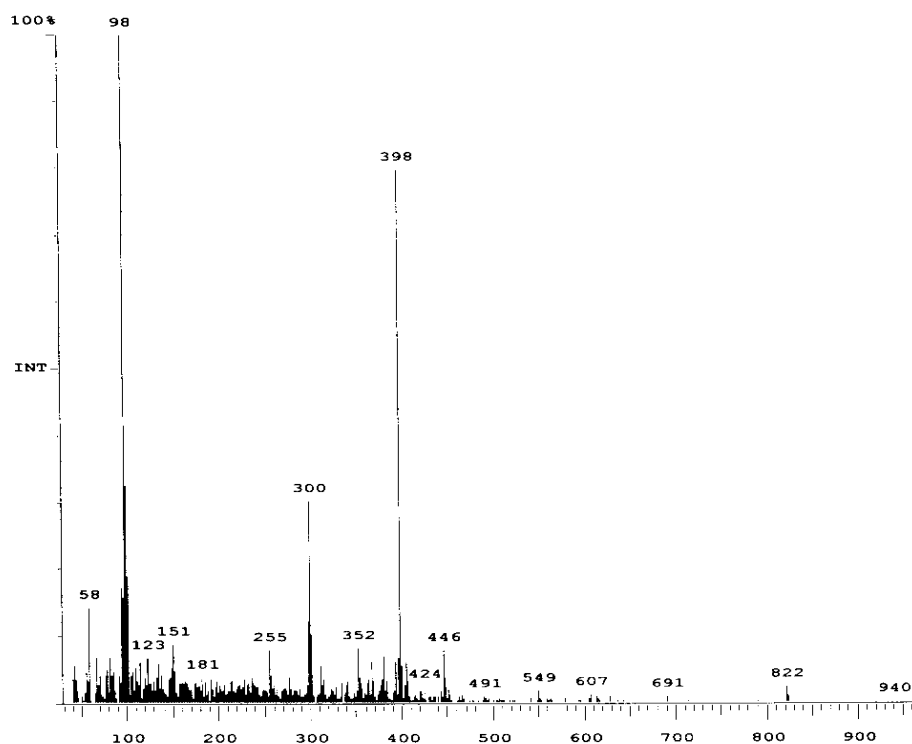


Figure 2.5 Mass Spectrum of Rifampicin

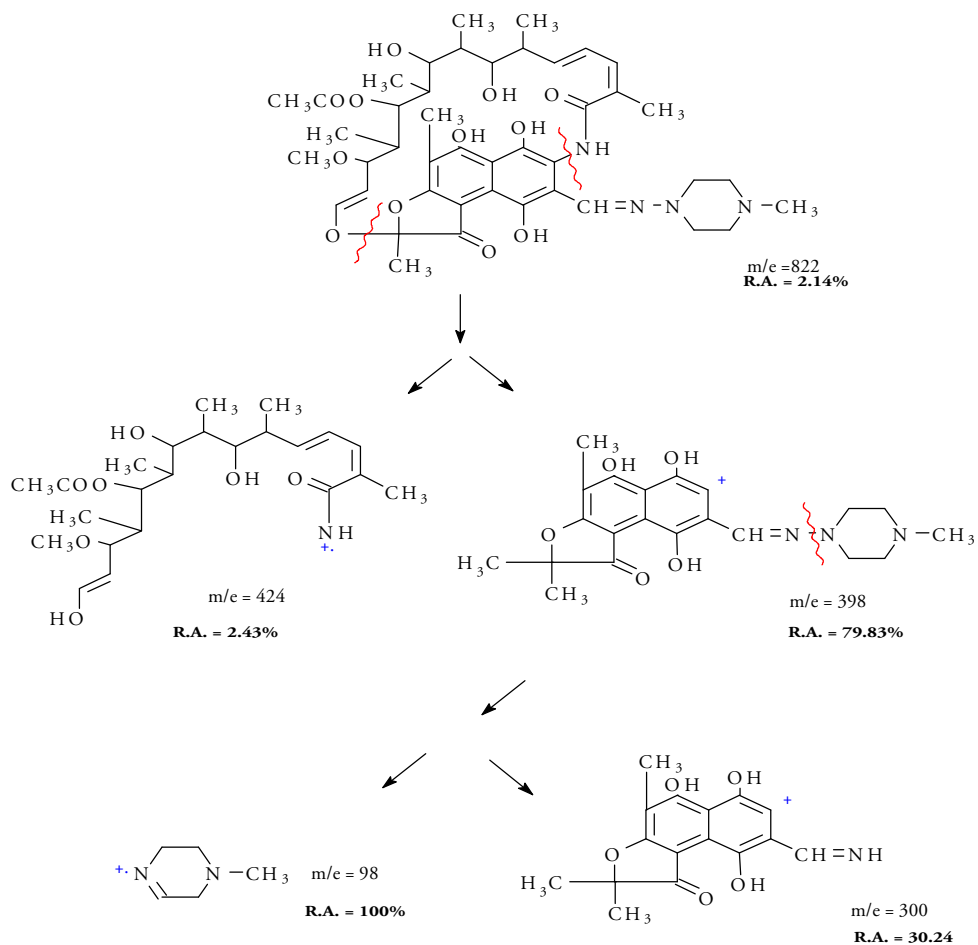


Figure 2.6 Rifampicin Fragmentation Pattern

2.2.1.5 RIFAMPICIN MELTING RANGE

Rifampicin melted with decomposition at 186 - 189°C which concurs with the value given in the literature of 187.2 °C [Gallo et al., 1974].

2.2.1.6 DIFFERENTIAL SCANNING CALORIMETRY THERMOGRAM OF RIFAMPICIN

The differential scanning calorimetry thermogram of rifampicin shows an onset at 180.70°C , peaking off at 194.53°C and ending at 201.16°C, corresponding to the melting of rifampicin with an enthalpy of rifampicin being 56.939J/g. The results are similar to the literature values [Gallo et al., 1974].

2.2.1.7 THIN LAYER CHROMATOGRAPHY OF RIFAMPICIN

Thin layer chromatography was conducted by using a silica gel GF₂₅₄ plate and the mobile phase of ethyl acetate and hexane in the ratio of 25 : 75 (v/v). The results clearly indicate that there are no other contaminants within the rifampicin sample, since only a single orange spot was found. The Rf value was approximately 0.66.

2.2.2 ISONIAZID

Isoniazid is the most commonly used generic name for pyridine-4-carboxylic acid hydrazide. Names including 4-pyridinecarboxylic acid hydrazide or pyridine-4-carboxyhydrazide are often used. Other popular generic names or abbreviations such as isonicotinohydrazide, isonicotinic acid hydrazide, isonicotinoylhydrazine, tubazid and isoniazidium can also be used. This odourless white (or colourless) crystalline powder is slightly sweet when first tasted, but turns bitter instantly. Its molecular weight is 137.14g/mol and has an empirical formula of $C_5H_5N_3O$. The structure of isoniazid is represented in figure 2.7.

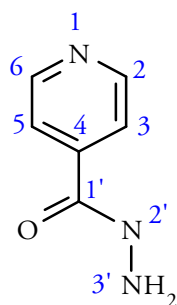


Figure 2.7 Chemical Structure of Isoniazid

2.2.2.1 ULTRAVIOLET SPECTRUM OF ISONIAZID

The UV-Vis spectrum is illustrated in figure 2.8 The absorption maxima are displayed in table 2.5. The calculated λ value agrees with the literature given value [Brewer et al., 1977].

Table 2.5 : UV-Vis Data of Isoniazid

UV-Vis Spectrum Data			
λ_{max} (nm) (Experimental)	λ_{max} (nm) (literature)	λ_{max} (nm) (Experimental)	λ_{max} (nm) (Literature)
262.2	265	4215	4300

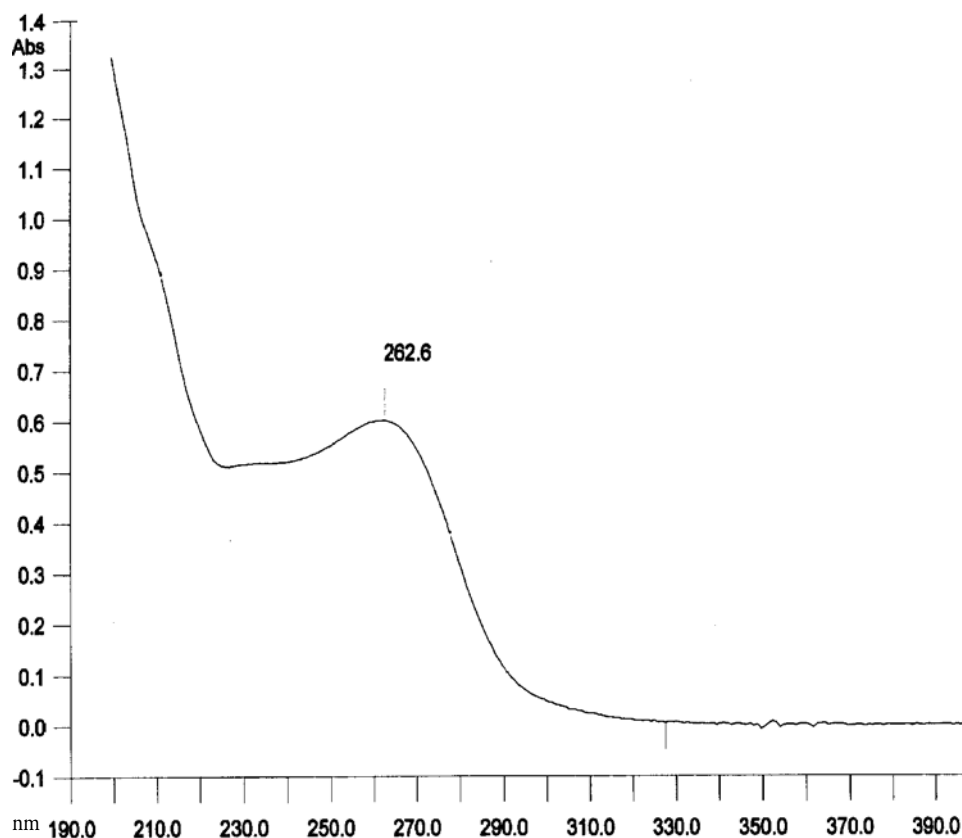


Figure 2.8 UV-Vis Spectrum of Isoniazid

2.2.2.2 *INFRARED SPECTRUM OF ISONIAZID*

The spectrum (figure 2.9) confirms the presence of the relevant functional groups. The results of which are interpreted and tabulated in table 2.6. The data obtained are similar to the isoniazid infrared spectrum given in the literature [Brewer et al., 1977].

Table 2.6 Infrared Results of Isoniazid

Infrared Spectrum Data		
<i>IR Absorption Band (cm⁻¹) (Experimental)</i>	<i>IR Absorption Band (cm⁻¹) (Literature)</i>	<i>Functional Groups</i>
various peaks 3300.0 - 3000.0	3300.0 - 3000.0	-NH, -NH bonded stretching
3174.3	3172	Aromatic C-H stretching
1667.2	1670	-C=O stretching
1661.1 and 1635	1640, 1610	-NH ₃ asymmetric bending
1603.3	1600	-C=N stretching
1556.7, 1492.3 and 1463.8	1560, 1500, 1465	Aromatic ring vibration

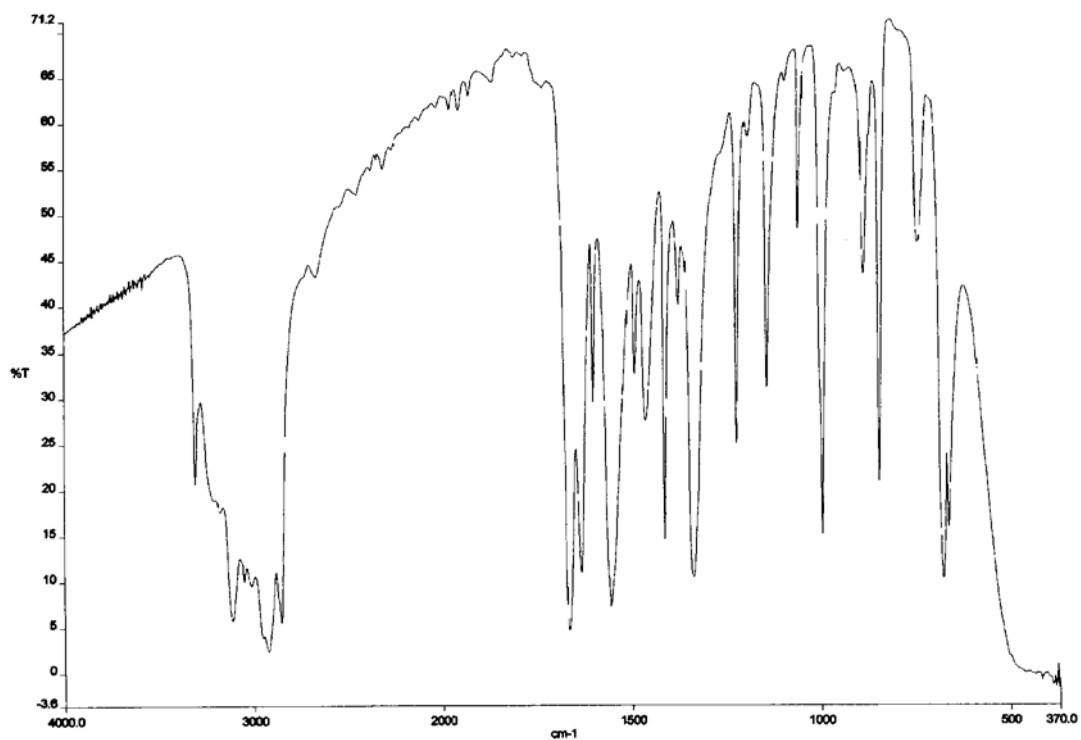


Figure 2.9 Infrared Spectrum of Isoniazid

2.2.2.3 NUCLEAR MAGNETIC RESONANCE SPECTRUM OF ISONIAZID

In order to prevent the deuterium-proton exchange, dimethylsulfoxide- d_6 was used as the solvent for ^1H NMR experiments (table 2.7). The concentration of the analyte was made up to approximately 25mg/ml. A ^{13}C NMR experiment (table 2.8) was also conducted on the same sample. Both the ^1H NMR and ^{13}C NMR spectra clearly identify that the analyte is isoniazid.

Table 2.7 ^1H NMR Spectrum Data of Isoniazid

^1H NMR Spectrum Data			
<i>Proton Represented</i>	<i>Multiplicity</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>
Hydrazino H (-NH ₂)	Singlet	4.60	4.60
Aromatic H	Double Doublet	7.72, 8.70	7.73, 8.70
CO-NH	Singlet	10.08	10.15

Table 2.8 ^{13}C NMR Spectrum Data of Isoniazid

^{13}C NMR Spectrum Data		
<i>Carbon Represented</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>
Hydrazino	164.1	164.4
Heteroaromatic (C4, C3 and C5, C2 and C6)	121.4, 140.0, 150.1	121.6, 140.0, 150.0

2.2.2.4 MASS SPECTRUM OF ISONIAZID

The molecular ion peak at m/e of 137 corresponds to the 137.14 g/mol molecular mass of isoniazid stated in the literature [Brewer et al., 1977]. The fragmentation pattern of the isoniazid analyte also coincides with the possible fragmentation pathway of isoniazid (refer to figure 2.11), therefore the analyte is confirmed to be isoniazid. Furthermore, the fragmentation peaks of 137, 106, 78 and 51 have the relative abundance of 36.18, 41.62, 100 and 16.85% respectively. This could suggest that

although the molecular ion of isoniazid ($M/e = 137$) had a higher relative abundance than rifampicin molecular ion ($M/e = 822$ had only 2.14% relative abundance), isoniazid could still be quite unstable.

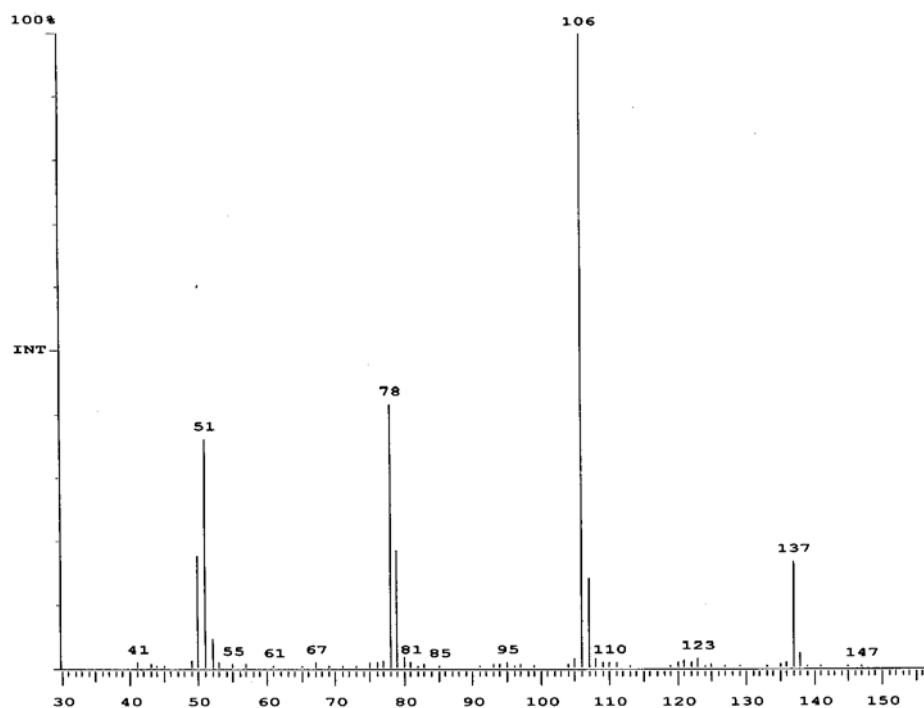


Figure 2.10 Mass Spectrum of Isoniazid

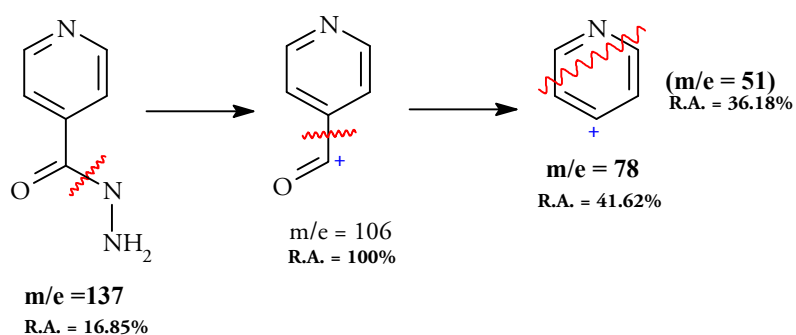


Figure 2.11 Fragmentation Pattern of Isoniazid

2.2.2.5 ISONIAZID MELTING RANGE

The melting point range of the isoniazid sample was between 171.0 to 175.5°C, which matched the literature given range of between 170.0 to 174.0°C [Brewer *et al.*, 1977] .

2.2.2.6 DIFFERENTIAL SCANNING CALORIMETRY THERMOGRAM OF ISONIAZID

The DSC thermogram displayed a sharp endothermic peak which started at 171.62°C, reaching the maxima at 175.20°C and ending at 179.85°C. The enthalpy of isoniazid was calculated at 237.728J/g. Minor decompositions of isoniazid at about 200°C could also be observed. The thermogram values coincided with the literature given values [Brewer *et al.*, 1977].

2.2.2.7 THIN LAYER CHROMATOGRAPHY OF ISONIAZID

Thin layer chromatography of isoniazid was conducted by using a silica gel F₂₅₄ with the mobile phase consisting of ethyl acetate, acetone, methanol and hexane in the ratio of 50 : 20 : 20 : 10 (v/v). Only one spot with the R_f value of 0.67 was observed which clearly indicated that there were no other UV-active contaminants within the isoniazid sample.

2.2.3 PYRAZINAMIDE

Pyrazinamide, the generic name of pyrazine-2-carboxamide, is a white, odourless crystalline powder with the empirical formula of C₅H₅N₃O and weight of 123.11g/mol [Felder *et al.*, 1983]. The structure of pyrazinamide can be found in figure 2.12.

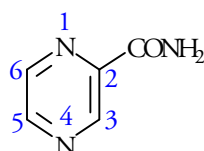


Figure 2.12 Chemical Structure of Pyrazinamide

2.2.3.1 ULTRAVIOLET SPECTRUM OF PYRAZINAMIDE

Table 2.9. and figure 2.13. show the results of pyrazinamide UV-Vis spectroscopy which is consistent with the literature reported values [Felder et al., 1983].

Table 2.9 UV-Vis Data of Pyrazinamide

UV-Vis Spectrum Data			
<i>max (nm)</i> <i>(Experimental)</i>	<i>max (nm)</i> <i>(literature)</i>	<i>(Experimental)</i>	<i>(Literature)</i>
208.0	209	8931	8765
267.7	269	8102	8036

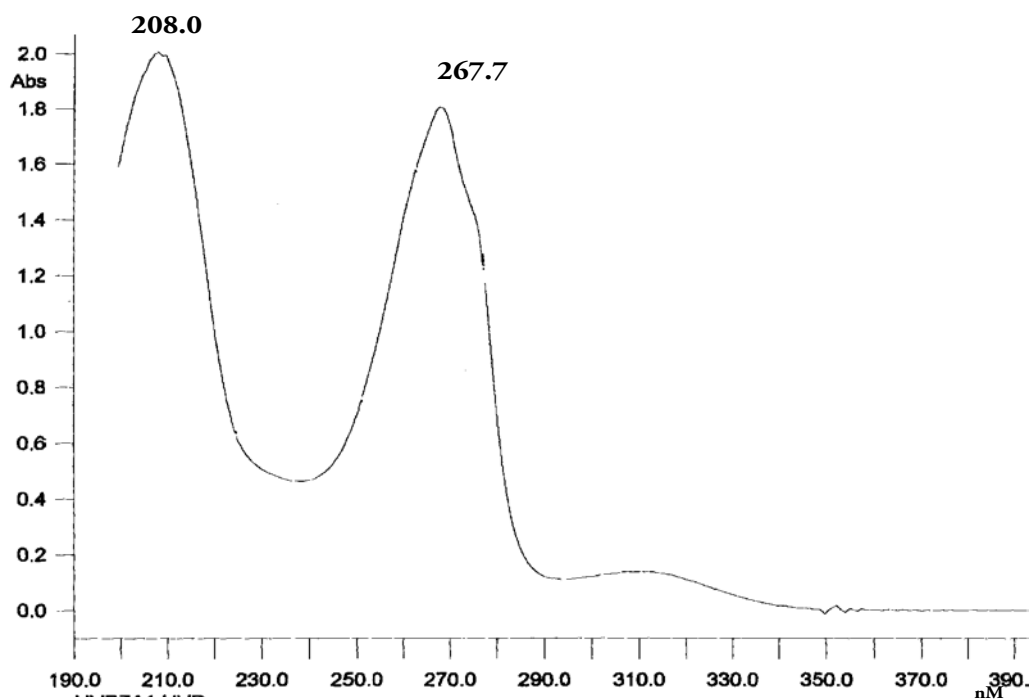


Figure 2.13 UV-Vis Spectrum of Pyrazinamide

2.2.3.2 INFRARED SPECTRUM OF PYRAZINAMIDE

The IR spectrum of the pyrazinamide confirms the presence of the relative functional groups (table 2.10) as the spectrum is given in figure 2.14. The spectrum corresponds to those in the literature values [Felder et al., 1983].

Table 2.10 Infrared Result of Pyrazinamide

Infrared Spectrum Data		
<i>IR Absorption Band (cm⁻¹) (Experimental)</i>	<i>IR Absorption Band (cm⁻¹) (Literature)</i>	<i>Functional Groups</i>
3445.2, 3295.6	3425, 3290	-NH, -NH bonded stretching
3174.3	3160	Aromatic C-H stretching
1711.1	1716	-C=O stretching
1603.1, 1615.1	1614	-NH ₃ asymmetric bending
1589.3	1582	-C=N stretching
1381.5, 1350.2	1382	Aromatic ring vibration
1182.1-783.6	1183-782	CH out of plane, NH ₂ rocking

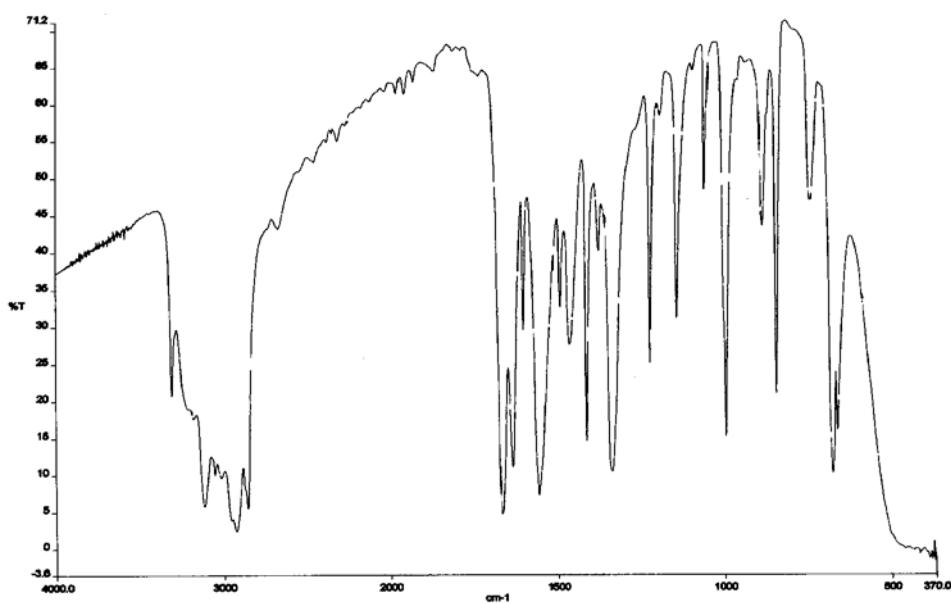


Figure 2.14 Infrared Spectrum of Pyrazinamide

2.2.3.3 NUCLEAR MAGNETIC RESONANCE SPECTRUM OF PYRAZINAMIDE

Both ^1H and ^{13}C NMR experiments were carried out in DMSO-d_6 . The interpretation of the peaks as well as the literature values [Felder et al., 1983] are tabulated in tables 2.11 and 2.12. After examining the peaks, the results confirmed the identity of the analyte to be pyrazinamide.

Table 2.11 ^1H NMR Spectrum Data of Pyrazinamide

^1H NMR Spectrum Data			
<i>Proton Represented</i>	<i>Multiplicity</i>	<i>(ppm) Experimental</i>	<i>(ppm) Literature [Felder, 1983]</i>
$-\text{CONH}_2$	Singlet	8.26, 7.87	8.25, 7.88
H-5	Quartet	8.73	8.71
H-6	Doublet	8.82	8.85
H-3	Doublet	9.18	9.21

Table 2.12 ^{13}C NMR Spectrum Data of Pyrazinamide

^{13}C NMR Spectrum Data		
<i>Carbon Represented</i>	<i>(ppm) (Experimental)</i>	<i>(ppm) (Literature)</i>
DMSO	39.44	40
C-5	143.2	143.2
C-3	143.6	143.6
C-2	145.1	145.0
C-6	147.3	147.3
C=O	165.0	165.0

2.2.3.4 MASS SPECTRUM OF PYRAZINAMIDE

The molecular ion at $m/e = 123$ of the pyrazinamide analyte corresponds with the molecular weight of the actual pyrazinamide (123.11g/mol) [Felder et al., 1983]. The fragmentation pathway is illustrated in figure 2.16. The fragment peaks of m/e of 123, 80 (base peak), 50 and 44 have the relative abundance of 19.84, 100, 4.81 and 33.13% respectively. The fragmentation pattern imply that pyrazinamide could readily lose its carboxamide group.

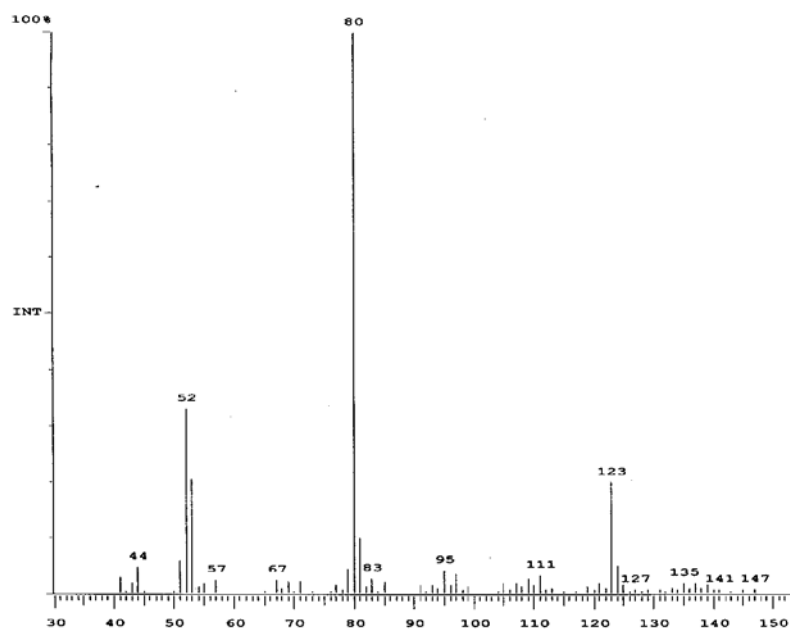


Figure 2.15 Relative Abundance of Pyrazinamide and the Fragments

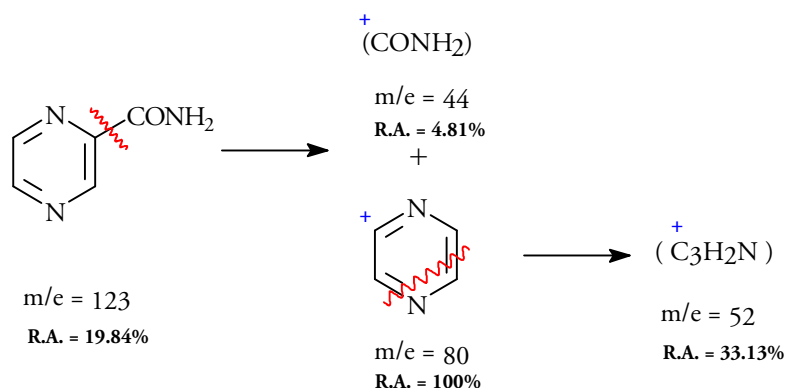


Figure 2.16 Pyrazinamide Fragmentation Pattern

2.2.3.5 PYRAZINAMIDE MELTING RANGE

Literature [Felder et al., 1983] states that the melting point of pyrazinamide ranges from 189.5 to 191.0°C. The melting point range of the pyrazinamide sample concurred with the melting point range of pyrazinamide stated in the literature (190.0 to 192.0°C).

2.2.3.6 DIFFERENTIAL SCANNING CALORIMETRY THERMOGRAM OF PYRAZINAMIDE

The pyrazinamide thermal curve displayed a sharp endothermic peak which started at 189.107°C, reaching the maxima at 191.700°C and ending at 192.889°C corresponding to pyrazinamide melting. The enthalpy of pyrazinamide is about 193.429J/g.

2.2.3.7 THIN LAYER CHROMATOGRAPHY OF PYRAZINAMIDE

Thin layer chromatography of pyrazinamide was conducted by using a silica gel plate F₂₅₄ and chloroform, methanol and conc. ammonia in the ratio of 20:20:1 (v/v) [Felder *et al.*, 1983] as the mobile phase. After careful examination of the plate, only one spot with R_f value of 0.51 was observed which clearly indicated that the purity of the sample was acceptable.

2.3 CONCLUSION

Identifying and characterising a particular organic compound has generally relied on the spectroscopic techniques such as UV, IR, NMR and MS, but thermal analyses (including melting point and DSC) and the thin layer chromatography has also been useful [McMurry, 1992]. The identity and the purity of all of the samples donated by Druggist Research Group of Pharmicare-Lennon[®] was confirmed because in the characterisation of rifampicin, isoniazid and pyrazinamide concurrence with the literature values occurred. The purity of the samples were further confirmed in the HPLC analyses described in the next chapter. The identity and the purity of each of the three batches used plays an important part for the solubility and the stability studies in subsequent chapters.

CHAPTER THREE

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION

3.1. INTRODUCTION

Analytical methods employed for the qualitative and the quantitative determination of pharmaceutical products and their metabolites or degradants play a significant role in pre-formulation studies. Both the evaluation and interpretation of various experimental data rely on a specific method. Therefore it is essential to develop an adequate method for a particular system to suit a particular analysis and this enables one to yield maximum efficiency regarding each related analysis performed. The method should be acceptable with respect to the regulatory requirements and reliable by incorporating statistical analyses to evaluate the performance. Most important of all, a method has to be validated for its effectiveness and appropriateness for the particular analysis undertaken. An adequate validation usually consists of the following parameters: specificity, accuracy, precision, linearity, reproducibility, ruggedness, limitation of detection and quantitation [Lang et al., 1991; Wahlich et al., 1990]. These parameters form part of the U.S.P. method validation.

Titrimetric and spectrophotometric techniques have been the analytical methods most commonly used for monitoring or analyzing drug/chemical substances. However, these techniques could be non-specific from time to time due to the fact that the result(s) may constantly be interfered with by the impurity(ies) presented within the sample(s) and cause a small degree of error. Therefore, a reduction of quantitative accuracy may be expected. Many modern analytical techniques depend on column chromatography due to the fact that these techniques have the ability of selectively separating a mixture of substances and quantifying them at the same time. Among all the column chromatographic techniques, high-performance liquid chromatography (HPLC) is by far the most popular, due to its versatility, efficacy, precision and speed [Skoog et al., 1992; Wahlich et al., 1990].

Over the past 10 to 15 years, HPLC has become one of the most dominant analytical separation tools in pharmaceutical, chemical and various other laboratories. According to the different affinity of each chemical for the column packing materials and the different affinity of the chemical for the mobile phase, the chemicals are separated accordingly and quantified. The analytical methods for HPLC are relatively easy to develop and validate. The HPLC quantitative analyses can be completed within a short period of time, while still yielding a high degree of accuracy [Chong et al., 1996]. Rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA) are the three key drugs which are mostly likely to be prescribed separately or in various combinations in the treatment of tuberculosis [Herfindal et al., 1992]. A number of reports have described the development of high performance liquid chromatographic methods for analyzing each of these drugs individually or in various combinations of two of the drugs . However, on examination [Argekar et al., 1996; Beretta et al., 1987; Clarke et al., 1991; Gaitode et al., 1991; Defilippi et al., 1994; Gaitode et al., 1990; Gidoh et al., 1981; Guillaumont et al., 1982; Gupta et al., 1988; Ishii et al., 1988; Jindal et al., 1994; Jindal et al., 1995; Lachau; *et. al.*, 1992; Karlagains et al., 1957; Ratti et al., 1981; Seifart et al., 1995; Shah et al., 1991; Shah et al., 1992; Su et al., 1989; Walobu et al., 1991; Walubo et al., 1994, Woo et al., 1987; Yamaoto et al., 1987; Yousseff et al., 1998 and Yusuff et al., 1991] the methods for the simultaneous analysis of isoniazid, pyrazinamide and rifampicin required the use of specialized columns, gradient elution and/or a complex mobile phases rendering them unsuitable, not cost-effective for large-scale industrial application. The difficulty in analysis of these three drugs in combination may be attributed to the polarity differences between rifampicin and isoniazid and pyrazinamide, as well as the similarity of polarity between isoniazid and pyrazinamide. The aim of this chapter is therefore to present the development and validation of a HPLC method that can be use for analyzing all three drugs in combination by using simple mobile phase compositions, a cost effective column and an isocratic elution system.

3.2 METHOD DEVELOPMENT EXPERIMENTS

Prior to the validation, a method for analysis of all three drugs in combination has to be developed with logical sequence. The method development phase is an essential component which provides the analyst with an opportunity to undertake some practical experiments that may help to identify the optimum chromatographic conditions. This includes adjusting and optimizing the mobile phase composition for a particular chromatographic column [Clarke et al., 1994].

3.2.1 CHROMATOGRAPHIC SYSTEM

The following chromatographic system (Set (a)) is selected:

High pressure pump	: Solvent delivery system Model 6000A (Waters [®] , Massachusetts)
Injector	: WISP Model 710B (Waters [®] , Massachusetts)
Detector	: Lambda max UV detector Model 481 (Waters [®] , Massachusetts)
Integrator and recorder	: Data Module (Waters [®] , Massachusetts)

3.2.2 CHROMATOGRAPHIC SYSTEM CONDITIONS

The following chromatographic system conditions were used:

Flow rate	: 1.0ml/min
Volume of injection	: 20µl
Wavelength of detection	: 260nm
A.U.F.S.	: 1.0
Instrumental noise rejection	: 1000 units
Area of rejection	: 100,000 units

3.2.3 CHOICE OF COLUMN AND MOBILE PHASE

The four different of types of columns investigated are listed below:

(C-a) = Nova-pak® C18, 3.9×150mm column, 4µm/60D, (Waters®, Massachusetts)

(C-b) = µ-bondapak™ C18, 4.6×250mm column a C18 guard column, 10µm/125D (Waters®, Massachusetts)

(C-c) = Bondex® C18, 3.9×300mm column, 10µm/60D (Phenomenex®, California)

(C-d) = Nova-pak® CN-HP C18, 3.9×150 column, 4µm/60D (Waters®, Massachusetts)

Column efficiency was assessed for these columns. The number of theoretical plates (N) was calculated according to the 5F methods. The 5F is the measure of column performance near the baseline as resolution is most critical near the peak base. This method is generally stringent and indicates the lowest plate count [Skoog, 1992; Waters®, 1998]. The 5F equation is expressed in equation 3.1

$$N = 25(V/w)^2 \quad \dots \dots \dots \quad \text{Eq. 3.1.}$$

Where N = number of theoretical plates
 V = distance to the peak (mm)
 W = peak width at 4.4% peak height (mm)

All of the four columns showed good column efficiency since the amount of theoretical plates in each column exceeded 12,500. A column with a plate count of above 6,000 is considered suitable for analyses [Waters®, 1998].

A F32 pH meter (Beckman[®], USA), HPLC filter system with 0.5mmFH-RP filter paper (Millipore[®], Ireland), and a Soniclean (Ultrasonic Engineering[®], RSA) were used for the preparation of various mobile phases. The four sets of mobile phases with different combinations of organic-aqueous ratio were used. The solvents used are listed below:

(a) = Methanol (MeOH) [BDH, England]

(b) = Acetonitrile (MeCN) [BDH, England]

(c) = Disodium orthophosphate (Na_2HPO_4) buffer solution. Na_2HPO_4 [Merck, Darmstadt] (0.02M of Na_2HPO_4 buffer solution obtained by dissolving 0.0568g of Na_2HPO_4 in 1000ml of HPLC water.)

(d) = Tetrabutylammonium hydroxide ($(\text{Bu})_4\text{NOH}$ or $t\text{BAH}$) [Merck, Darmstadt] (2.5ml of 20% $(\text{Bu})_4\text{NOH}$ was added to a 1000ml of volumetric flask and made up to volume with water to obtain 0.0005M of $(\text{Bu})_4\text{NOH}$ buffered solution.)

All of the mobile phases used were composed of the following: solvents (MeOH or MeCN) and buffered solutions (Na_2HPO_4 or $(\text{Bu})_4\text{NOH}$) were measured accurately to the required ratio (v/v or in % ratio). The combinations were further adjusted with orthophosphoric acid (H_3PO_4) [BDH, England] to the particular pH, then filtered and degassed.

Table 3.1 Mobile Phase Adjustment ($\text{MeOH} : \text{Na}_2\text{HPO}_4$)

Mobile Phase ($\text{MeOH} : \text{Na}_2\text{HPO}_4$)					
Mobile phase		Column Type	Rt (min)		
Ratio [a : c]	pH		Rifampicin	Isoniazid	Pyrazinamide
80 : 20	4.5	C -a	1.73	0.83	0.83
75 : 25	4.5	C -a	2.4	0.91	0.92
70 : 30	4.5	C -a	2.9	1.03	1.04
65 : 35	4.5	C -a	3.32	1.06	1.08
60 : 40	4.5	C -a	6.05	1.11	1.12

After examining the above table, this set of mobile phases (MeOH : Na₂HPO₄) was found to be unsuitable for the combination analysis of rifampicin, isoniazid and pyrazinamide since the retention times of isoniazid and pyrazinamide are similar resulting in lack of resolution between the two drugs. Because of the different polarity compared to methanol, an attempt was made to separate isoniazid and pyrazinamide by replacing methanol with acetonitrile.

Table 3.2 Mobile Phase Adjustment (MeCN : Na₂HPO₄)

Mobile Phase (MeCN : Na ₂ HPO ₄)					
Mobile Phase		Column Type	Rt (min)		
Ratio [b : c]	pH		Rifampicin	Isoniazid	Pyrazinamide
60 : 40	3.7	C -a	2.14	0.95	1.04
60 : 40	3.7	C -b	6.2	2.8	3.15
60 : 40	3.7	C -c	4.93	2.61	2.75
60 : 40	3.7	C -d	1.78	1.3	1.27
50 : 50	3.7	C -a	3.92	1.02	1.06
50 : 50	3.7	C -b	11.59	2.77	3.28
50 : 50	3.7	C -c	7.44	2.53	2.78
50 : 50	3.7	C -d	2.12	1.32	1.32
40 : 60	3.7	C -b	13.27	2.89	3.37
40 : 60	3.7	C -c	>20.0	2.66	2.93
40 : 60	3.7	C -d	2.87	1.34	1.35

Although a small degree of separation between isoniazid and pyrazinamide was found by using this set of mobile phase combinations (MeCN : Na₂HPO₄), however, adequate base-line resolution was not achieved. Therefore further investigations and changing of mobile phase compositions was required.

Table 3.3 Mobile Phase Adjustment (MeOH : tBAH)

Mobile Phase (MeOH : tBAH)					
<i>Mobile</i>		<i>Column</i>	<i>Rt (min)</i>		
<i>Ratio [a : d]</i>	<i>pH</i>		<i>Type</i>	<i>Rifampicin</i>	<i>Isoniazid</i>
75 : 25	3.7	C-a	3.34	1.2	1.39
75 : 25	3.7	C-b	8.12	3.75	3.68
75 : 25	3.7	C-c	7.89	3.44	3.45
75 : 25	3.7	C-d	3.46	1.29	1.26
70 : 30	3.7	C-a	2.1	0.99	0.99
70 : 30	3.7	C-b	10.56	3.42	3.46
70 : 30	3.7	C-c	10.22	3.33	3.36
70 : 30	3.7	C-d	2.88	1.17	1.18
65 : 35	3.7	C-a	4.11	1.51	1.7
65 : 35	3.7	C-b	14.45	3.46	3.58
65 : 35	3.7	C-c	14.22	3.15	3.26
65 : 35	3.7	C-d	3.45	2.1	2.78

These observations clearly shown that this set of mobile phase combinations (MeOH : tBAH) was not suitable for the application to this project. Total separation between isoniazid and pyrazinamide was not achieved. However, comparing to table 3.3 to 3.1, the degree of separation between isoniazid and pyrazinamide has increased. This showed that tBAH could contribute the separation between isoniazid and pyrazinamide, therefore, further investigation employing MeCN and tBAH was carried out and the results are listed in table 3.4.

Table 3.4 clearly indicates that this set of mobile phase compositions demonstrated analytical usefulness if employing C-b (4.6×250mm μ -bondapakTM C18 plus a C18 guard column, 10 μ m/125D (Waters[®], Massachusetts) as the analytical column. Smaller increments of the solvent-aqueous ratio were carried out (refer to table 3.5.). The results showed that the mobile phase which consists of 42.5% of MeCN and 57.5% tBAH produced the best separation, but the mobile phase consisting of 45 : 55 and 55 : 45 of MeOH : tBAH showed reasonable separation. The effect of pH adjustment could further optimize the separation. Therefore the a set of experiments were performed based on pH variation and the results are tabulated in table 3.6.

Table 3.4 Mobile Phase Adjustment (MeCN : tBAH)

Mobile phase Adjustment (MeCN : tBAH)					
Mobile phase		Column Type	Rt (min)		
Ratio = [b : d]	pH		Rifampicin	Isoniazid	Pyrazinamide
40 : 60	3.7	C-a	1.71	1.3	1.34
40 : 60	3.7	C -b	13.49	3.15	3.71
40 : 60	3.7	C -c	9.87	2.94	3.18
40 : 60	3.7	C -d	2.51	1.60	1.65
45 : 55	3.7	C-a	1.68	1.09	1.12
45 : 55	3.7	C -b	8.15	3.08	3.63
45 : 55	3.7	C-c	6.99	3.01	3.10
45 : 55	3.7	C-d	2.22	1.56	1.58
50 : 50	3.7	C -a	1.60	1.05	1.10
50 : 50	3.7	C -b	6.14	2.72	3.3
50 : 50	3.7	C -c	5.15	2.55	2.81
50 : 50	3.7	C -d	1.77	1.35	1.35
60 : 40	3.7	C-a	1.24	1.05	1.06
60 : 40	3.7	C -b	4.28	2.61	3.42
60 : 40	3.7	C-c	4.02	2.12	2.18
60 : 40	3.7	C-d	1.53	1.16	1.17

Table 3.5 Mobile Phase Adjustment (MeCN : tBAH with Column (C-b))

Mobile Phase (MeCN : tBAH with Column (C-b))						
MeCN (%)	(Bu) ₄ NOH (%)	pH	Column Type	Rt (min)		
				Rifampicin	Isoniazid	Pyrazinamide
50	50	3.7	C-b	6.14	2.82	3.3
47.5	52.5	3.7	C-b	7.27	3.02	3.47
45	55	3.7	C-b	8.15	3.08	3.63
42.5	57.5	3.7	C-b	9.02	3.19	3.70
40	60	3.7	C-b	13.49	3.15	3.71

3.2.4 METHOD OPTIMIZATION

The same chromatographic conditions as for the mobile phase development stage 5 were applied except pH was altered. Table 3.6. below shows the effect of pH on the above selected mobile phase.

Table 3.6 Mobile Phase Optimization (pH Alteration)

pH Alteration						
<i>MeCN</i> (%)	<i>tBAH</i> (%)	<i>pH</i>	<i>Column</i> <i>Type</i>	<i>Rt (min)</i>		
				<i>Rifampicin</i>	<i>Isonaizid</i>	<i>Pyrazinamide</i>
42.5	57.5	3.70	C-b	9.02	3.19	3.7
42.5	57.5	3.50	C-b	9.39	3.17	3.69
42.5	57.5	3.40	C-b	9.85	3.11	3.63
42.5	57.5	3.20	C-b	10.68	2.92	3.56
42.5	57.5	3.10	C-b	10.97	2.90	3.54
42.5	57.5	3.00	C-b	11.68	2.95	3.47
42.5	57.5	2.85	C-b	12.02	2.89	3.45

All of the mobile phase conditions listed in tables 3.5 and 3.6 produced acceptable separation results, this proved that the method showed acceptable ruggedness. The best separation was produced by using the mobile phase consisting of 42.5% : 57.5%, MeCN: tBAH (0.0002M), v/v, with a final pH of 3.10, but the pH range of 3.10 to 3.40 was also acceptable. After six injections, the retention time periods were found to be 2.85±0.01, 3.54±0.01 and 10.97±0.01 min for RIF, INH and PZA respectively when using the above conditions with pH set at 3.1.

3.3 METHOD VALIDATION

For the method validation, an additional HPLC system (Set (b)) is also used.

High pressure pump	: SpectraSERIES isocratic pump P100 (Therma Separation Products, Florida)
Injector	: M7125 20 μ l fixed loop (Rheodyne Inc., CA, USA)
Detector	: SpectraSERIES UV detector UV100 (Therma Separation Products, Florida)
Recorder	: Rikadenki chart recorder (Rikadenki, Tokyo)

3.3.1 STANDARD SOLUTION PREPARATION

Two sets of analyte solutions were prepared as follows: (For Set (a) HPLC system) 0.05g of INH accurately weighed was dissolved in 100ml of water to obtain a 0.5mg/ml stock solution (same procedure was applied to PZA), while 0.02g of RIF was transferred into a 100ml volumetric flask and made up to volume with water to obtain 0.2mg/ml stock solution. (For Set (b) HPLC system) 0.02g of INH, 0.012g of PZA and 0.014g of RIF were transferred to three 100ml of volumetric flasks and the contents were dissolved in water to obtain 0.2, 0.12 and 0.14mg/ml stock solutions respectively. These stock solutions were further diluted to various concentrations which are listed in table 3.7.

3.3.2 PRECISION, ACCURACY, LINEARITY AND REPRODUCIBILITY

Precision is the description of the closeness of replicate determinations of a standard (an analyte with known concentration) by an assay while accuracy is the closeness of the determined value to the true value. The accuracy and the precision should be determined using a minimum of 6 (excluding blank sample) determinations per each concentration. The standard solutions were injected and analyzed 6 times. The coefficient of variation (CV) must be calculated. In order to produce acceptable reproducibility, the mean coefficient of variation value should not exceed $\pm 15\%$

of the actual value (except during limits of quantitation study where the mean value must be within 20% of the actual value) [Dagar *et al.*, 1995; Edwardson *et al.*, 1990]. The data are listed in table 3.7.

It is necessary to use an adequate amount of standards in order to sufficiently define the relationship between concentration and the response. This response should be reproducible. Each particular concentration should correspond to only one response to enable the construction of a standard curve. Most standard curves can be classified as either exhibiting a linear or non-linear relationship. In this validation, a linear response is required [Dagar *et al.*, 1995; Edwardson *et al.*, 1990]. The linearities of both set (a) and (b) HPLC systems can be found in table 3.8 and the related standard curves are presented in figures 3.1 to 3.3.

Table 3.7 Coefficients of Variation (CV) of Rifampicin, Isoniazid and Pyrazinamide

Precision Data								
Set (a)								Mean CV
RIF	Conc. (mg/ml)	0.02	0.04	0.05	0.08	0.10	0.25	0.19
	CV (%)	0.04	0.42	0.23	0.00	0.26	0.18	
INH	Conc. (mg/ml)	0.02	0.04	0.05	0.08	0.10	0.14	0.28
	CV (%)	0.26	0.15	0.43	0.26	0.17	0.38	
PZA	Conc. (mg/ml)	0.01	0.02	0.05	0.06	0.10	0.12	0.23
	CV (%)	0.19	0.39	0.09	0.05	0.39	0.25	
Set (b)								
RIF	Conc. (mg/ml)	0.02	0.04	0.06	0.10	0.15	0.20	0.07
	CV (%)	0.17	0.11	0.05	0.06	0.00	0.09	
INH	Conc. (mg/ml)	0.02	0.06	0.08	0.10	0.20	0.25	0.10
	CV (%)	0.18	0.18	0.00	0.15	0.18	0.06	
PZA	Conc. (mg/ml)	0.02	0.06	0.08	0.10	0.20	0.25	0.16
	CV (%)	0.23	0.25	0.11	0.02	0.09	0.15	

Table 3.8 Linearity Results for Rifampicin, Isoniazid and Pyrazinamide

Linearity Data				
Drug	Instrument	Gradient	y intercept	Correlation Coefficient (r^2)
RIF	“Set (a)”	144672497.25 units/mg	-535.57 units	0.9999
INH	“Set (a)”	171655612.60 units/mg	250.68 units	0.9999
PZA	“Set (a)”	224671655.34 units/mg	116.75 units	0.9998
RIF	“Set (b)”	55.26 mm/mg	0.11 mm	0.9996
INH	“Set (b)”	115.23 mm/mg	-0.15mm	0.9995
PZA	“Set (b)”	169.44 mm/mg	0.13 mm	0.9996

Low coefficients of variation (CV) and good correlation coefficients (r), were produced for both HPLC systems. This indicates that the level of precision and accuracy for the method were satisfactory. Furthermore, good linearity was found. However, the set (a) HPLC system produced better results compared to set (b) HPLC system. This may be attributed to the use of auto-sampler.

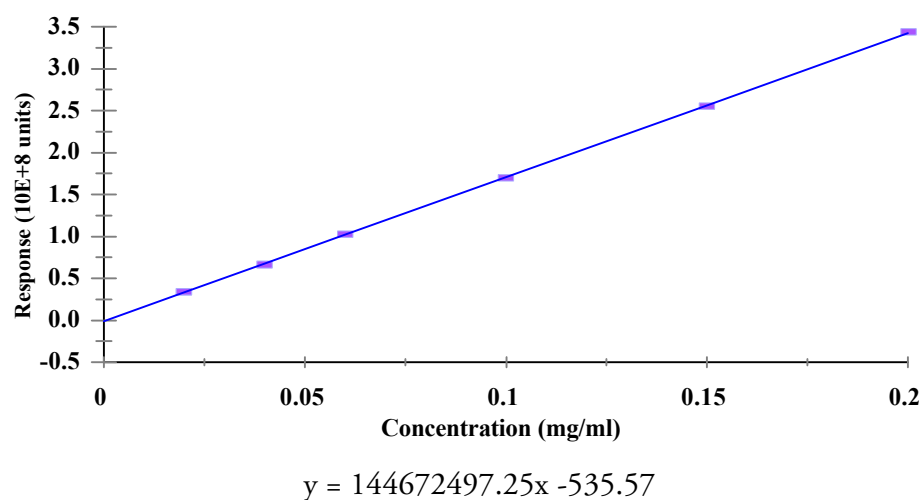


Figure 3.1. Rifampicin Standard Curve (Set (a) HPLC System)

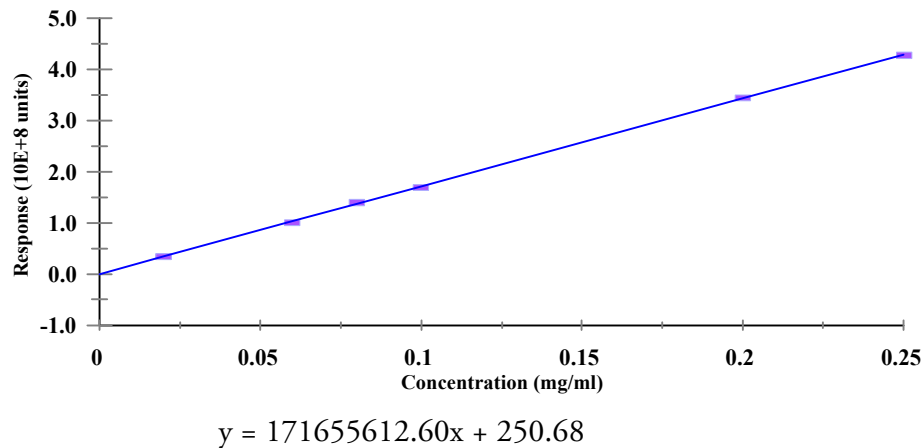


Figure 3.2. Isoniazid Standard Curve (Set (a) HPLC System)

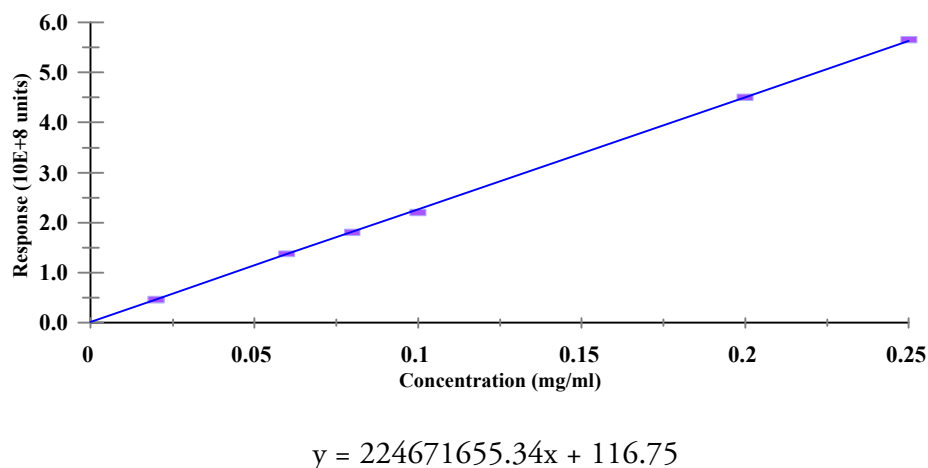


Figure 3.3. Pyrazinamide Standard Curve (Set (a) HPLC System)

3.3.3. RUGGEDNESS

A 48-hour stability trial was carried out on the standard solutions (1.0 mg/ml) of rifampicin, isoniazid and pyrazinamide. This is an attempt to produce the ruggedness of the stock solution. Two sets of stock solutions were placed either at $25 \pm 1^\circ\text{C}$ or in the fridge ($5 \pm 1^\circ\text{C}$) and protected from any light source. The stabilities of the drug solutions are shown in figures 3.4. to 3.6. where the concentration of drug remaining against time plotted (the blue curve represents the drug stability in room temperature whereas the brown curves represent the refrigerated drug standard).

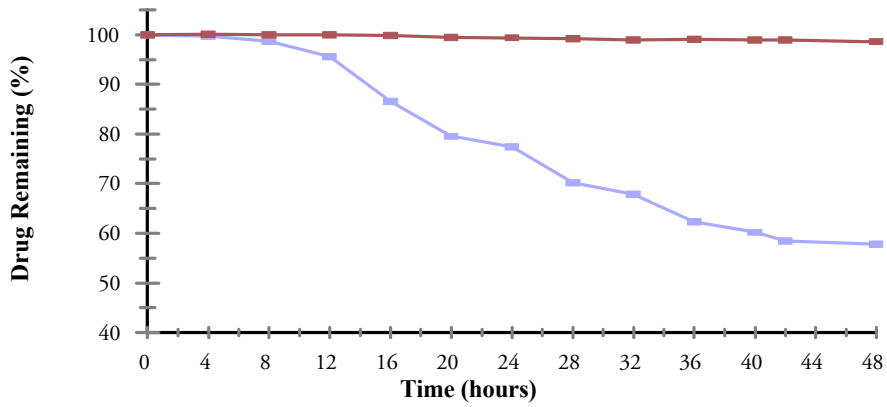


Figure 3.4 Stability-Time Curves of Rifampicin Stock Solution

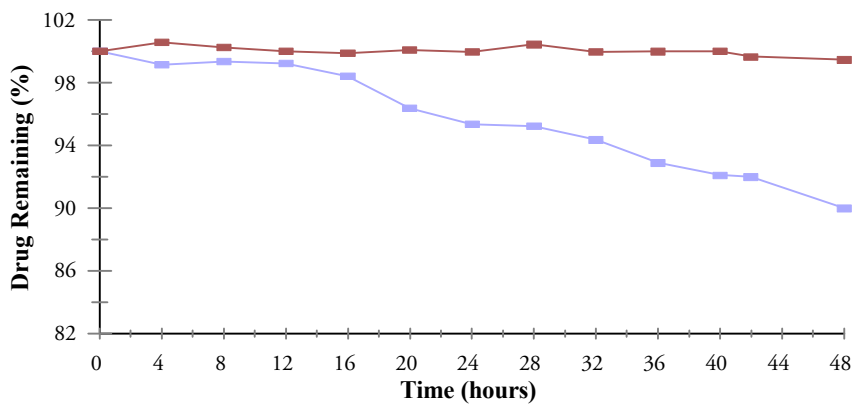


Figure 3.5 Stability-Time Curves of Isoniazid Stock Solution

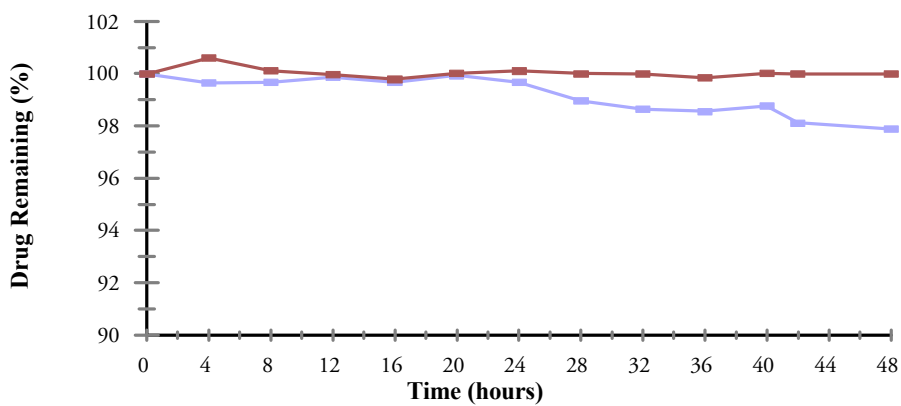


Figure 3.6 Stability-Time Curves of Pyrazinamide Stock Solution

The Stability-time curves clearly indicates that rifampicin is unstable in the aqueous solution at room temperature. A 40% loss of the total amount of the rifampicin occurs after 48 hours. This effect was less dramatic for both isoniazid and pyrazinamide. The standards were deemed to be reliable only if the analysis was carried out within 8-12 hours after preparation.

Ruggedness of the mobile phase was clearly demonstrated in the results listed tables 3.5 and 3.6 and in sections 3.2.3 and 3.2.4. Although the best separation was produced by using the mobile phase consisting of 42.5% : 57.5%, MeCN: tBAH (0.0002M) with pH of 3.1, this mobile phase could still produce acceptable results even when the ratio of mobile phase was varied by $\pm 2.5\%$ and the pH was altered by about ± 0.6 unit.

3.3.4 SPECIFICITY

The specificity demonstrates the ability of a method to satisfactorily measure the analyte and minimize the interferences from both the exogenous and endogenous sources. Typical exogenous interference are solvent impurities and detergents from incomplete glassware washing which could easily be avoided. However, the endogenous interferences such as analyte impurities and degradants could cause a significant degree of error. Therefore relevant stress studies under oxidative, heat, UV, acid and base stressing and observation are required to be carried out. [Clarke *et al.*, 1994].

The HPLC chromatograms of the specificity studies are given with the photodiode-array spectra in the figures below (figures 3.10 to 3.15). The photodiode-array experiments were conducted with a Sepectra Gold[®] Photodiode-array system (Beckman, UK) to investigate the integrity of the drug peaks. These chromatograms were plotted as absorbance of the specific drug peak region (which will be listed in the corresponding tables) against a range of wavelength (190 to 400nm). Photo-diode-array technique was employ in order to clarify the purity of the peak. The pattern of each UV spectrum of an individual drug is unique, therefore the shape of the UV spectra should maintain almost an identical fraction (except differ in their intensities). Therefore, the diode-array scan of an particular elution peak in different time region should also reproduce a similar argument. The impurities within the elution peak would cause an alteration of the shape of the diode-array scan.

3.3.4.1 UNSTRESSED SAMPLE

The HPLC (figure 3.7 (a)) and the photodiode-array (figure 3.8 (a-c)) chromatograms are presented. In the HPLC chromatogram, the peak at retention times of 2.85, 3.54 and 10.97 minutes represent isoniazid, pyrazinamide and rifampicin respectively. The photodiode-array chromatograms were obtained by scanning at four different points (listed in table 3.9) of each of the drug peaks. The photodiode-array spectra confirm the integrity of the three drug peaks. The conditions of oxidation, heat, UV, acid and base are used and peak integrity monitored in order to ensure accurate results during stability studies.

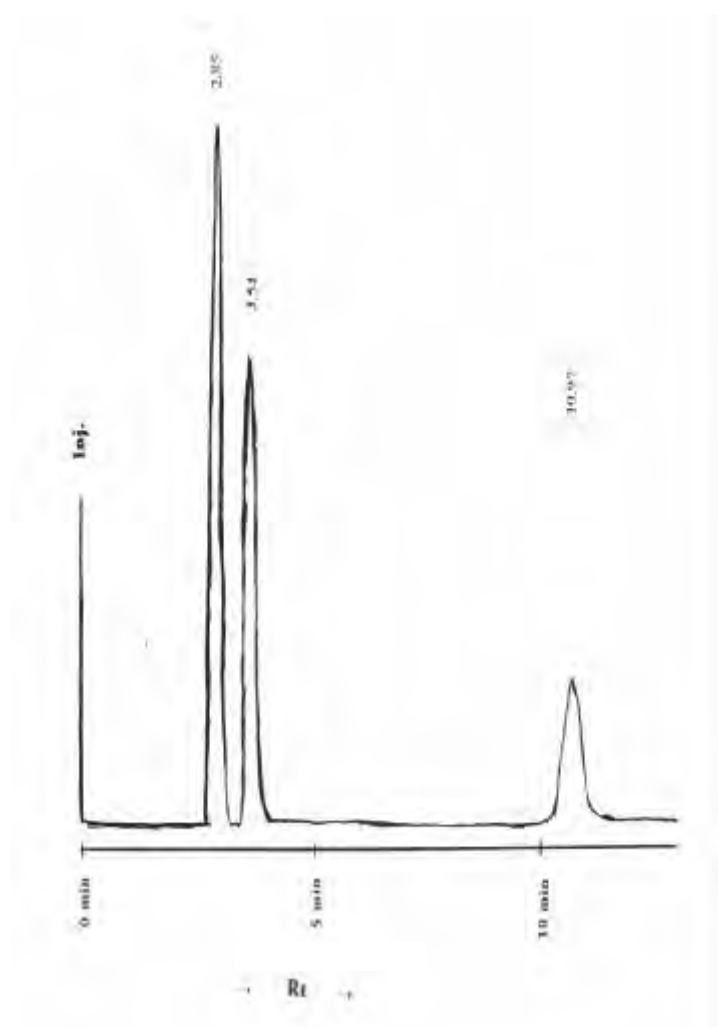
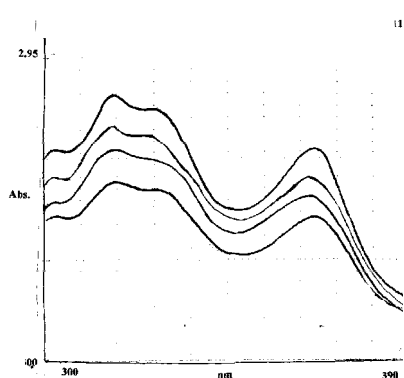


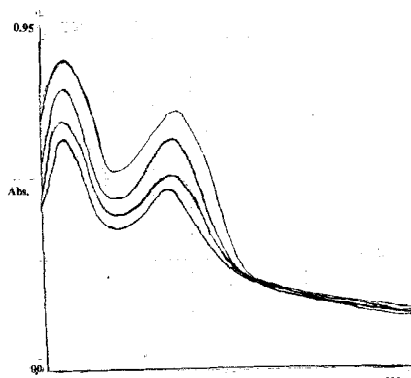
Figure 3.7 The HPLC Chromatograms of Unstressed RIF, INH and PZA

Table 3.9 Photodiode-array Scans at Various Retention Times

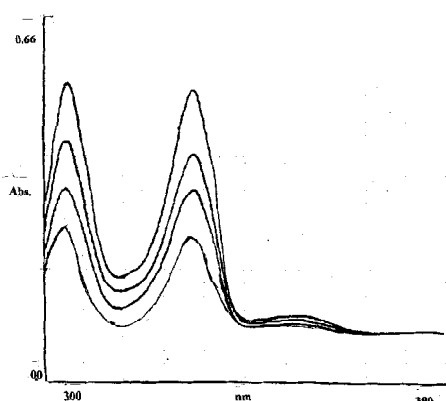
Photodiode-array Scans Time Region for Figure 3.8		
<i>RIF (Rt)</i>	<i>INH (Rt)</i>	<i>PZA (Rt)</i>
<i>min</i>	<i>min</i>	<i>min</i>
10.91-10.93	2.81-2.82	3.52-3.53
10.93-10.96	2.82-2.84	3.55-3.57
10.97-10.99	2.83-2.86	3.50-3.56
10.99-11.02	2.85-2.87	3.51-3.52



(a)



(b)



(c)

Figure 3.8 The Photodiode-array spectra of (a) RIF, (b) INH and (c) PZA

3.3.4.2 OXIDATIVE STRESSING

0.5ml of the hydrogen peroxide (Univar, Krugersdorp, RSA) was added to 9ml of rifampicin, isoniazid and pyrazinamide stock solutions to form a 5% hydrogen peroxide solution and the solution stored at room temperature for 72 hours. A number of degradant peaks were detected on HPLC analysis are shown in the figure 3.9. Figure 3.10.(a-c) and table 3.10. represent the data of the photodiode-array scan of the rifampicin, isoniazid and pyrazinamide peaks and the scan regions.

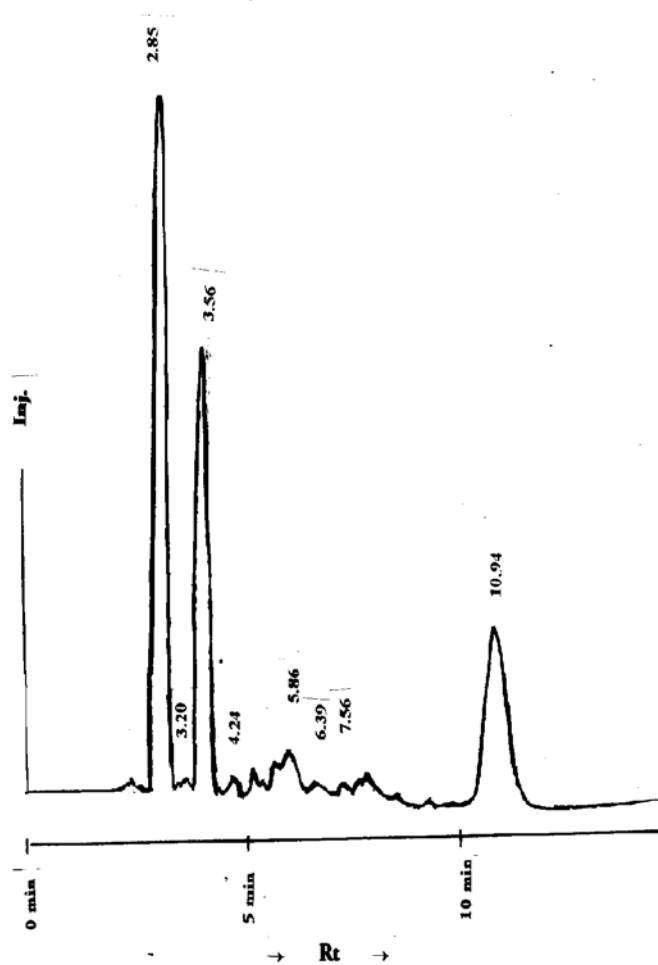


Figure 3.9 The HPLC Chromatogram of Oxidative Stressed RIF, INH and PZA

Table 3.10 Photodiode-array Scans at Various Retention Times

Photodiode-array Scans Time region for Figure 3.10		
<i>RIF (Rt)</i>	<i>INH (Rt)</i>	<i>PZA (Rt)</i>
<i>min</i>	<i>min</i>	<i>min</i>
10.92-10.94	2.81-2.82	3.52-3.53
10.93-10.95	2.80-2.84	3.52-3.55
10.96-10.99	2.84-2.87	3.56-3.60
11.05-11.06	2.85-2.88	3.60-3.63

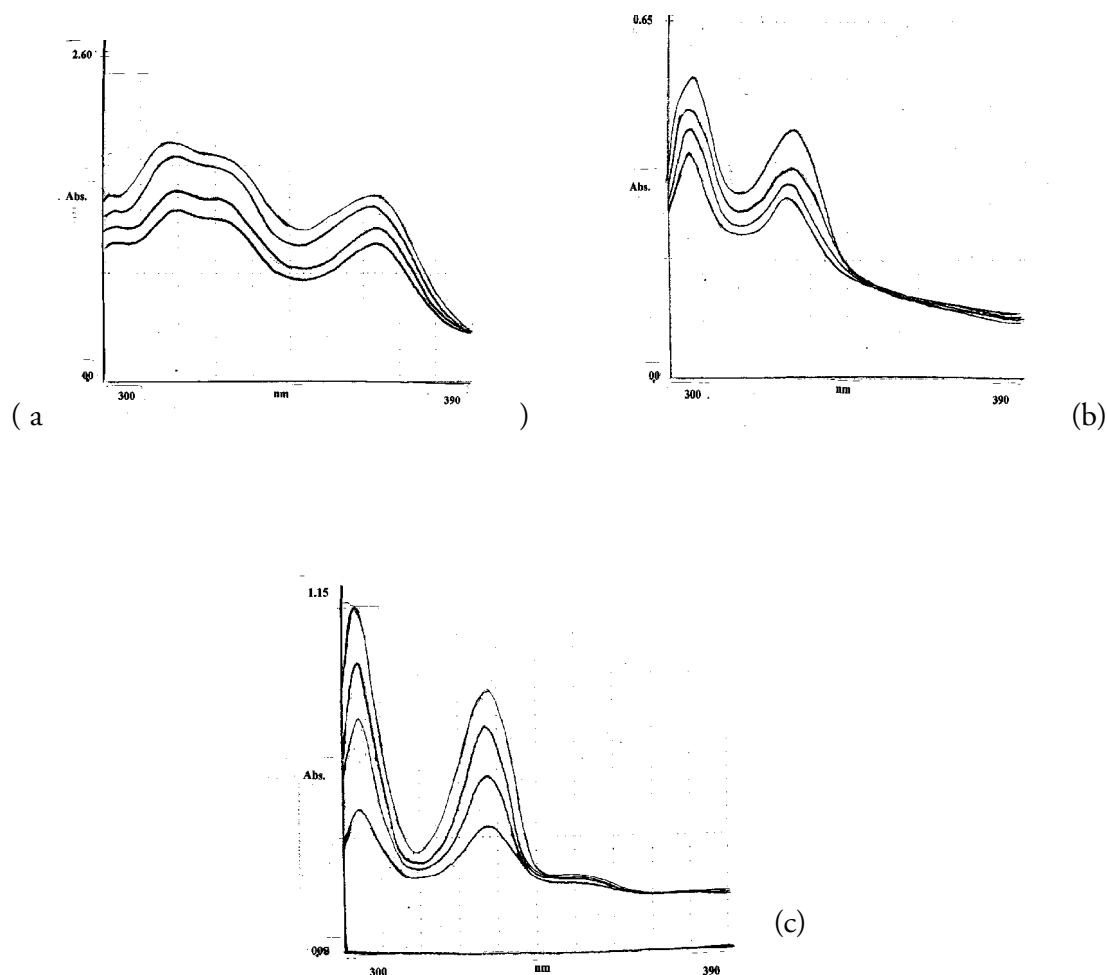


Figure 3.10 Photodiode-array chromatograms of Oxidative Stressed (a) RIF, (b) INH and (c) PZA

3.3.4.3 HEAT STRESSING

Rifampicin, isoniazid and pyrazinamide stock solutions were allowed to reflux for 30 minutes. Figures 3.11 and 3.12 represent the HPLC chromatogram and the photodiode-array spectra of the heat stressed sample confirming the integrity of the drug peaks under these conditions.

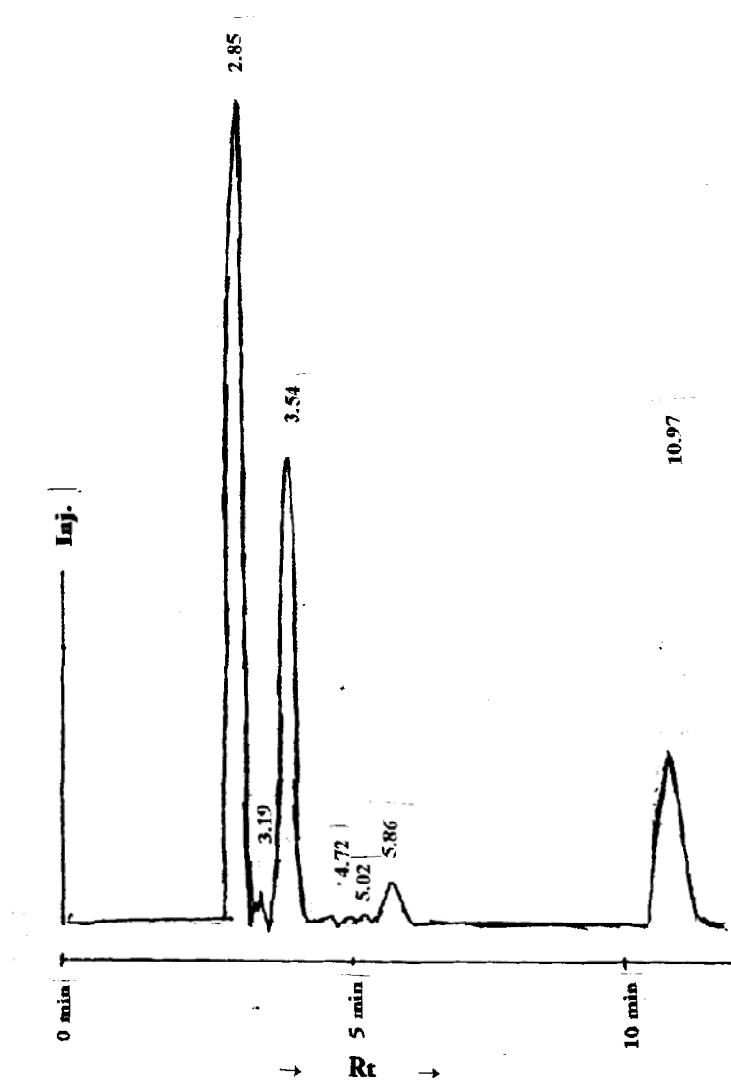
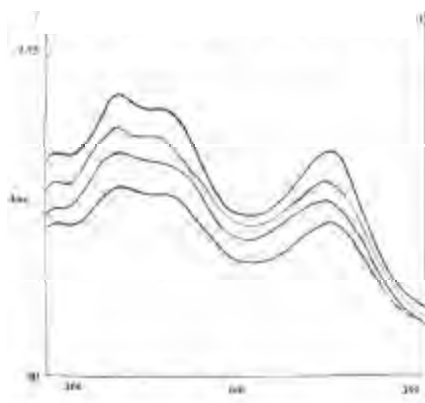


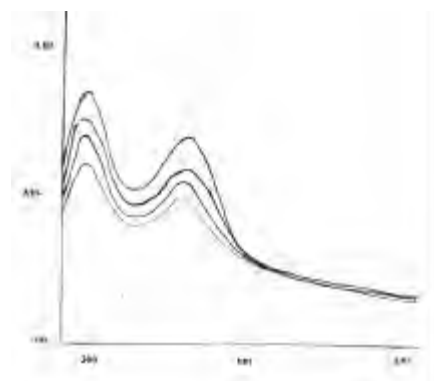
Figure 3.11 The HPLC Chromatogram of Heat Stressed RIF, INH and PZA

Table 3.11 Photodiode-array Scans at Various Retention Times

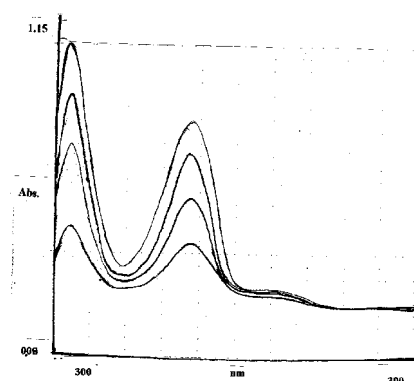
Photodiode-array Scans Time Region for Figure 3.12		
<i>RIF (Rt)</i>	<i>INH (Rt)</i>	<i>PZA (Rt)</i>
<i>min</i>	<i>min</i>	<i>min</i>
10.90-10.92	2.80-2.82	3.51-3.55
10.94-10.96	2.82-2.84	3.53-3.54
10.96-10.98	2.85-2.87	3.54-3.56
10.98-11.00	2.88-2.89	3.55-3.57



(a)



(b)



(c)

Figure 3.12 The Photodiode-array Spectra of Heat Stressed (a) RIF (b) INH and (c) PZA

3.4.4.4 ULTRAVIOLET LIGHT STRESSING

The HPLC chromatogram represented by figure 3.13 containing rifampicin, isoniazid and pyrazinamide was obtained after the solution which contains all three drugs was subjected to a ultraviolet lamp (15W, 254nm) for 7 days. The photodiode-array results can be examined in figure 3.14(a-c).

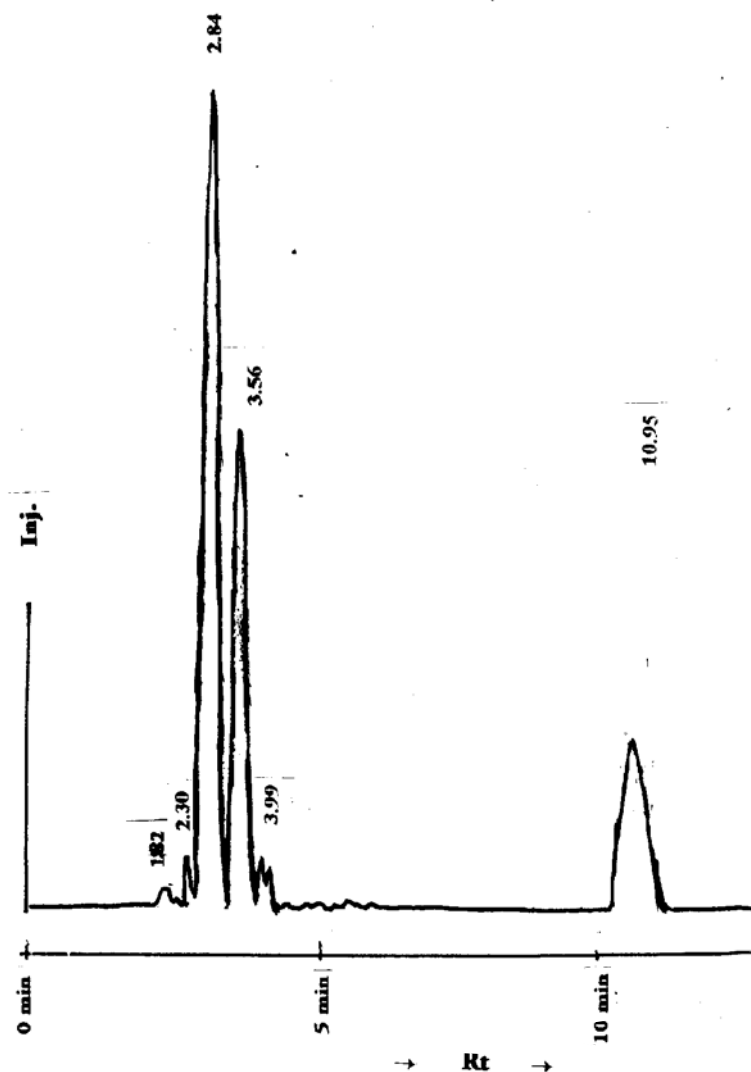
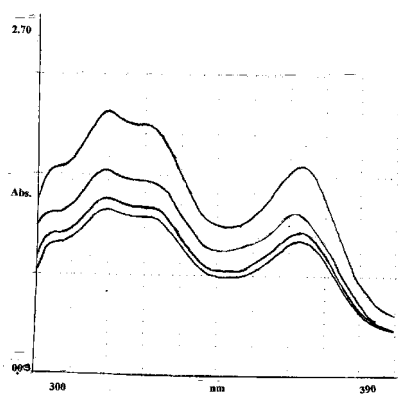


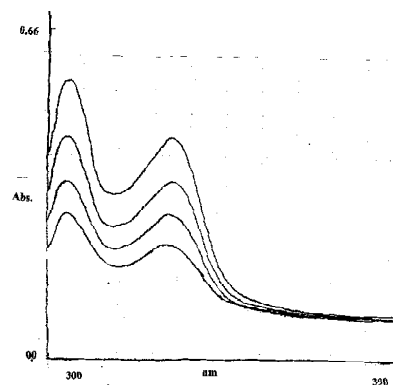
Figure 3.13 The HPLC Chromatogram of UV Stressed RIF, INH and PZA

Table 3.12 Photodiode-array Scans at Various Retention Times

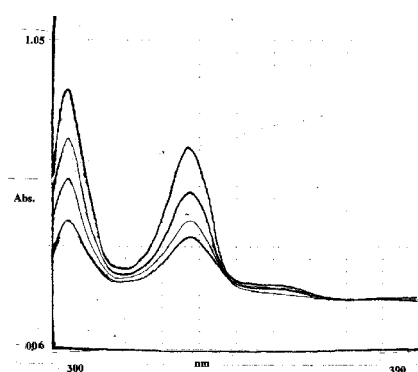
Photodiode-array Scans Time Region for Figure 3.14		
RIF (Rt)	INH (Rt)	PZA (Rt)
min	min	min
10.92-10.94	2.81-2.82	3.52-3.53
10.92-10.94	2.83-2.84	3.53-3.54
10.97-10.99	2.84-2.85	3.54-3.55
10.98-11.01	2.85-2.86	3.55-3.57



(a)



(b)



(c)

Figure 3.14 The Photodiode-array Spectra of UV Stressed (a) RIF, (b) INH and (c) PZA

3.3.4.5 ACID STRESSING

Exact amount of 2 ml of 5.0 M HCL solution was added 8ml of solutions containing rifampicin, isoniazid and pyrazinamide to produce 0.1M solutions. and further were refluxed for 30 minutes. Figure 3.15. represents the HPLC chromatogram and the figure 3.16.(a and b) photodiode-array of the acid stressed sample. However, since rifampicin had degraded completely, hence, concluded that rifampicin is highly sensitive to low pH media.

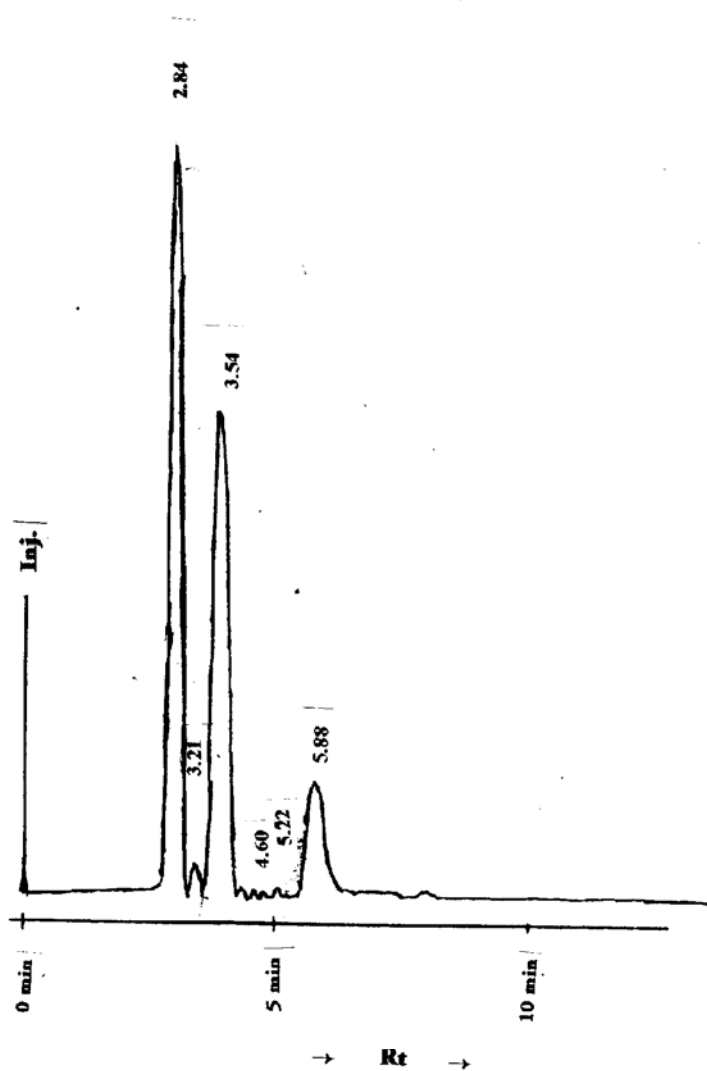
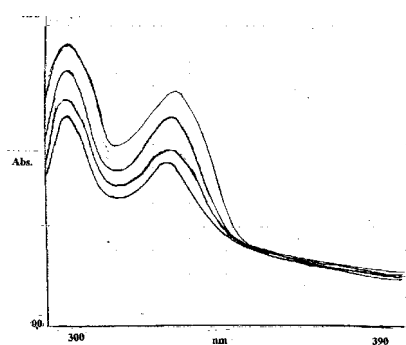


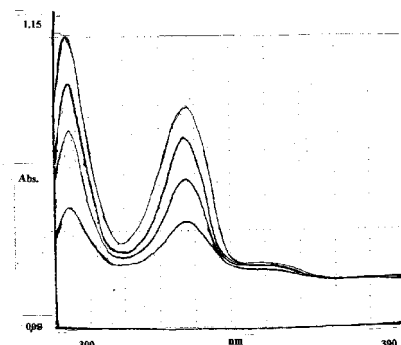
Figure 3.15 The HPLC Chromatogram of Acid Stressed RIF, INH and PZA

Table 3.13 Photodiode-array Scans at Various Retention Times

Photodiode-array Scans Time Region for Figure 3.16		
RIF (Rt)	INH (Rt)	PZA (Rt)
min	min	min
Completely Degraded	2.81-2.82	3.52-3.53
	2.83-2.86	3.54-3.55
	2.88-2.89	3.56-3.59
	2.84-2.85	3.58-3.61



(a)



(b)

Figure 3.16 The Photodiode-array Spectra of Acid Stressed (a) INH and (b) PZA

3.3.4.6 BASE STRESSING

A total amount of 4 ml of 2.5 M NaOH solution was added to 6ml rifampicin, isoniazid and pyrazinamide stock solution respectively and further were refluxed for 30 minutes. Figure 3.17 represents the HPLC chromatogram of the studies and the correlated photodiode-array chromatograms are shown in figure 3.18(a-b). However, rifampicin was completely degraded, the elution pattern of the degradants indicate that the use of this mobile phase in the basic media is not suitable for rifampicin analysis. Therefore, the aqueous media of the pre-formulation studies should not exceed pH 7.0.

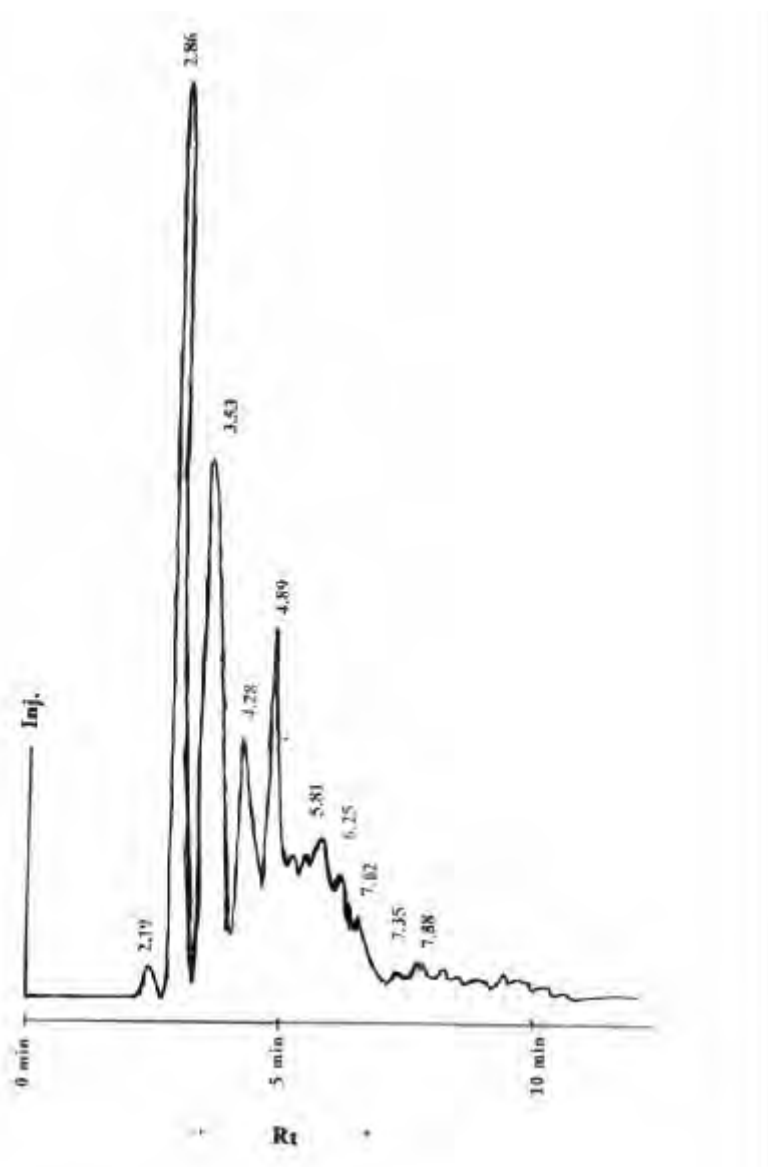


Figure 3.17 HPLC Chromatogram of Base Stressed RIF, INH and PZA

Table 3.14 Photodiode-array Scans at Various Retention Times

Photodiode-array Scans Time Region for Figure 3.18		
RIF (Rt)	INH (Rt)	PZA (Rt)
min	min	min
Completely Degraded	2.81-2.82	Contaminated
	2.83-2.85	
	2.84-2.88	
	2.89-2.90	

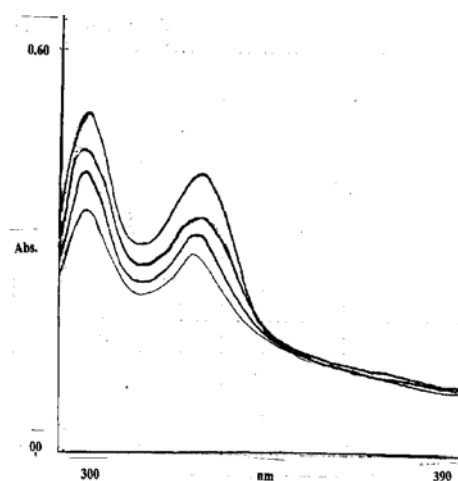


Figure 3.18 The Photodiode-array Spectrum of Base Stressed INH

3.3.5 LIMITS OF DETECTION AND QUANTITATION

Limit of detection (LOD) is defined as the lowest concentration of an analyte that the analytical process can reliably differentiate from background noise level. The LOD for rifampicin, isoniazid and pyrazinamide was determined by diluting the stock solutions of known concentration until the ratio analyte response signal became three times that of the noise whereas the limit for quantitation is the lowest concentration of an analyte which can be measured with an acceptable degree of confidence. The LOQ of rifampicin, isoniazid and pyrazinamide was performed by diluting these solutions until the signal-to-noise ratio was greater than 3 and the accuracy and precision of the response was less than 10%. IUPAC and the serial dilution method are the two methods used for determination of LOQ and LOD [Wahlich et al., 1990].

The IUPAC method of LOD and LOQ determinations were performed using Set (a) HPLC system with the equation 3.2. and the estimations are listed in table 3.15.

$$C(Q) = K(s/S) \dots\dots\dots \text{eq. 3.2.}$$

- where
- C = smallest concentration (three injections)
 - Q = amount
 - K = constant (for LOD, K = 3 and for LOQ, K = 10)
 - s = standard deviation of analytical blank signal (i.e. baseline noise) 20 injections
 - S = slope of response *versus* concentration curve

Serial dilution method was performed by diluting rifampicin, isoniazid and pyrazinamide stock solutions until the set (a) HPLC system was unable to observe the parent peak. The LOD and LOQ were calculated and are listed in table 3.9.

Table 3.15 LOD and LOQ Estimations

LOD and LOQ			
<i>HPLC system</i>	<i>Drug</i>	<i>IUPAC method estimation</i>	<i>Serial dilution method estimation</i>
Set (a)	RIF	0.133µg/ml	0.200µg/ml
Set (a)	INH	0.111µg/ml	0.150µg/ml
Set (a)	PZA	0.137µg/ml	0.150µg/ml
Set (b)	RIF	0.207µg/ml	0.250µg/ml
Set (b)	INH	0.133µg/ml	0.175µg/ml
Set (b)	PZA	0.148µg/ml	0.175µ/ml

3.4. CONCLUSION

An effective formulation of rifampicin, isoniazid and pyrazinamide in combination can only be achieved if the solubility and stability is known. The development and validation of an effective and robust analytical method is necessary to monitor these parameters. Acceptable resolution was observed between the drugs and their degradants. The detector responses were linear over the respective concentration ranges. The above method which is specifically designed for these particular analyses, is sensitive, precise, rapid and involves good robustness. This method can be used for routine analysis of a combination of rifampicin, isoniazid and pyrazinamide in quality control and development laboratories. The same method will be applied for monitoring the solubilities and stabilities of rifampicin, isoniazid and pyrazinamide in chapters 4 to 7.

CHAPTER FOUR

SOLUBILITY AND STABILITY STUDIES

4.1 INTRODUCTION

The objective of the project is a liquid formulation which contains 75, 50 and 250mg of rifampicin, isoniazid and pyrazinamide respectively in not more than a 10ml aqueous-based solvent. The solubility of the drugs has to be investigated and solubilizing agents should be employed if the solubility of a particular drug is found to be below the desired level. The common interpretation of the solubility data is as per the Higuchi and Connors phase-solubility method [Higuchi, *et al.*, 1965]. Moreover, the aqueous stabilities of the drugs should also be examined and stabilizing agents should be employed if the aqueous stability of the drugs is found to be below the regulatory or pharmacopoeial requirement. The stability trials generally followed the ICH and FDA Guidelines [Cartensen; 1995]. In this chapter, the terminologies are reviewed and some of the fundamental but necessary concepts that would facilitate the explanation in chapters 5, 6 and 7 are also discussed.

4.1.1 SOLUBILITY STUDIES

The solubility is a unique characteristic physical property of each chemical component. Higuchi, *et. al.* state that “the equilibrium solubility of a chemical substance in a given solvent, at given temperature and pressure, is a quantity characteristic of the substance, and may, therefore, be utilized as a criterion of identity and purity.” [Higuchi, *et al.*, 1965]. The Higuchi and Connors phase-solubility analysis technique is commonly used in the assessment of the solubility of the chemical component.

A phase is a homogenous portion of matter and there may be more than one phase presented in a pharmaceutical formulation. The active drug ingredients are considered as components which are

constituents of a system whose concentration is capable of independent variation. The phase-solubility technique enables researchers to determine the solubility distributions of various components in a specific phase or in various phases if necessary. When a component exhibits a solubility in excess of that expected for the pure compound, the additional quantity may be expected to precipitate out unless an additional amount of solvent is introduced or an additional quantity of solubilizing agent(s) is (are) applied. For this project, various types of phase-solubility diagrams have to be discussed and these phase-solubility diagrams may be categorized as either non-interacting or interacting [Higuchi, et al., 1965].

4.1.1.1 PHASE-SOLUBILITY OF NON-INTERACTING COMPONENTS

On addition of the non-interacting components at constant temperature and pressure, the resulting solubility diagram is illustrated in figures 4.1. and 4.2. Figure 4.1. represents a constant increment of the component weight as the amount of solvent is increased constantly. The slope obtained shows the solubility of the specific component whereas figure 4.2. illustrates the solubility of each pure component in respect of an additional pure component in the solvent. By definition, the solubility of the component (S) should not be affected when adding a second component (Z) if these two components are non-interactive [Higuchi, et al., 1965].

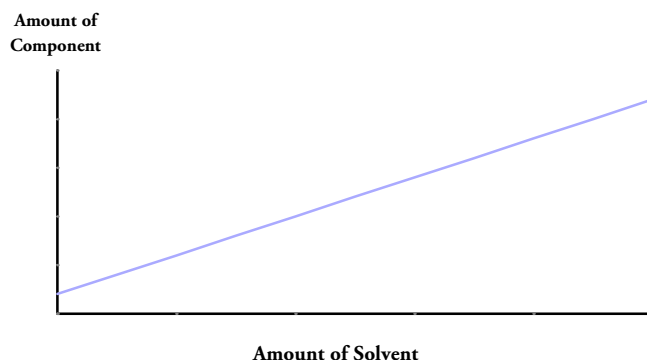


Figure 4.1 Component Versus Solvent Solubility Curve

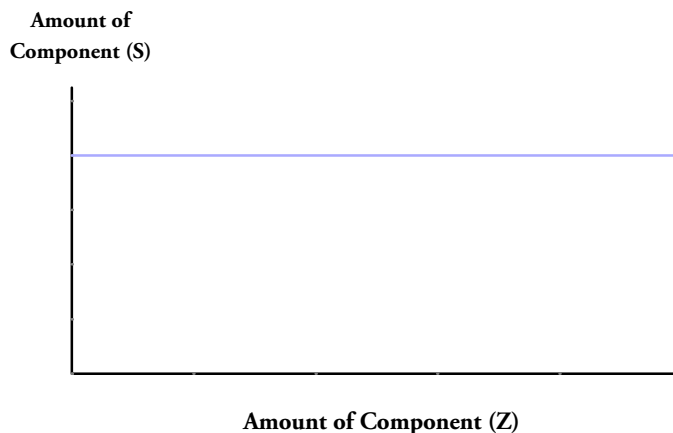


Figure 4.2 Phase Solubility Curve of Non-Interacting Components

4.1.1.2 PHASE-SOLUBILITY OF THE INTERACTING COMPONENTS

Some of the chemicals may not be influenced by each other regarding their solubilities, but in reality, many systems, perhaps most systems do not exhibit such ideal behaviour. Assume a closed system with constant temperature, pressure and the amount of the solute, but for two soluble components, various possible types of solubility phenomena can be found. These possibilities are represented in figures 4.3 to 4.5 and the curves are constructed as follows: plotting the solubility expressed in molar concentration of the first component on the y-axis versus the quantity of the additional second component on the x-axis.

In the presence of the second component, the solubility of the first component can either be enhanced or reduced if interaction occurs. The ideal behaviour of the interacting component is represented in figures 4.3 to 4.5.

Figure 4.3 demonstrates the effect of the component in the presence of a solubilizing agent. The solubility of the component increases in three different ways as the quantity of the solubilizing agent increases and these different types of curves are labelled as A_p , A_S and A_N . It would be desirable if one or more than one solubilizing agent(s) could be found to increase the solubility of rifampicin and/or

pyrazinamide resulting in an A_p curve. Reduction of the solubility of the first component could also occur when adding a second component. The reduction can be expressed in terms of B_S and B_N curves which are demonstrated in figure 4.4. Another event such as demonstrated in figure 4.5 can also occur. All of the above mentioned phase-solubility diagrams may be important for the future interpretation of the results obtained from any necessary phase-solubility experiments [Higuchi, et al., 1965] .

Figure 4.3 shows that the solubility of the first component, designated as S has clearly increased with the addition of the second component, designated as Z. The type A diagrams indicate the formation of soluble complexes between the first component, assigned as S, and the second component Z, thereby increasing the total amount of S in solution. The following statement can be made concerning the type A diagrams: A linear increase in solubility is represented by line A_S , while positive or negative curvature in the line is indicated as A_p and A_N respectively and the explanations are as follows:

1. If all complexes formed follow the thermodynamic first order in respect to Z, which means the components ratio can be simplified as SZ, S_2Z , S_3Z , S_4Z , ..., S_nZ , then a type A_S diagram is expected. The interaction between salicylic acid and tetramethylfumaramide, tetramethylisophthalamide, tetramethylsuccinamide or tertamethylphthalamide [Higuchi, et al., 1965] are a few examples.
2. The type A_p diagram will be produced if complexes formed are higher order than those in Z, such as SZ_2 , SZ_3 , SZ_4 , SZ_5 , ..., SZ_n . The interaction of oxytetracycline and nicotinamide is one of the well known examples of a type A_S interaction [Higuchi, et al., 1965] .
3. The origin of the type A_N is uncertain and this may be due to the alteration in the nature of every particular solvent in the presence of large concentrations of S or Z , thus leading to change in the complex formation constant. Self-association of components at higher concentrations may also effect the complexation [Higuchi, et al., 1965].

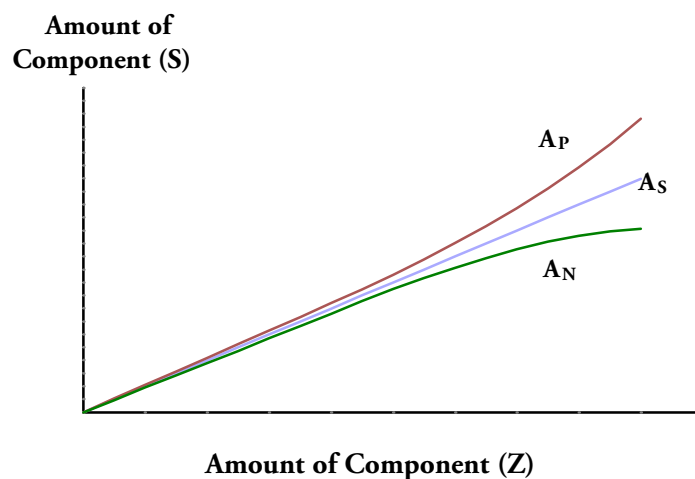


Figure 4.3 The Solubility Enhancement Phase-Solubility Curves

The second major class of phase-solubility diagrams is illustrated by the curves in figure 4.4 which demonstrates the reduction of the solubility of the first component by the presence of the second component. The curves B_S and B_N , follow the exact same principle as A_S and A_N , except instead of forming a soluble complex like the type A curves, the type B curves are the result of insoluble complex formation between the two components [Higuchi, et al., 1965].

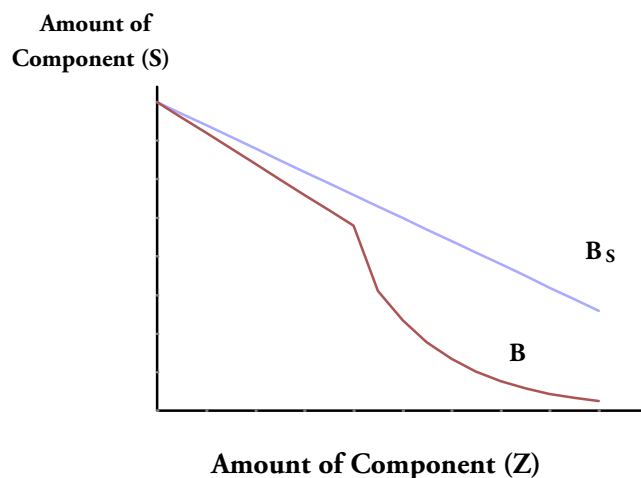


Figure 4.4 Two Different Types of Solubility Reduction

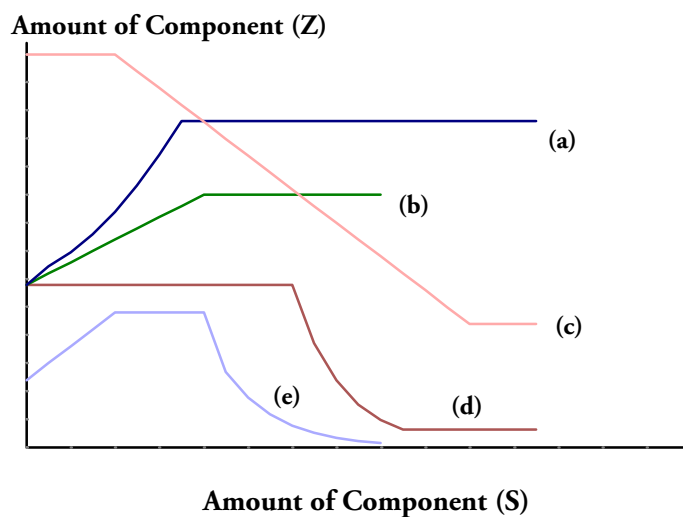


Figure 4.5 Few of the Possibilities of Plateau Exhibition

The type A diagram sometime may exhibits a plateau [in (a) and (b)of Figure 4.5]. This is mainly due to the solubility of the S and Z complex in the system having reached the maximum level. The interaction between caffeine and benzocaine clearly demonstrat the case (a), whereas the interaction phase-solubility curve of theophylline and 1-hydroxy-2-naphthoate follows the case (b) behavior. A type B curve can sometimes be followed after the formation of the plateau such as in the case of (c), the interaction between sacrosine anhydride against 2,5-dihydroxybenzoic acid, (d), the interaction between 2,5-dihydroxybenzoic acid and butyl theophylline and (e), the interaction between 1,3-dimethylbenzoyleneurea and catechol which are the best examples. This is due to the change in the complexation between the two components and the occurrence of the insoluble precipitates. Sometimes the formation of the second plateau can be found after the solubility reduction such as in case (c). [Higuchi, et al., 1965] .

4.1.1.3 GENERAL PHASE-SOLUBILITY METHODOLOGY

The experimental protocol of each of the phase-solubility studies will be specified in each section, but in general, the method consists of two steps:

Step (1)

- (i) 20ml water was saturated with the drug while constantly shaking for a period of 72 hours at $25 \pm 2^{\circ}\text{C}$ in a shaker water bath.

or

- (ii) The solubilizing/suspending agents were accurately weighed out and dissolved in 20ml of water before saturating with the drug(s) and shaken for a 12 hours at $25 \pm 2^{\circ}\text{C}$. This method, applicable to all the solubility studies is based on the experimental observation of sections 4.2.1 and 4.2.2 Section 4.2.1 has shown that the aqueous solubility of the drugs reached approximately 95% of the maximum level within 12 hours and in the same period of time, the degradation reached about 5% for rifampicin and 1% for isoniazid and pyrazinamide. This method allowed maximum drug dissolution, yet without generating too many degradants compared to a 72-hour shaking period.

Step (2) Applicable to all solubility studies

All the flasks were covered tightly to prevent evaporation of the solution. A layer of tin foil was further applied to prevent any light penetration causing unwanted drug photodegradation. The Whatman[®] filter papers (W&R Balston, UK) were saturated (by immersing the filter paper into the sample solution) before filtering the sample solution to avoid adsorption. The samples were analyzed within 6 hours to prevent any additional degradation taking place, since the sample starts to degrade after 8 hours, based on the experimental observation of section 3.4.4. Each of the solubility experiments were conducted at least in triplicate. The results are summarized and expressed as the curves indicating either the amount of drug (in mg) dissolved against time (for the type (i)

solubility studies) or the amount of drug (in mg) dissolved against the amount of solubilizing agent/suspending agent used (in %) (for the type (ii) solubility studies).

4.1.2 STABILITY STUDIES

The objective of stability testing is to provide information on how the quality of a formulation varies with a period of time under the influence of several environmental factors, such as pressure, temperature, light and humidity, hence enabling a reasonable recommendation to be made. In the pharmaceutical field, the term “stability” comprises several concepts which include both the chemical and physical properties of a specific formulation. However, in the pre-formulation stage, the stability studies target only the chemical stability of the active drug ingredients which in this case are rifampicin, isoniazid and pyrazinamide.

During the pre-formulation stage, the normal and accelerated stability studies were performed. The intention behind the normal studies is to show whether the drug substance will remain within specification during a certain recommended time period, termed as the re-test period, while under the recommended storage conditions. The accelerated studies are defined as the studies undertaken to demonstrate the stability characteristics of the drug substance under the influence of various extrinsic factors. These studies are performed on the samples from the same batch under various conditions which include temperature increments as an accelerating factor. Accelerated studies are conducted to provide a set of data which enable the characterization of the intrinsic stability of the drug substance, establishing degradation pathways, evaluating the rate kinetics and identifying the basic decomposition products termed as the degradants.

The *in vitro* degradations of rifampicin, isoniazid and pyrazinamide in aqueous media have not been determined nor reported. Sections 4.1.2.1 to 4.1.2.2 report on the degradation in an aqueous medium.

4.1.2.1 RIFAMPICIN DEGRADATION PATHWAY PREDICTION

During the metabolism of rifampicin, three routes of degradation are suggested in figure 4.6. Rifampicin may undergo reversible hydrolysis to yield 3-formylrifamycin SV [a.1.]. This degradation occurs readily in the presence of hydrogen atoms, desacetylrifampicin [a.2.] is the lost of the C-25 acetate. Both of these degradants still act as an antituberculars agents. However, in the presence of oxygen, rifampicin is oxidized to form rifampin quinone [a.3.] which is inactive, due to the fact that the free hydroxyl groups on the C-1 and C-8 are essential for the binding of the drug to the bacteria [Foye, et al., 1995]. These routes of rifampicin degradation (termed scheme (I) degradation) could possibly be present within the aqueous media. The four oxygen atoms at the C-1, C-8, C-21 and C-23 positions of rifampicin serve as the essential structural activity requirement to bind with the RNA-polymerases of *M. tuberculosis* to prevent further cell multiplication but the azomethine-piperazine side chain serves only to increase the aqueous solubility of rifampicin. [Hansch, et al., 1990].

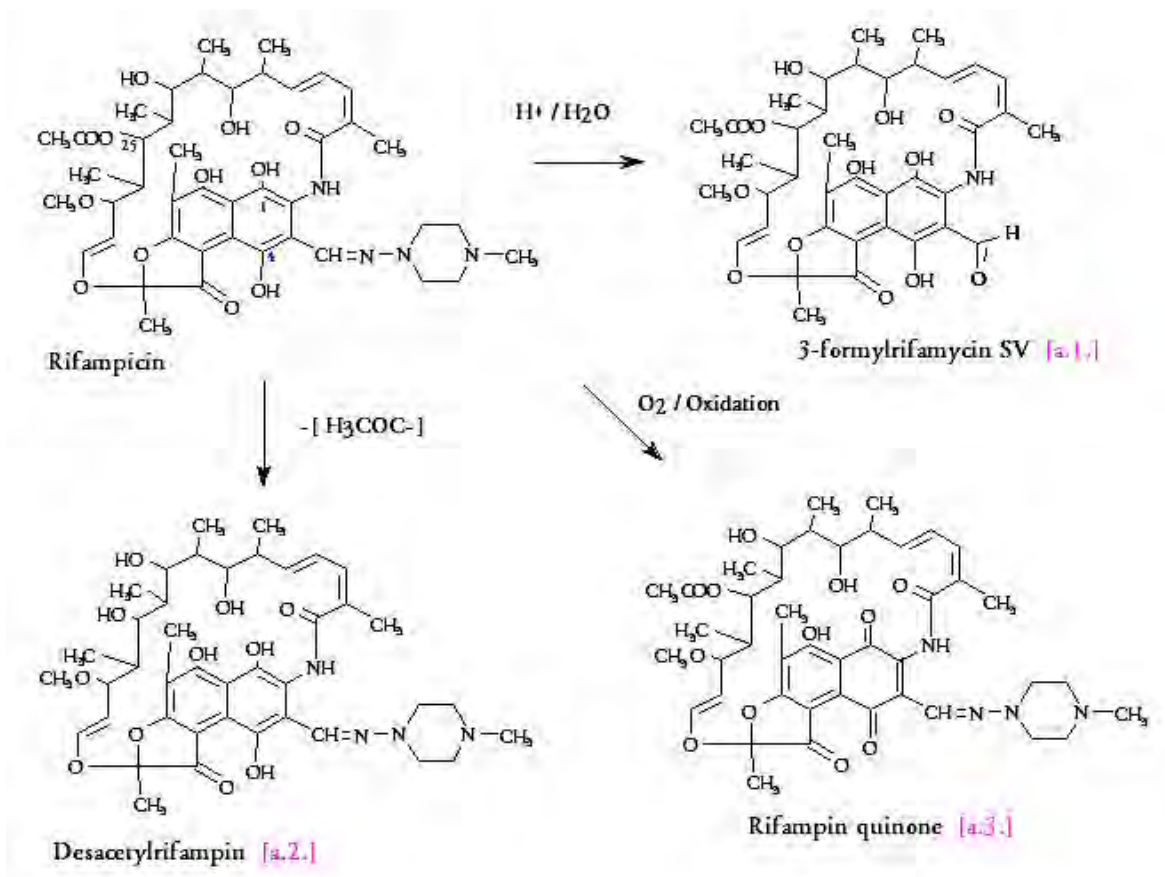


Figure 4.6 Rifampicin Metabolic Pathway [Degradation Scheme (I)]

The mass spectral fragmentation of rifampicin also suggests an alternative route of rifampicin degradation [listed in the figure 4.7 scheme (II)] and both routes of degradation can take place. However, Scheme (II) degradation can occur when the highly energized electrons occur as an external initiating factor, whereas the Scheme (I) degradation of rifampicin can occur naturally in the aqueous solvent with atmospheric oxygen present or just the H^+ and the OH^- ions. Therefore, scheme (I) degradation should take preference. But the whole process is still not certain unless the degradants are isolated.

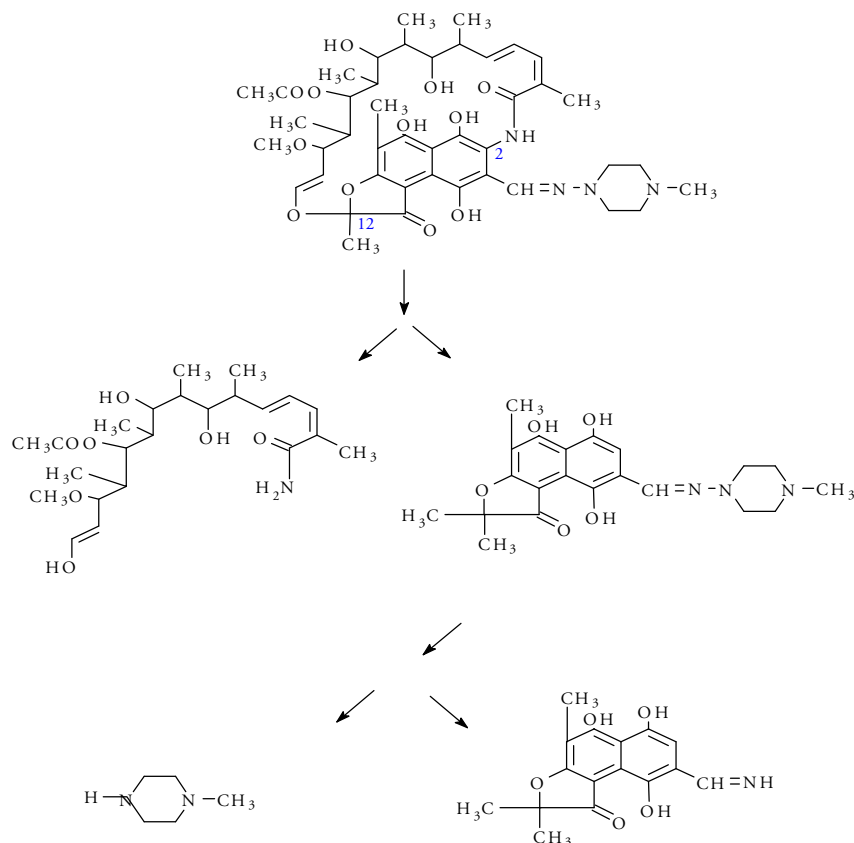
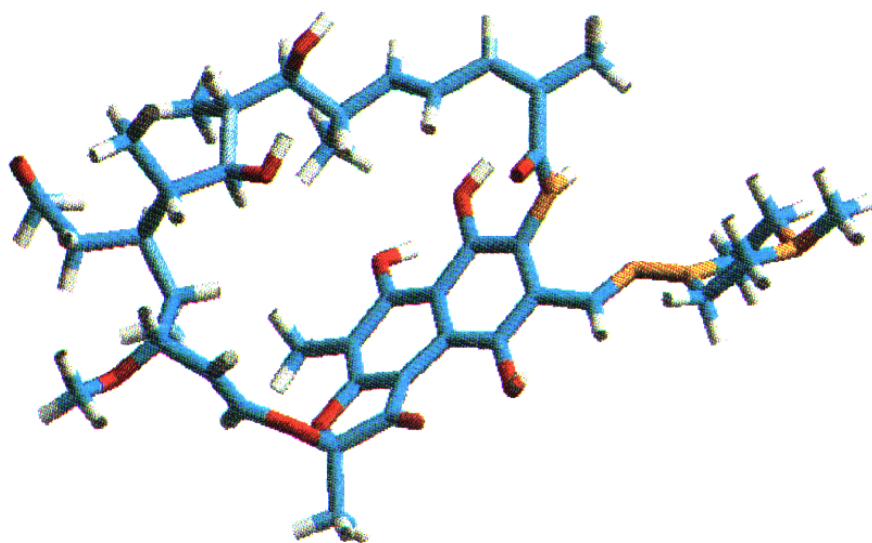


Figure 4.7 Rifampicin Mass Spectral Fragmentation [Degradation Scheme (II)]

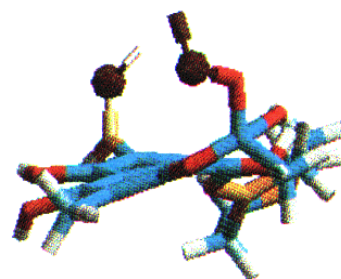
Force field molecular simulation is a sophisticated tool which allows the scientist to investigate various intricate problems. The Cerius[®] II Force Field Simulation program was used to simulate the 3 dimensional models of the drugs. The structure of the drugs was manually constructed and investigation of the model with the lowest potential energy was carried out by a 100,000 frames simulation. The results are illustrated in figures 4.8, 4.11 and 4.12. Figure 4.8(a). shows a cylindrical model of the lowest energy conformation of rifampicin when stored at 25EC. Figure 4.8(b) and (c). represent the conjugated

aromatic rings of rifampicin. Although the sp_2 orbitals preferred to be planer, these aromatic rings appear to be highly distorted. The oxidation route of figure 4.6 results in a quinone [a.3.] and the cleavage of the C1 and C4 carbonyl groups on the quinone [a.3.] could release the torsional strain. Therefore, the oxidative rifampicin degradation appears to be logical. (Keys: Blue =Carbon, Red = Oxygen, Orange= Nitrogen, Whitel/Light Grey = Hydrogen, Brown = R' and R")

(a)



(b)



(c)

Figure 4.8 Computer Generated Models of Rifampicin (a) whole molecule, the Conjugated Aromatic Rings (b) "Side" View and (c) "Back" View

4.1.2.2 ISONIAZID AND PYRAZINAMIDE DEGRADATION PATHWAY

The major degradation pathway of isoniazid in the presence of aqueous solvent should involve the cleavage of hydrazine which is one of the isoniazid metabolic pathways [Foye, et al., 1995]. The degradation yields the isonicotinic acid [b.1] which may further breakdown to a pyridine base [b.2].



Figure 4.9 Isoniazid Degradation Pathway

Pyrazinamide should degrade in a similar manner as isoniazid. In the presence of water, pyrazinamide should form pyrazinecarboxylic acid [c.1], sometimes referred as the pyrazinoic acid, which may further lose its carboxylic group to yield pyrazine [c.2] [Foye, et al., 1995].

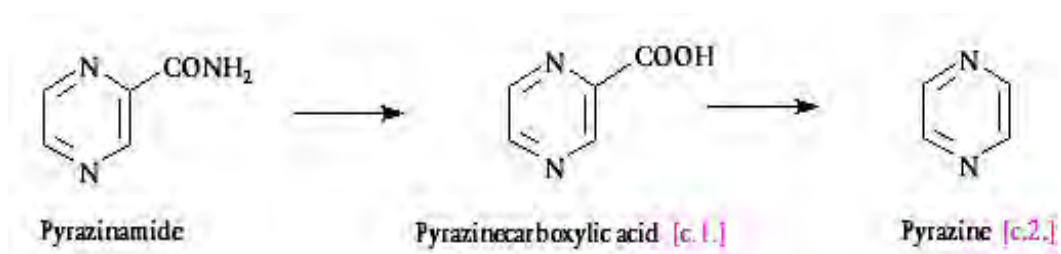


Figure 4.10 Pyrazinamide Degradation Pathway

The carboxylic degradants ([c.1] and [b.1]) of isoniazid and pyrazinamide have the bactericidal activity and the nitrogen atom serves only as the lipophilicity enhancement which allows the drugs to be transported through the waxy layer of the bacterial membrane [Hansch, et al., 1990].

The molecular models of isoniazid and pyrazinamide were generated by the Cerius® II Force Field Simulation. At 25EC, the conformations of isoniazid and pyrazinamide with the minimum energy are demonstrated in figures 4.11 and 4.12 with both top and side view of the molecules. Both isoniazid and pyrazinamide appear to be flat and therefore the torsional strain does not play a part in the degradation of these two drugs.

Keys: Blue = Carbon, Red = Oxygen, Orange = Nitrogen, White/Light Grey = Hydrogen

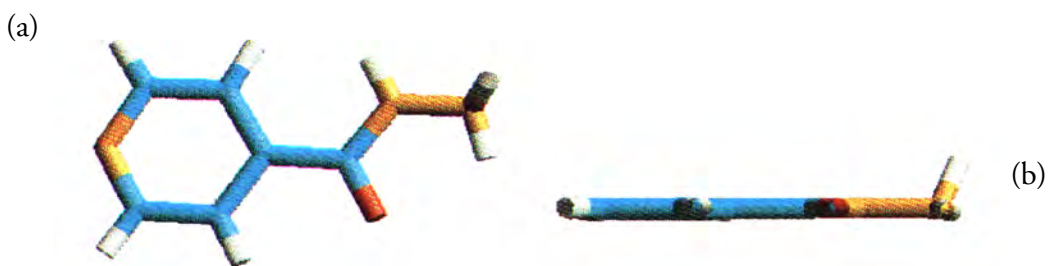


Figure 4.11 Computer Generated Models of Isoniazid (a) “Top” View and (b) “Side” View



Figure 4.12 Computer Generated Models of Pyrazinamide (a) “Top” View and (b) “Side” View

4.1.2.3 *GENERAL STABILITY STUDIES METHODOLOGY*

Both the ICH and FDA indicate that the stability indicating methodology should be properly validated and described in sufficient detail to permit validation by the ICH and FDA laboratories. The FDA guidelines further state that generally for the oral solution or suspension to meet the regulation, various characteristics such as the appearance, pH, colour, odour, should be examined. Viscosity and redispersibility of the formulation are the two important physical considerations for a suspension. The formulation should fulfill the requirements of 90% of drug remaining for 12 months on storage at 25EC \pm 2EC and 60 \pm 5% relative humidity and 6 months when subjected to a condition of 40EC \pm 2EC with 75 \pm 5% relative humidity [Cartensen, 1995]. The former specification provides the long term stability data and the latter specification is considered as an accelerated test which provides the assessment of the possible damage caused by occasional excursions from the kinetic mean temperature. However, for the accuracy and convenience of this project, the solutions/suspensions will be stored in closed ampoules which are not affected by the relative humidity factor. The samples were thus tested at 25EC \pm 2EC, 40EC \pm 2EC, and 60EC \pm 2EC dry heat environment. The 60EC \pm 2EC accelerated condition may provide further information on the drug stability at high temperatures since the temperature may exceed 40EC in some areas of Africa. The samples were stored (in triplicate) at these temperatures for 7 days and the analyzed daily by HPLC (chapter 3) to determine the percentage of the drug(s) remaining in order to establish a stability-time curve.

4.1.3. KARL FISCHER METHOD FOR ANALYZING THE WATER CONTENT

The moisture in the air can be absorbed by the solubilizing/stabilizing agents resulting in an error in the phase-solubility or stability experiment. The Karl-Fischer method was employed to determine the water content of each solubilizing/stabilizing agents before any experiment was undertaken. The principle behind the Karl Fischer method for water determination is just a simple titration. The water is converted stoichiometrically in the presence of sulfur dioxide, methanol and a base by addition of iodine. Use of a Mettler DL18 Karl Fischer titrator (Mettler, Switzerland) allowed the analysis of trace amount of water content in parts per million. The instrument combines the advantage of both volumetric and coulometric titration to produce a very accurate result.

4.2 EXPERIMENTAL RESULTS AND DISCUSSION

4.2.1 BASIC SOLUBILITY STUDIES

20ml of water was saturated with the rifampicin, isoniazid or pyrazinamide while constantly shaking for a period of 72 hours at $25 \pm 2^{\circ}\text{C}$ in a shaker water bath. The experimental data were obtained by HPLC analysis for a 72-hour period. The dissolution curves of the saturated rifampicin, isoniazid and pyrazinamide solutions are presented in figures 4.13 to 4.15. It was found that within 12 hours the drugs dissolved to the maximum concentration without initiating a substantial amount of degradation. Therefore the other subsequent solubility studies (from chapters 5 to 7) will employ the 12 hour saturation method.

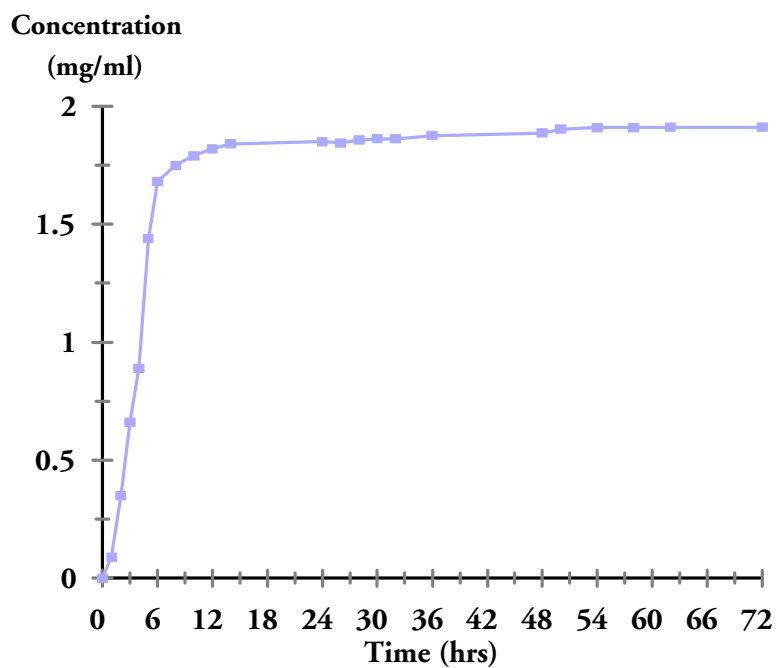


Figure 4.13 Solubility-Time Curve of Rifampicin

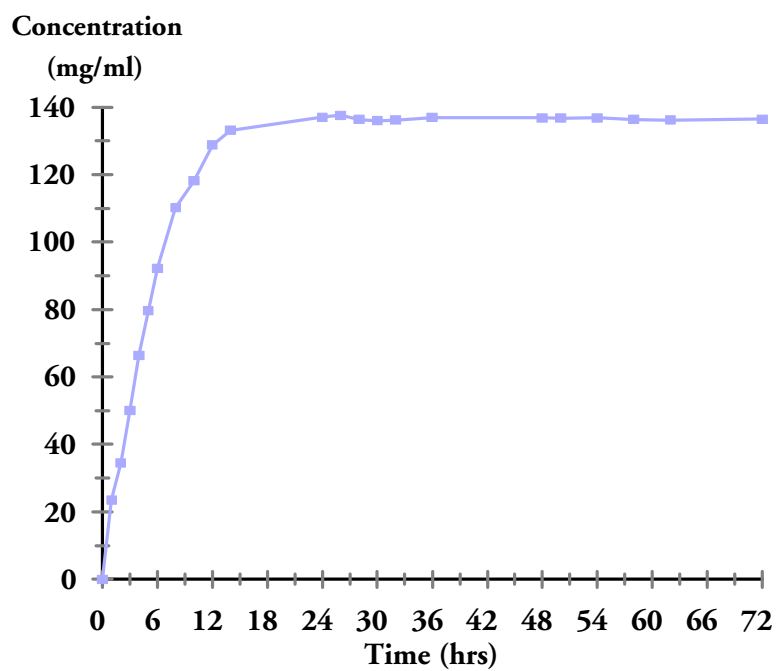


Figure 4.14 Solubility-Time Curve of Isoniazid

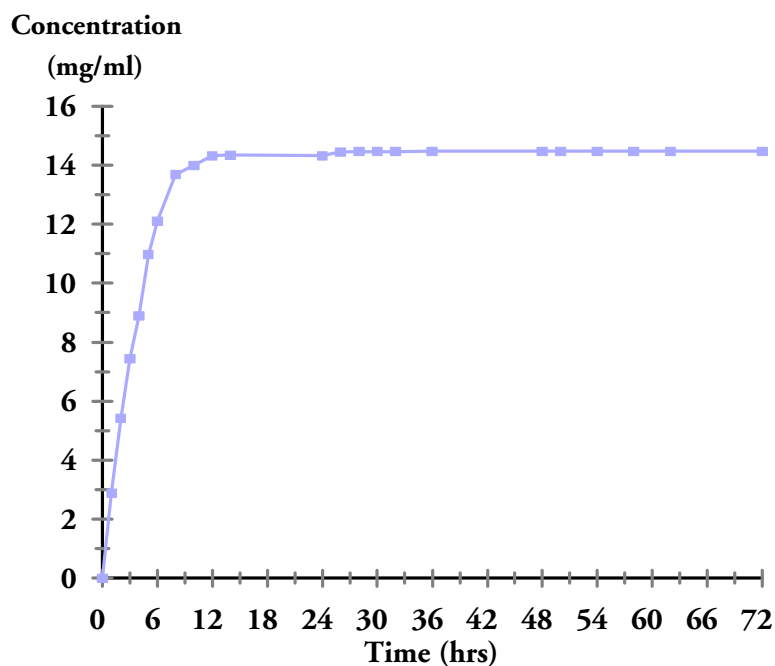


Figure 4.15 Solubility-Time Curve of Pyrazinamide

4.2.2 SOLUBILITY STUDIES OF THE DRUGS IN AT VARIOUS pHs WITH OR WITHOUT THE PHOSPHATE BUFFER

In case of oral formulations, the drug is expected to be exposed to a range of pHs in the body. The high basicity of the duodenum and the highly acidic environment in stomach may enhance the degradation of the drug to a certain extent. In this section, the effect of altering pH of the solvent is investigated. Not only would the results indicate the extent of absorption, the data would also indicate the suitable pH for the maximum drug solubility and stability and may therefore may be beneficial to the future formulation studies. Addition of buffering agents were also investigated. These experiments should provide a basic idea on the solubility and stability profiles of each individual drug in the presence of the buffering agent.

A total amount of 0.72g of disodium hydrogen orthophosphate dodecahydrate was accurately weighed and dissolved in a 1000ml volumetric flask with water to produce a 0.02M buffer solution. A Beckman M32 pH meter (Beckman, USA) was used the pH values measurement when adding dropwise hydrochloric acid (HCl) or sodium hydroxide (NaOH) into Na₂HPO₄ buffered solution to achieve a range of pH values from 2-10. The effect of the unbuffered solvent was also examined. Drugs were added to these solvents until saturation and analyzed by HPLC . The data are listed in table 4.1.

Table 4.1 Solubilities of Rifampicin, Isoniazid and Pyrazinamide in Various pH Buffered Solutions and Unbuffered Water at pH 7.1

Solubility of the Drugs at Various pH Values			
	Solubilities (mg/ml)		
Buffered Solvent	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
pH = 2.00 ± 0.01	83.23 ± 0.10	130.22 ± 0.80	14.26 ± 0.12
pH = 3.00 ± 0.01	2.75 ± 0.04	131.14 ± 0.55	14.22 ± 0.36
pH = 4.00 ± 0.01	2.36 ± 0.08	130.61 ± 0.93	14.36 ± 0.47
pH = 5.00 ± 0.01	2.35 ± 0.05	129.56 ± 0.33	14.42 ± 0.33
pH = 6.00 ± 0.01	2.31 ± 0.02	129.58 ± 0.98	14.28 ± 0.33
PH = 7.00 ± 0.01	2.18 ± 0.04	128.26 ± 1.01	14.39 ± 0.66
pH = 8.00 ± 0.01	3.56 ± 0.07	130.22 ± 0.08	14.29 ± 0.50
pH = 9.00 ± 0.01	4.16 ± 0.03	127.45 ± 0.22	14.41 ± 0.14
pH = 10.00 ± 0.01	5.39 ± 0.08	129.45 ± 0.41	14.32 ± 0.25
Unbuffered			
pH = 7.01 ± 0.01	1.82 ± 0.02	128.80 ± 0.66	14.33 ± 0.40

4.2.3 STABILITY STUDIES OF THE DRUGS AT VARIOUS pHs WITH OR WITHOUT THE PHOSPHATE BUFFER

0.05mg/ml drug solutions at different pH values were used for the stability studies and analyzed by HPLC.

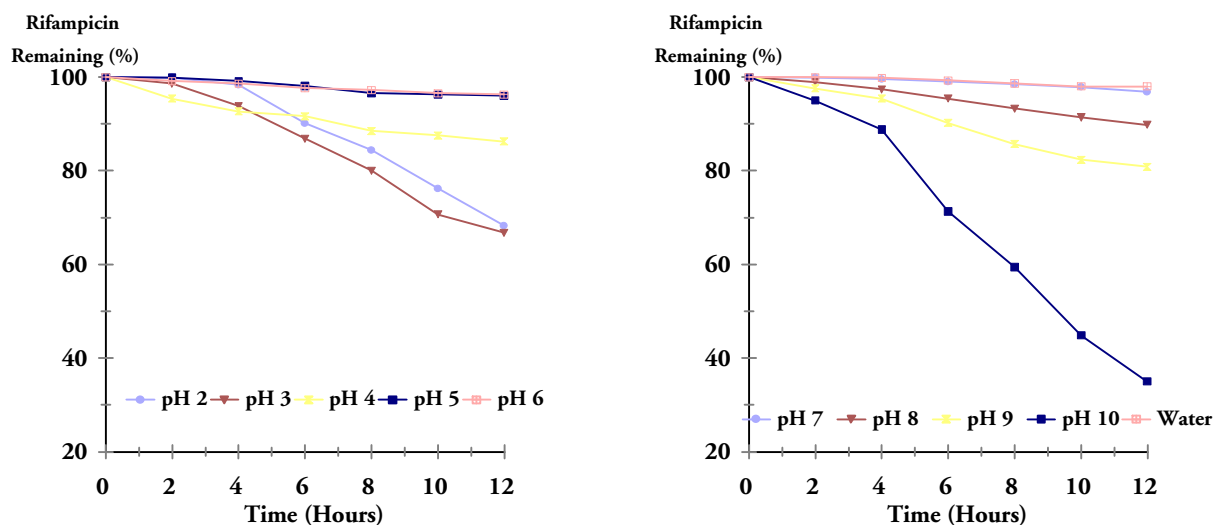


Figure 4.16 Rifampicin pH Stability Profiles

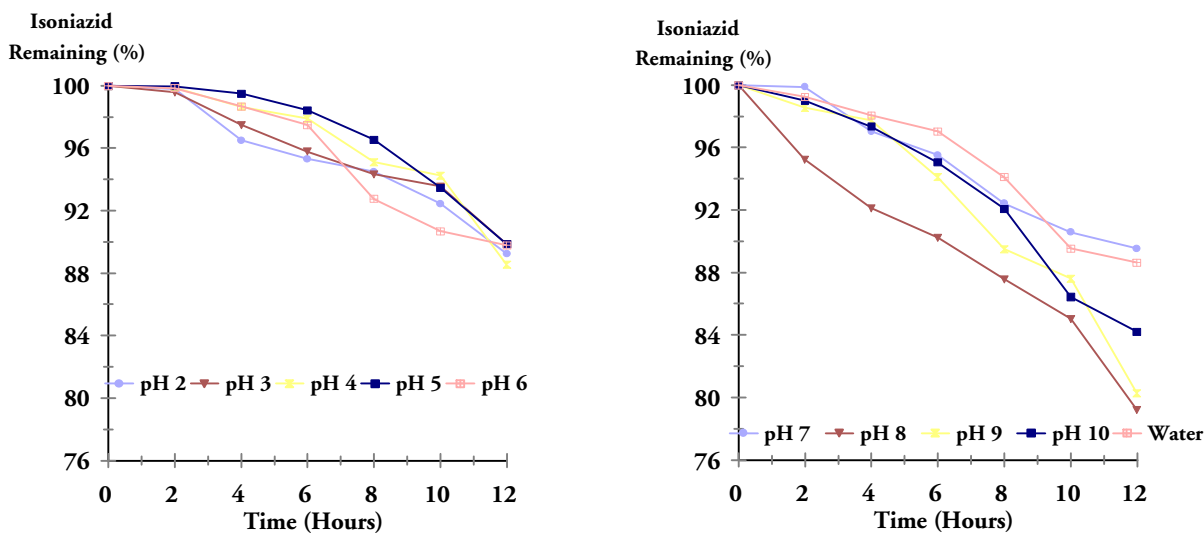


Figure 4.17 Isoniazid pH Stability Profiles

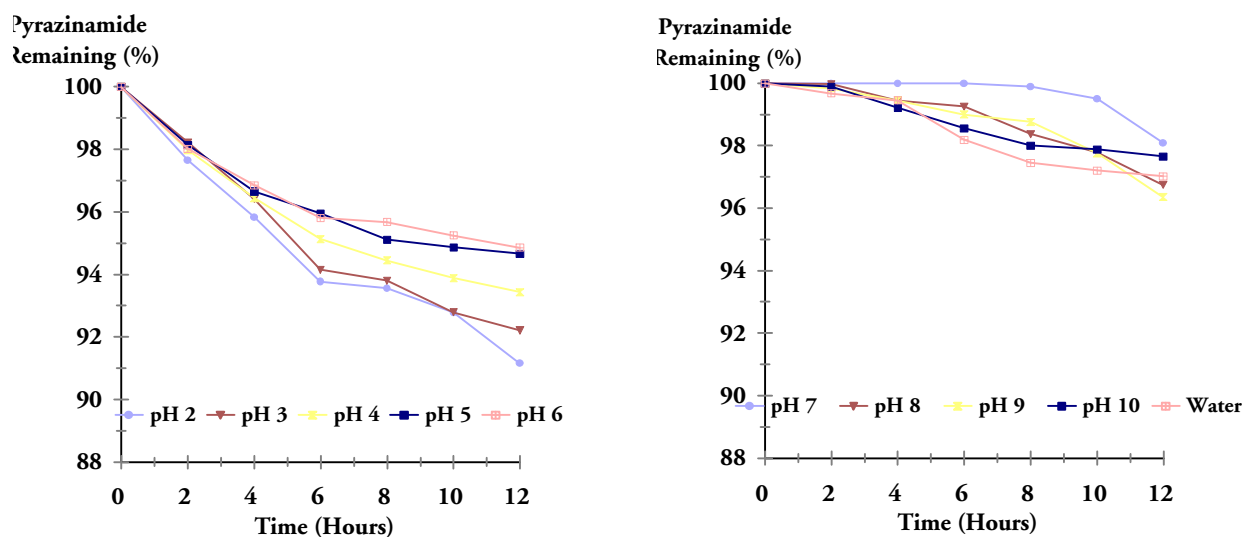


Figure 4.18 Pyrazinamide pH Stability Profiles

4.3 CONCLUSION

The aim of this chapter was to put forward a few of the fundamental but important concepts which would facilitate the presentation of chapters 5 to 7. These concepts were obtained from the Higuchi and Connors phase-solubility method [Higuchi, 1965] and the ICH and FDA Guidelines [Cartensen, 1995].

In sections 4.1.2.1 to 4.1.2.2, the predicted major degradation products of rifampicin were 3-formyl rifamycin SV [a.1.] and 1-amino-3-methyl piperazine when stored in the acidic medium and it may also oxidize with the atmospheric oxygen in the presence of OH⁻ ions yielding a rifampicin quinone [a.3.]. No specific report was found concerning the effect of altering solvent pH on isoniazid and pyrazinamide stabilities. However, there is a possibility of an amide to yield carboxylic acid while undergoing hydrolysis [McMurry, 1992], isoniazid and pyrazinamide could be hydrolyzed to isonicotinic acid [b.1.] and 2-pyrazinecarboxylic acid [c.1.], respectively, through either acidic or basic hydrolysis.

For the solubility studies, it was found that the optimum dissolution period was approximately 10-12

hours for the drugs to reach a equilibrium solubility, without generating excessive degradants. Any shorter period of time could result in insufficient dissolution, while a longer period of time would initiate an extra amount of the unwanted degradants. The experiment provides the optimum time period for phase-solubility studies.

A quantitative study of rifampicin stability in solvents at various pH values has been reported by Jindal, *et. al.* and Prankerd, *et. al.* for a narrow range (pH 2 to 9) and in the presence of ascorbic acid as an antioxidant. Their results indicate that rifampicin is most stable at pH 5 [Prankerd, et al., 1992; Jindal, et al., 1994]. This coincides with the experimental data which clearly show that rifampicin yields a maximum stability while stored in solvents with a pH of 5 to 7. This is due to the fact that the rifampicin has the pK_a value of 1.7 and pK_b value of 7.9.

Isoniazid has three pK_b values, 2.0, 3.5 and 10.8. This explains why isoniazid degraded slightly faster in the acidic medium than the mild alkaline environment. The stability of pyrazinamide was found to be relatively similar throughout the experimented pH range. This may due to the fact of pyrazinamide has a pK_b of 0.5 and the value is already below the pH range experimented, therefore no substantial the difference between the profiles occurred. Overall, pyrazinamide was found to be much more stable than isoniazid in aqueous medium.

It was also observed that the disodium hydrogen phosphate buffer did not have any significant effect on the drug stability, although Jindal, *et al.* [Jindal, et al., 1994] report that the phosphate buffer has an adverse effect on rifampicin stability. It is therefore acceptable to perform further solubility and stability studies while using unbuffered water as the solvent.

Rifampicin, isoniazid and pyrazinamide were found to be more stable in water, however, their solubility profile indicated that the solubility of rifampicin and pyrazinamide was below the desired concentration. Only about 18.2 mg of rifampicin and 143,3mg of pyrazinamide will dissolve in a 10ml aqueous formulation where as 75mg of rifampicin and 250mg of pyrazinamide are the amounts required for each dose. Therefore, certain solubilizing agents should be incorporated and it would be desirable that these agents act in enhancing the stability.

CHAPTER FIVE

SOLUBILITY AND STABILITY STUDIES OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE IN THE PRESENCE OF VARIOUS SURFACTANTS

5.1 INTRODUCTION

In the previous chapter, the solubility of rifampicin and pyrazinamide were found to be below the required dosage. Therefore, incorporating a solubilizing agent could be a solution to the problem. A surfactant is the term used to describe chemicals having surface-active properties and is commonly employed to increase the aqueous solubility of the less polar chemicals. Surfactants consist of a very diverse group of chemicals which are widely used within the pharmaceutical sciences for various functions in different formulations. However, due to the large variety of surfactants, only the three surfactants requested by Pharmacare-Lennon[®] were chosen as the solubilizing agent. The solubility and the stability of the drugs in the presence of these surfactants was studied.

The chemical structure of surfactant is characterized by the presence of both hydrophilic and lipophilic groups. The stronger hydrophilic properties are contributed by the functional groups such as $-\text{OSO}_2\text{ONa}$, $-\text{COONa}$ and $-\text{SO}_3\text{Na}$. The weaker hydrophilic groups include $-\text{OH}$, $-\text{O}-$, $-\text{C}=\text{O}$, $-\text{CHO}$, $-\text{NO}_2$, $-\text{NH}_2$, $-\text{NHR}$, $-\text{N}_2\text{R}$, $-\text{CN}$, $-\text{CNS}$, $-\text{COOH}$, COOR , $-\text{OPO}_3\text{OH}_2$, $-\text{OS}_3\text{O}_2\text{H}$, $-\text{Cl}$, $-\text{Br}$ and $-\text{I}$. These groups may interact with the polar part of the drugs, whereas the hydrocarbons such as the alkyl, aryl and alicyclic groups are excellent hydrophobic functional groups. These groups are lipophilic and may interact with the non-polar part of the drugs. For a surfactant to function properly, there should be a fine balance between the hydrophilicity and the lipophilicity within the molecule itself. Variation of the two groups may result in a significant activity change [Mathews et al., 1990; McMurry, 1992 and Wilson et al., 1977] .

Surfactant molecules may disperse themselves at the interface where the liquid phase meets another phase. The adsorbed surfactant may form a monomolecular film at the liquid-liquid interface of immiscible liquids. The surfactant molecule may then direct its hydrophilic portions towards the aqueous phase and surround the non-aqueous liquid with the lipophilic end. This phenomenon reduces the interfacial tension between the two liquids. These types of the surfactants are considered as a solubilizing agents [Mathews et. al, 1990 and Wilson et. al, 1977].

During the degradation of rifampicin, carbocations were formed in the intermediate stage. Hammond postulated that any factor which may have stabilizing effect on the carbocation should increase the rate of the reaction. The stability contribution properties of solvents have not been fully characterized. It is the relationship to the polarity which is generally expressed as the dielectric constant that indicates the ability of a particular solvent to behave as an insulator of electric charges. The solvent-drug molecule interaction may play an important role in either stabilizing or destabilizing of the drugs, especially in the case of rifampicin (refer to 4.1.2.1) Solvents with higher dielectric constants (with water having a dielectric constant of 80.4) tend to result in more degradation and vice versa. [McMurry, 1992] Incorporating surfactants could decrease the dielectric constant of the solvent and possibly could lead to the successful development of a paediatric formulation. For the purpose of this project, only the solubilizing surfactants which may enable the reduction of the dielectric constant, ϵ , of the solvent are taken into consideration.

Poloxamers, the solubilizing agents investigated belonging to the nonionic surfactants showed a reduction of the solvent dielectric constant. This class is the poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) triblock copolymers which are sometimes designated as the POE-POP-POE with the formula of $\text{H}(\text{O}-\text{CH}_2-\text{CH}_2)_a-(\text{O}-\text{CHMe}-\text{CH}_2)_b-(\text{O}-\text{CH}_2-\text{CH}_2)_a-\text{CH}_3$. Poloxamer is fairly tasteless and may be used orally (about 250mg) as a laxative [USPDI, 1991]. The ethylene and propylene groups serve as the lipophilic functional groups, while the high electronegativity oxygen atoms behave as the hydrophilic groups. These oxyalkylene polymers have a unique way of being designated where the name is always followed by a number code. The first two digits, when multiplied by 100, correspond to the approximate average molecular weight of the polyoxypropylene portion of the molecule and the third digit, when multiplied by 10, corresponds to the percentage by weight of the polyoxyethylene portion [Reynolds et. al, 1989]. The two poloxamers used, poloxamer 188 and 407 (BF Goodrich, Germany) are also known as Lutrol[®] F68 and F127

commercially. Poloxamer 188 and 407 have the POE chain of 12 and 67 and the molecular weight of 8350 and 11500, respectively. They are freely soluble in aqueous solvents.

2,4-hexadienol, also known as sorbitol (Elabtec, R.S.A.), is a surfactant with a slightly weaker solubilizing effect since it is an unsaturated hydrocarbon compound. Its chemical formula is $\text{CH}_3\text{CH}=\text{CHCHCOOH}$. This polyhydric alcohol has only half the sweetening power of sucrose and it occurs naturally in many fruits and vegetables. Sorbitol can be prepared commercially by the reduction of glucose and acts as a bulk sweetening agent. [Reynolds et. al, 1988]

Sorbitol has humectant and stabilizing properties and is widely employed in various pharmaceutical and cosmetic products. This creamy white powder with a faint characteristic odour is soluble in most solvents. The approximation of solubilities at 20°C is as follows, 700 parts in water, 10 parts in alcohol, 20 parts in ether and 150 parts in fats or fatty acids [Reynolds et. al, 1988].

Sorbitol is poorly absorbed from the intestinal tract following oral administration. It is metabolized by sorbitol dehydrogenase in the liver to fructose or converted directly by aldose reductase to glucose. It is therefore not hazardous to humans. Some of the excipients may well cause fungal contamination by promoting fungal growth, hence, applying sorbitol may be beneficial since it has a pK_a of 4.8 and has fungistatic effect even at the concentration as low as 0.05 percent. However, with a high dose of approximately 20 to 50g, sorbitol acts as an osmotic laxative.

In this chapter, the solubilities and the stabilities of rifampicin, isoniazid and pyrazinamide (and the different combination of these drugs) in the presence of poloxamer 188, poloxamer 407 and sorbitol will be studied with the intention of producing a 10ml aqueous formulation that contains 75mg of rifampicin, 100mg of isoniazid and 250mg of pyrazinamide

5.2 EXPERIMENTAL RESULTS AND OBSERVATIONS

5.2.1 KARL FISCHER EXPERIMENT

The water content of poloxamer 188 and 407 (BF Goodrich, Germany) was determined by Karl Fischer titration (refer to section 4.1.3.) with n=5 analyses. The mean water content for poloxamer 188 and 407 was approximately 3.2 and 4.9% (w/w). All the experiments related to poloxamer 188 and 407 were calculated accordingly. A 70% sorbitol solution (Elabtec, R.S.A.) was used in all experiments.

5.2.2 SOLUBILITY STUDIES OF RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE POLOXAMER 188

In order to produce a cost effective formulation, the amount of the expensive solubilizing agent such as poloxamer should be minimized. The poloxamer 188 concentration range employed below serves as a basic trial for investigating the effect of poloxamer 188 on the solubility of rifampicin, isoniazid and pyrazinamide. 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g of poloxamer 188 was accurately weighed out, dissolved in 20ml of water and saturated with the analyte (individual drug, any two drug combinations or the three drugs in combination). The samples were shaken in a 25 ± 2°C water bath for 12 hours, followed by the HPLC analyses of the sample supernatant (refer to section 4.1.1.3). The solubility curves of the drugs in the presence of six different concentrations of poloxamer 188 solution (2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 % (w/v)) were constructed in figures 5.1 to 5.3 with the phase-solubility curves classification listed in table 5.1.

The y-axis indicates the concentration (mg/ml) of the particular drug at the particular condition while the x-axis indicates the concentration (% (w/v)) of the poloxamer 188 present.

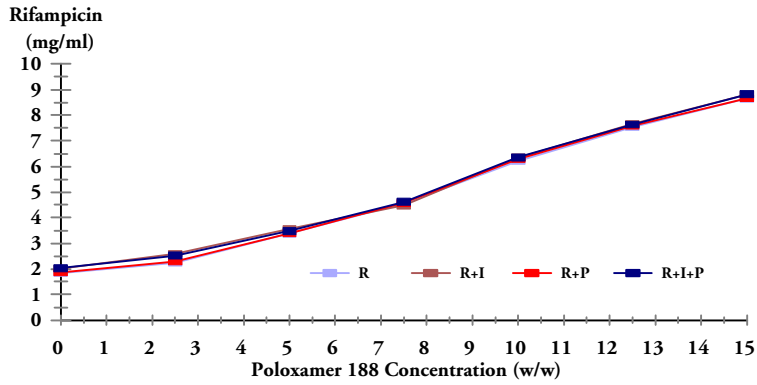


Figure 5.1 Phase-Solubility Curves of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Poloxamer 188

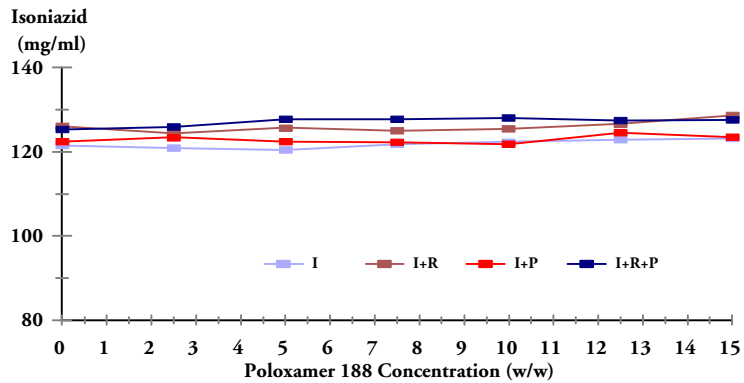


Figure 5.2 Phase Solubility Curves of Isoniazid (INH, INH with RIF, INH with PZA and INH with Both RIF and PZA) in the Presence of Poloxamer 188

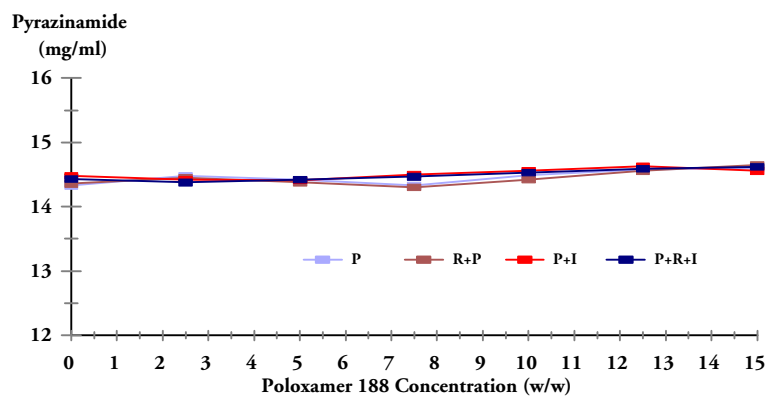


Figure 5.3 Phase Solubility Curves of Pyrazinamide (PZA, PZA with RIF, PZA with INH and PZA with Both RIF and INH) in the Presence of Poloxamer 188

Table 5.1 Classification of Poloxamer 188 Phase-Solubility Curves

Phase-Solubility Curves			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
<i>Poloxamer 188 only</i>	A _p	Minor	Insignificant
<i>Poloxamer 188 + RIF</i>	A _p	A _s	minimal
<i>Poloxamer 188 + INH</i>	A _p	alteration	alteration
<i>Poloxamer 188 + PZA</i>	A _p	on	on
<i>Poloxamer 188 + all three Drugs</i>	A _p	solubility	solubility

In the presence of approximately 12.5% of poloxamer 188, the solubility of rifampicin increased from 1.82mg/ml to about 7.54mg/ml which satisfies the requirement of 75mg per 10ml formulation. However, the solubility of pyrazinamide was not enhanced to a substantial level in the presence of poloxamer 188. In the presence of 15% poloxamer 188 solution, the solubility of pyrazinamide is approximately 14.66mg/ml which does not meet the required concentration for the formulation (25mg/ml).

5.2.3 STABILITY STUDIES OF RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE OF POLOXAMER 188

20ml of water was used to dissolve 1.0, 2.0, or 3.0g of poloxamer 188 to make up 5.0 10.0 and 15.0% (w/v) of poloxamer 188 solution. These concentrations were chosen so that amount of poloxamer 188 present and the stability of the drugs could be correlated. Each of the poloxamer 188 solutions was saturated with either rifampicin, isoniazid, pyrazinamide, rifampicin and isoniazid, rifampicin and pyrazinamide, isoniazid and pyrazinamide or all of the above mentioned drugs. All of the solutions were either purged with nitrogen or stored in air. This produced a total of 42 different solutions which were stored in ampoules with 3 sets of these 42 solutions produced in order to study the effect of the temperature on the stability of the drugs. The experiment was conducted in triplicate. The results are summarized and expressed in figures 5.4. to 5.21.

The following points were observed:

Nitrogen flushing enhanced the rifampicin stability up to 15 % (5.4 to 5.6 and 5.13 to 5.15).

An increase in poloxamer 188 concentration increases the stability of rifampicin slightly (maximum of 2%, refer to figures 5.5 and 5.13 to 5.15), but the stability of rifampicin is still poor after 7 days, degradation reached about 16% at 25EC, 33% at 40EC and 78% at 60EC for the ampoules without nitrogen flushing. Even with nitrogen flushing, approximately 6% at 25EC, 19% at 40EC and 62% at 60EC degradation was observed.

Isoniazid and pyrazinamide reduced the stability of rifampicin (refer to figures 5.13 to 5.15) And in addition, isoniazid and pyrazinamide seemed to reduce the stability of each other in the ratio of about 1% to 1%.

Nitrogen purging did not play any significant role in the degradation profiles of isoniazid and pyrazinamide (refer to figures 5.7 to 5.12).

A minor enhancement (about 1.8%) on the stability of isoniazid was experienced when the Poloxamer 188 concentration was increased, but the effect was not as obvious as in the case of pyrazinamide stability.

Note : For figures 5.4 to 5.12

The y-axis indicates the remaining percentage of that particular drug in the presence of poloxamer 188, the x-axis represents the day of analysis whereas the numerical figure on the z-axis indicates the percentage of poloxamer 188 present and the letter (N) represents the nitrogen flushing.

Note : For figures 5.13. to 5.21.

The y-axis indicates the total percentage remaining of that particular drug in the presence of poloxamer 188 after 7 days, the x-axis suggests the condition under which particular drug or combination of drugs was stored in “air” or “nitrogen”.

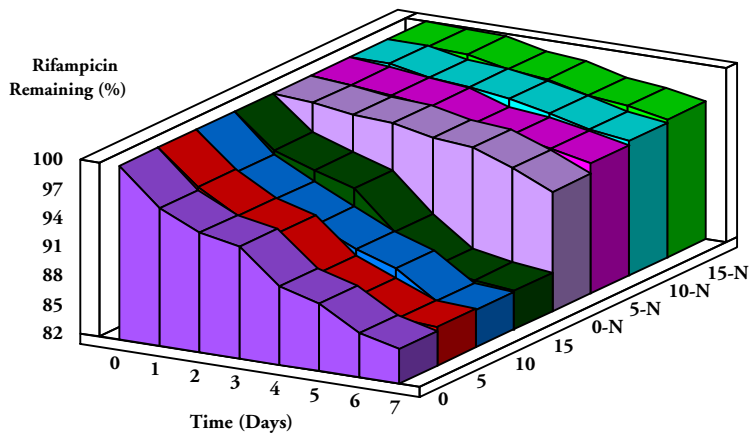


Figure 5.4 Stability Profiles of Rifampicin in the Presence of Poloxamer 188 at 25EC

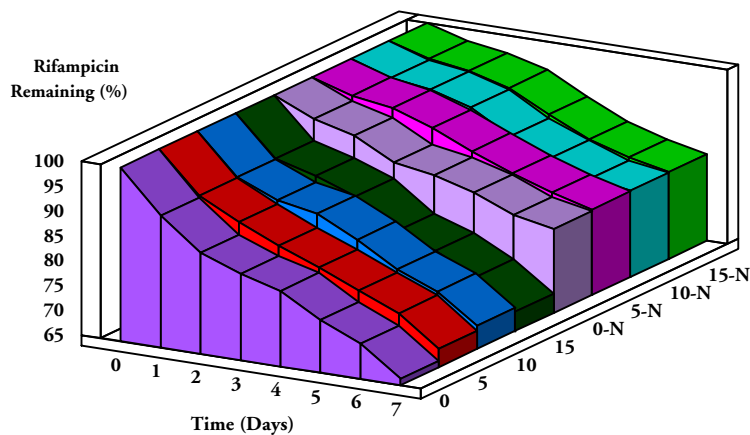


Figure 5.5 Stability Profiles of Rifampicin in the Presence of Poloxamer 188 at 40EC

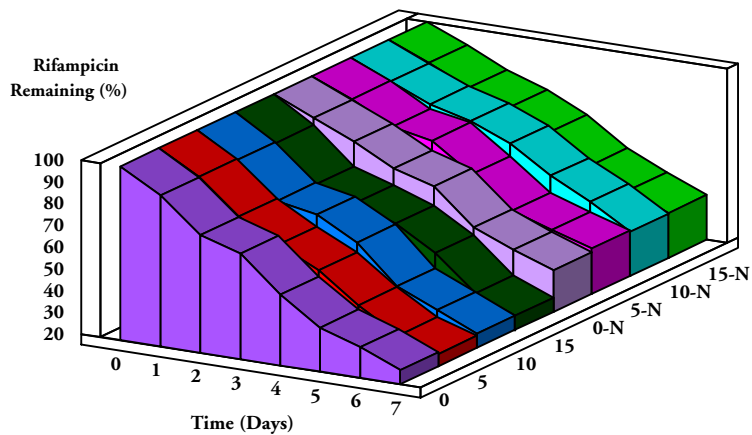


Figure 5.6 Stability Profiles of Rifampicin in the Presence of Poloxamer 188 at 60EC

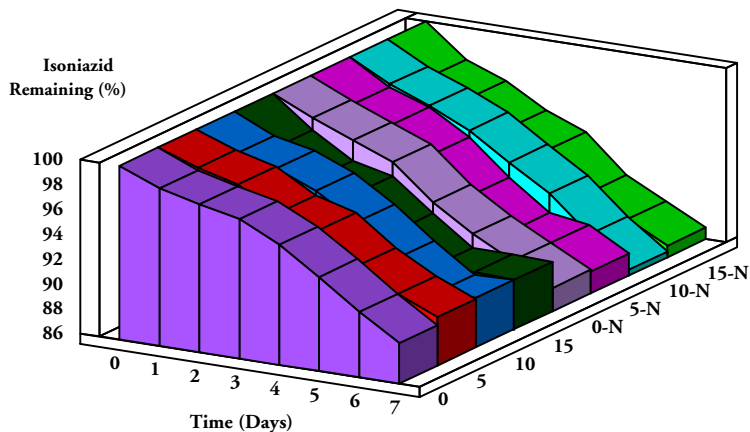


Figure 5.7 Stability Profiles of Isoniazid in the Presence of Poloxamer 188 at 25EC

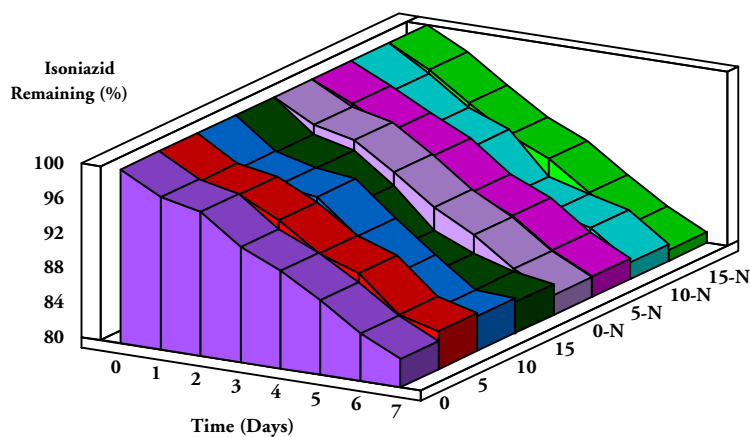


Figure 5.8 Stability Profiles of Isoniazid in the Presence of Poloxamer 188 at 40EC

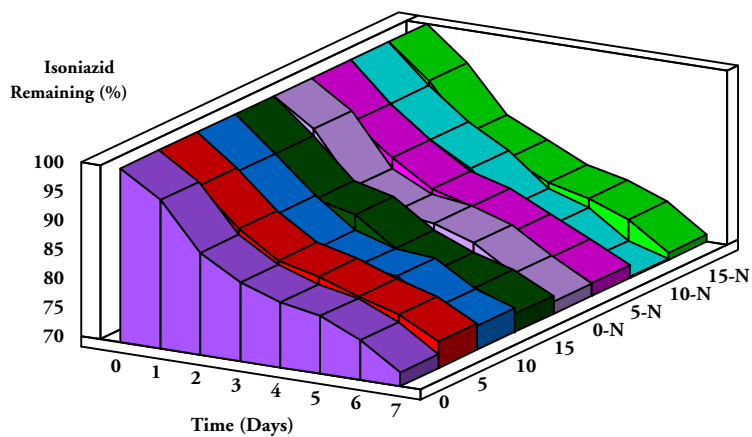


Figure 5.9 Stability Profiles of Isoniazid in the Presence of Poloxamer 188 at 60EC

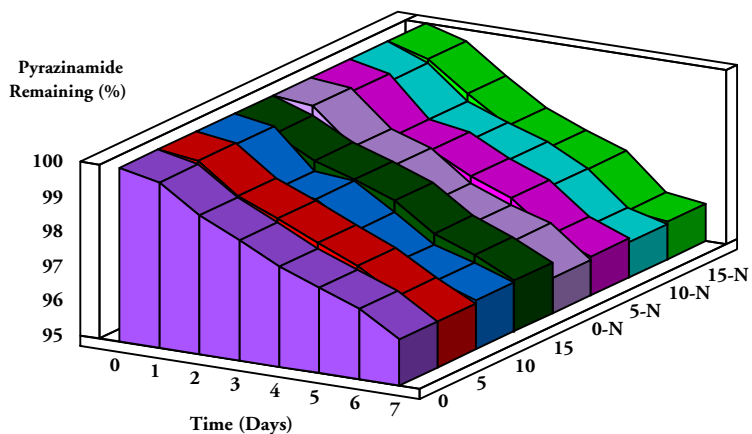


Figure 5.10 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 188 at 25°C

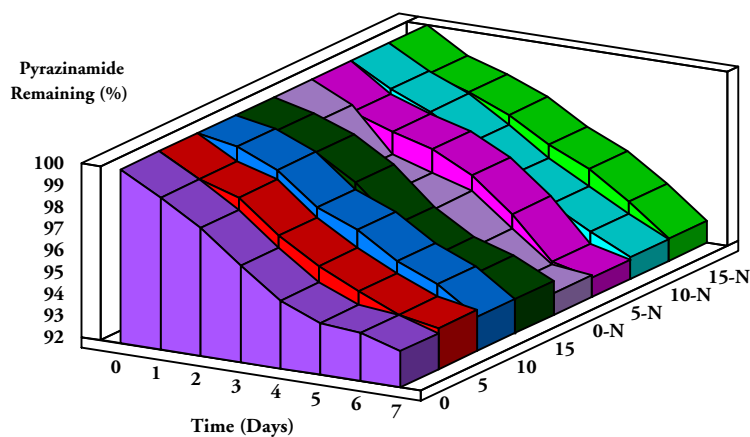


Figure 5.11 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 188 at 40°C

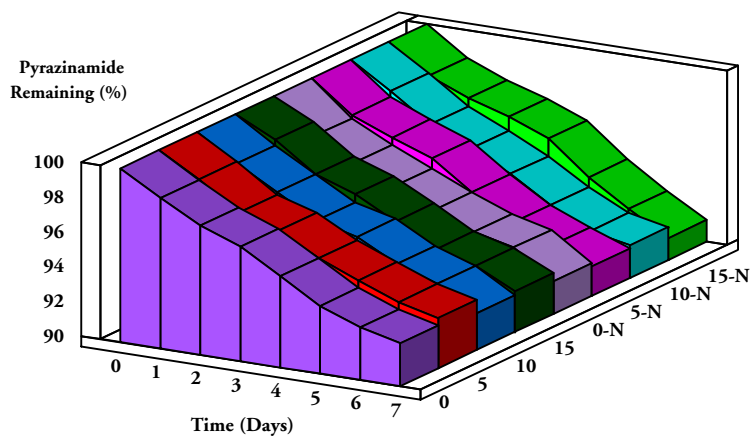


Figure 5.12 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 188 at 60°C

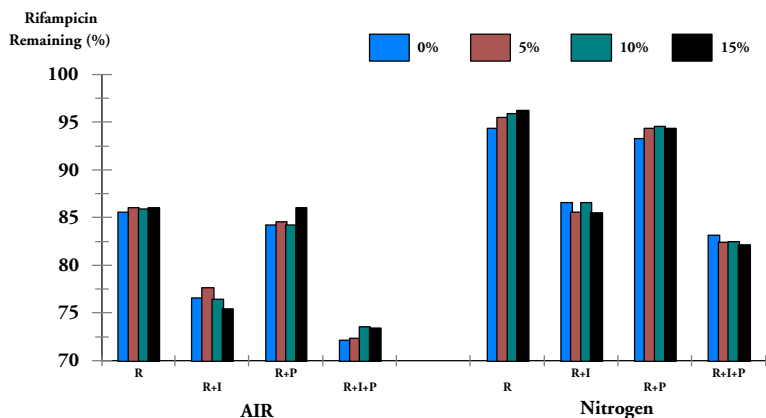


Figure 5.13 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 188 at 25EC

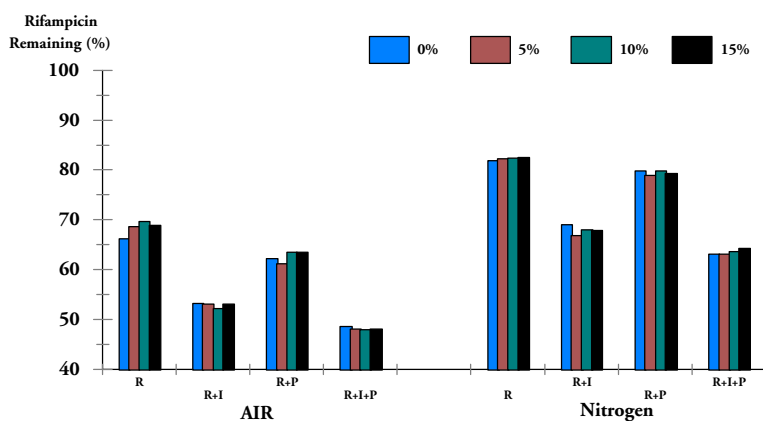


Figure 5.14 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 188 at 40EC

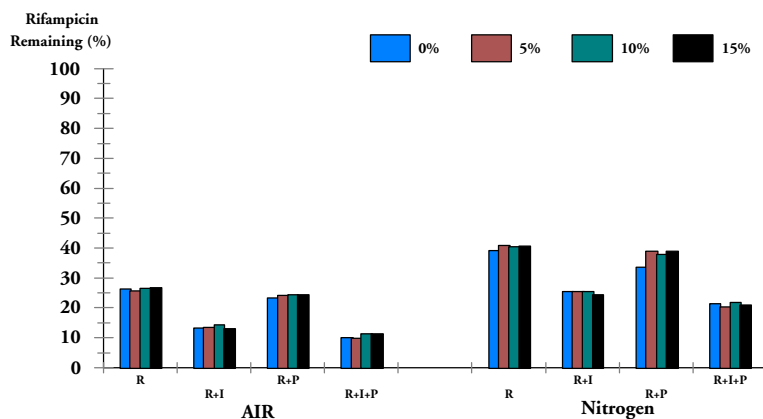


Figure 5.15 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 188 at 60EC

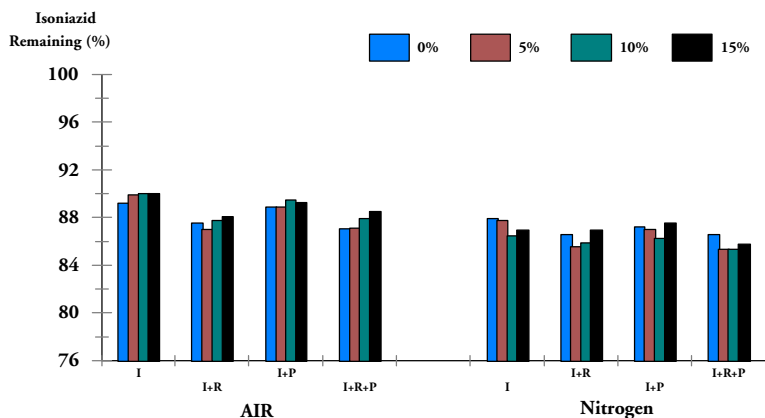


Figure 5.16 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 188 at 25EC

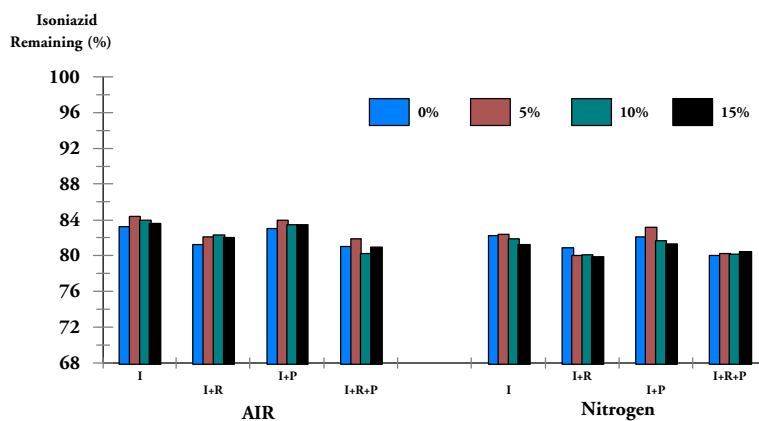


Figure 5.17 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 188 at 40EC

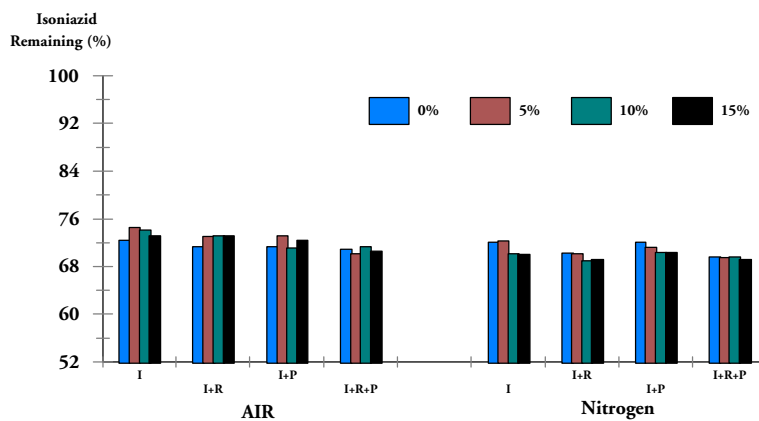


Figure 5.18 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 188 at 60EC

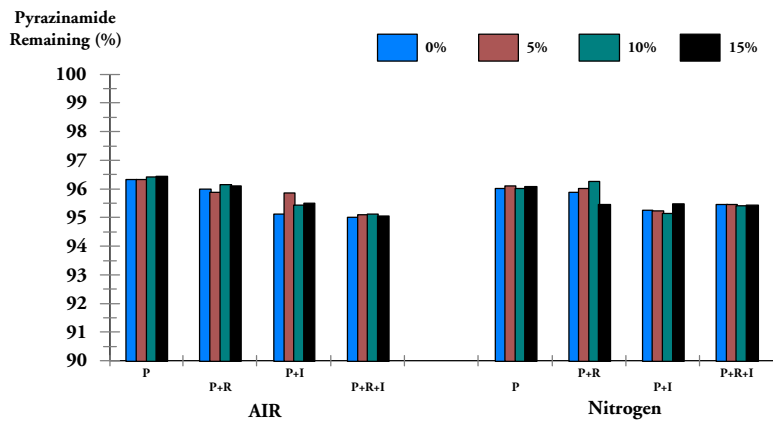


Figure 5.19 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 188 at 25EC

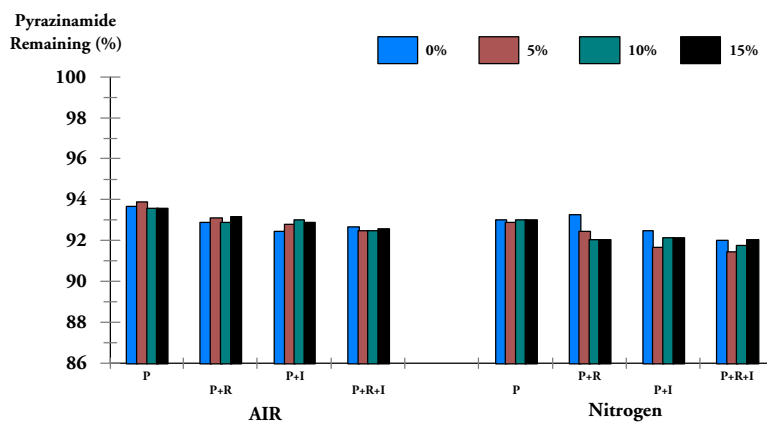


Figure 5.20 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 188 at 40EC

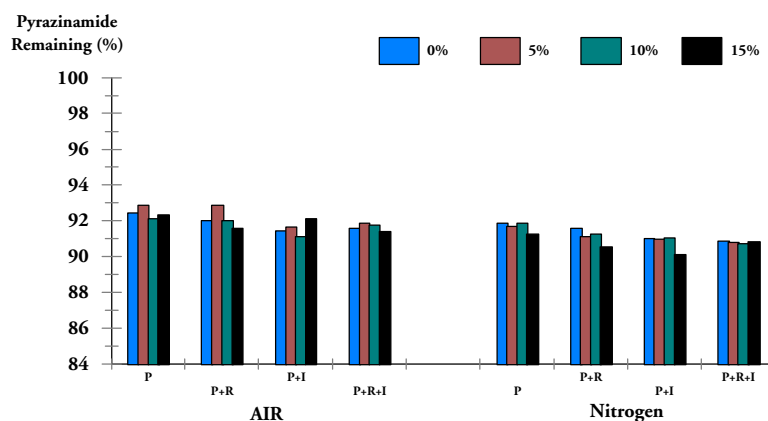


Figure 5.21 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 188 at 60EC

5.2.4 SOLUBILITY STUDIES OF RIF, INH, PZA, RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE OF POLOXAMER 407

The method used in section 5.2.2 was repeated except poloxamer 188 was replaced with poloxamer 407. The solubility curves of the drugs in the presence of six different concentrations of a poloxamer 407 solution (2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 % (w/v)) were constructed in figures 5.22 to 5.24 with the y-axis indicating the concentration (mg/ml) of the particular drug condition and x-axis indicating the concentration (% w/v) of the poloxamer 407 present. The phase-solubility curves data are listed in table 5.2.

Note : The abbreviations in figures 5.1 to 5.3:

R= Rifampicin / I= Isoniazid / P= Pyrazinamide

Table 5.2 Classification of Poloxamer 407 Phase-Solubility Curves

Phase-Solubility Curves			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
<i>Poloxamer 407 only</i>	A_N	Insignificant minimal alteration on solubility	A_P
<i>Poloxamer 407 + RIF</i>	A_N		A_P
<i>Poloxamer 407 + INH</i>	A_N		A_P
<i>Poloxamer 407 + PZA</i>	A_N		A_P
<i>Poloxamer 407 + all three Drugs</i>	A_N		A_P

The solubility of rifampicin in the presence of 7.5% of poloxamer 407 is approximately 7.8mg/ml which reaches the required concentration for formulation. The solubility of pyrazinamide was also enhanced by 2mg/ml with 15% of poloxamer 407. However, 16mg/ml of pyrazinamide is still far below the desired concentration of 25mg/ml.

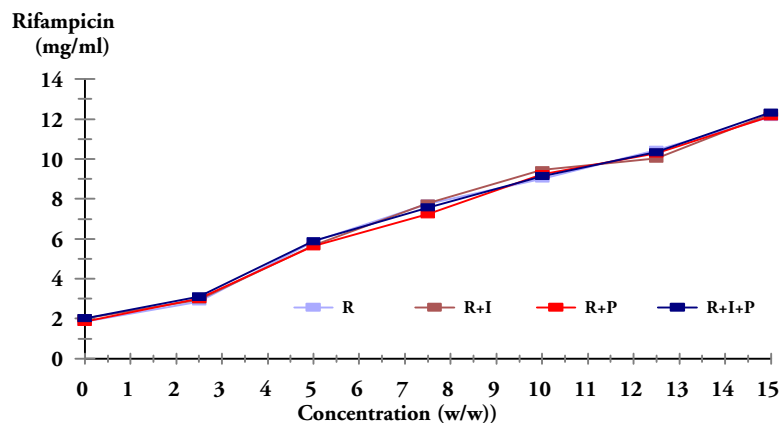


Figure 5.22 Phase-Solubility Curves of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Poloxamer 407

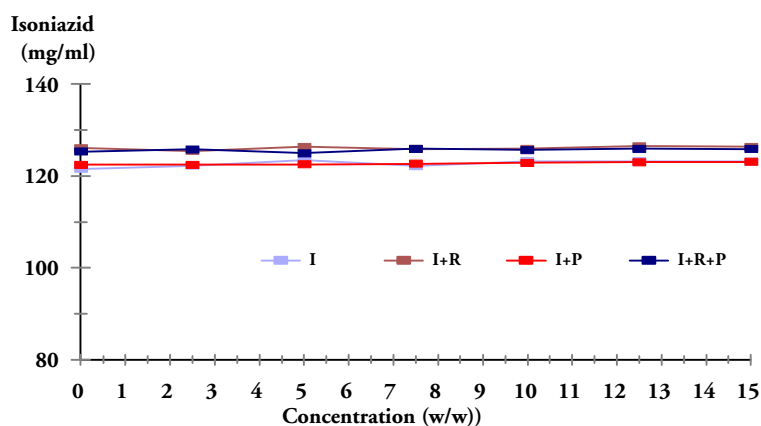


Figure 5.23 Phase-Solubility Curves of Isoniazid (INH, INH with RIF, INH with PZA and INH with Both RIF and PZA) in the Presence of Poloxamer 407

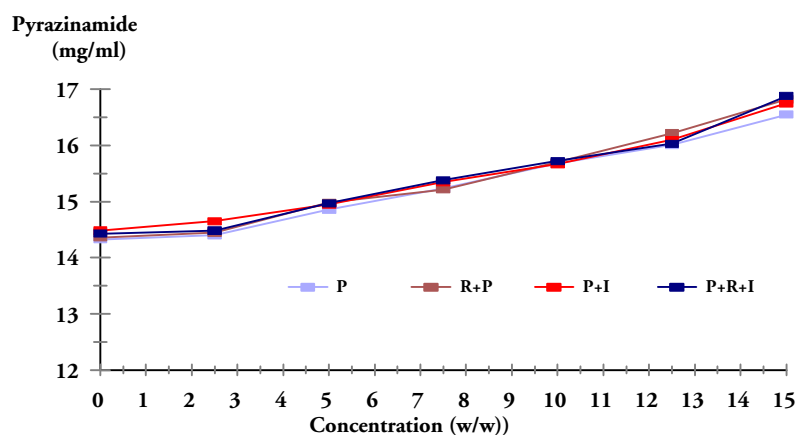


Figure 5.24 Phase-Solubility Curves of Pyrazinamide (PZA, PZA with RIF, PZA with INH and PZA with Both RIF and INH) in the Presence of Poloxamer 407

5.2.5 STABILITY STUDIES OF RIF, INH, PZA RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE POLOXAMER 407

All of the 3 sets of the 42 solutions were produced as per the same method stated in section 5.2.3 and stored in three ovens with different temperatures of 25, 40 and 60EC. Daily HPLC analyses were conducted in triplicate. The results are summarized and expressed in figures 5.25 to 5.42.

The following observations were made:

Rifampicin stability was enhanced in the presence of nitrogen up to 17% (figures 5.25 to 5.27). The presence of poloxamer 407 failed to produce a significant stabilizing effect on rifampicin, but with increasing amounts of poloxamer 407 an increase in rifampicin stability was observed. This is noticeable especially in the 25 and 40EC oven where either INH or/and PZA was/were present together with rifampicin (figures 5.34 to 5.35).

Both isoniazid and pyrazinamide experienced a stability reduction effect of about 2% when 5 % of poloxamer 407 was present (figures 5.28 to 5.33), however, this stability effect was reduced as the concentration of poloxamer 407 increased to 15 %. A total of 4% reduction was observed (figures 5.37 to 5.38 and 5.40 to 5.41).

Rifampicin, isoniazid and pyrazinamide destabilised each other when in combination (figure 5.34 to 5.42).

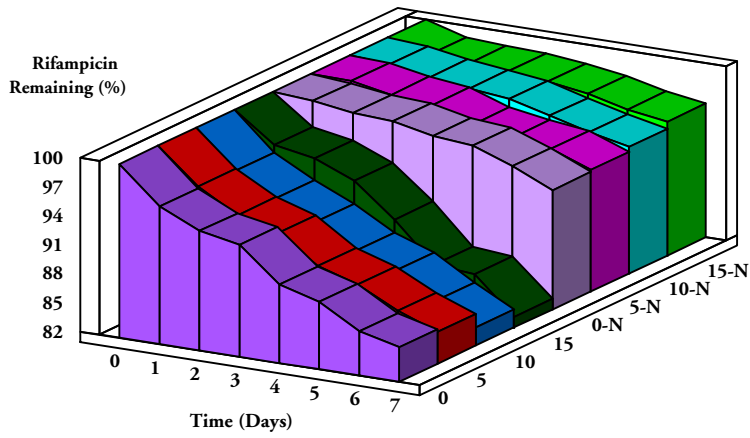


Figure 5.25 Stability Profiles of Rifampicin in the Presence of Poloxamer 407 at 25EC

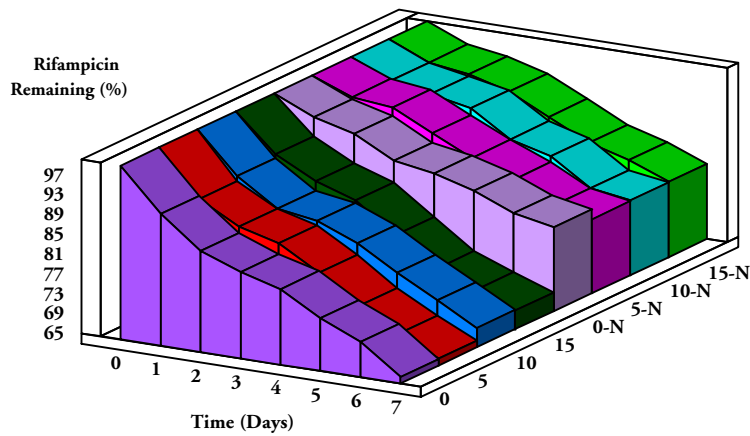


Figure 5.26 Stability Profiles of Rifampicin in the Presence of Poloxamer 407 at 40EC

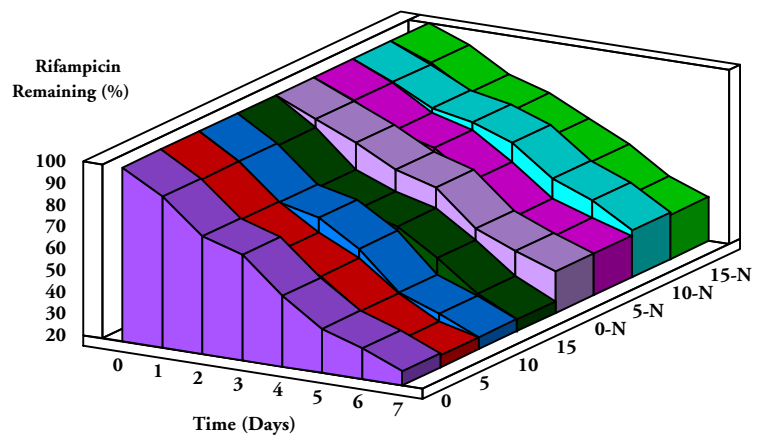


Figure 5.27 Stability Profiles of Rifampicin in the Presence of Poloxamer 407 at 60EC

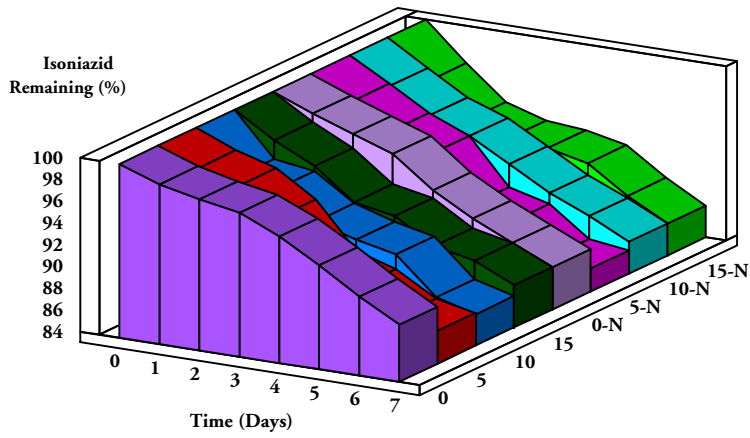


Figure 5.28 Stability Profiles of Isoniazid in the Presence of Poloxamer 407 at 25EC

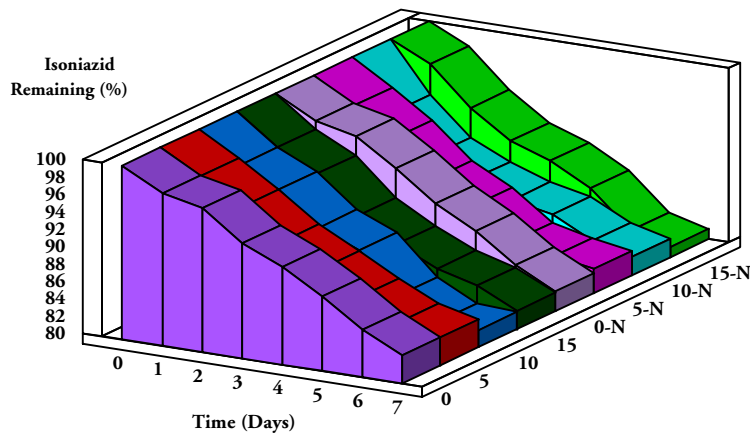


Figure 5.29 Stability Profiles of Isoniazid in the Presence of Poloxamer 407 at 40EC

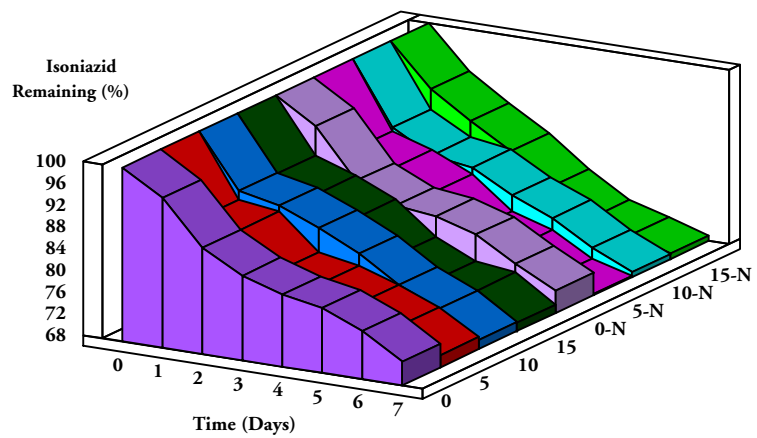


Figure 5.30 Stability Profiles of Isoniazid in the Presence of Poloxamer 407 at 60EC

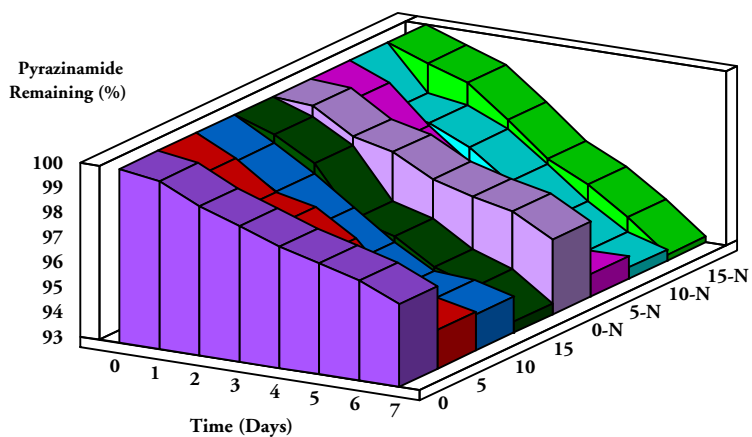


Figure 5.31 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 407 at 25°C

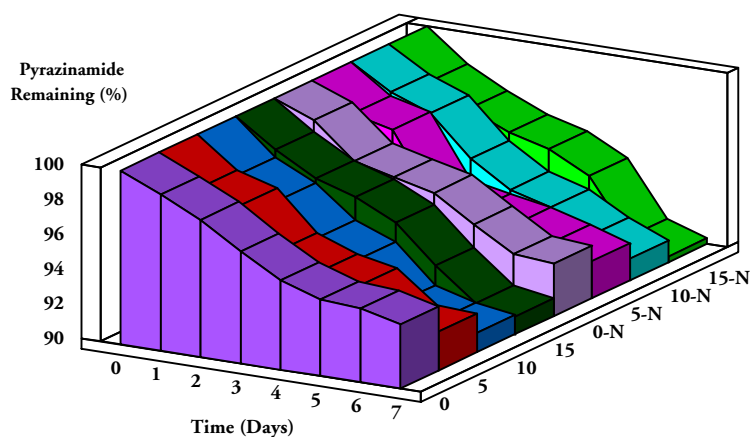


Figure 5.32 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 407 at 40°C

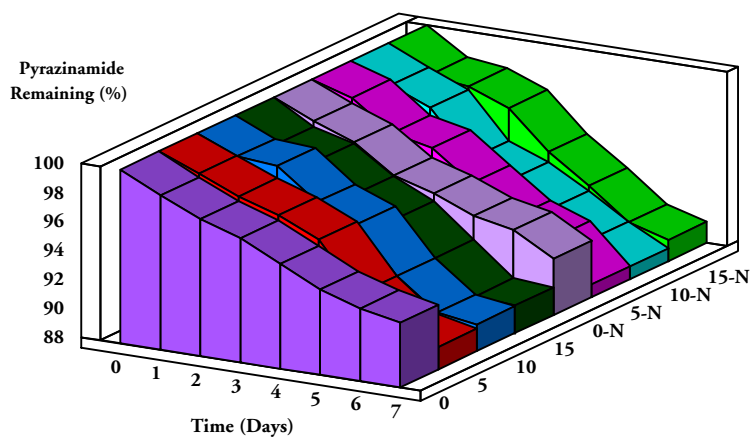


Figure 5.33 Stability Profiles of Pyrazinamide in the Presence of Poloxamer 407 at 60°C

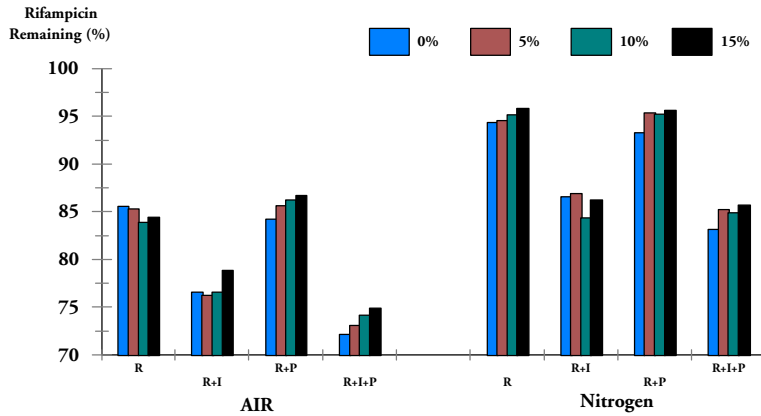


Figure 5.34 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 407 at 25EC

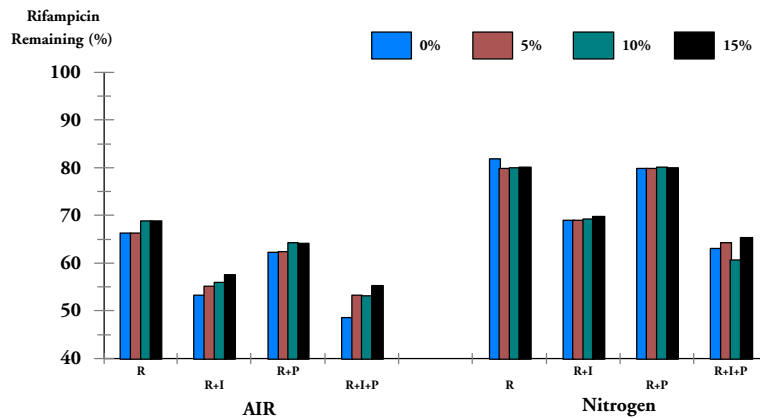


Figure 5.35 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 407 at 40EC

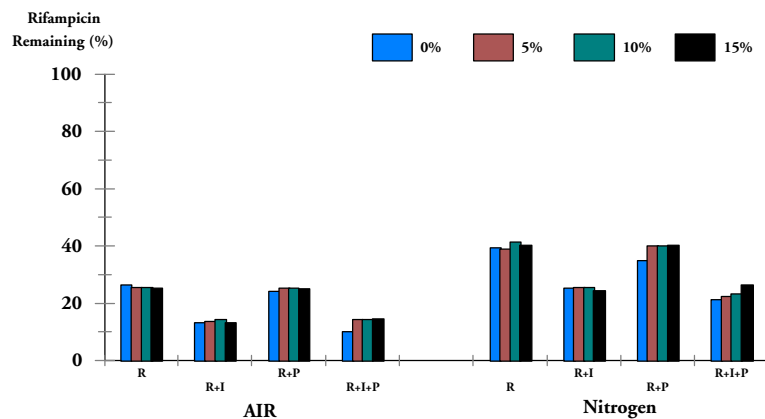


Figure 5.36 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Poloxamer 407 at 60EC

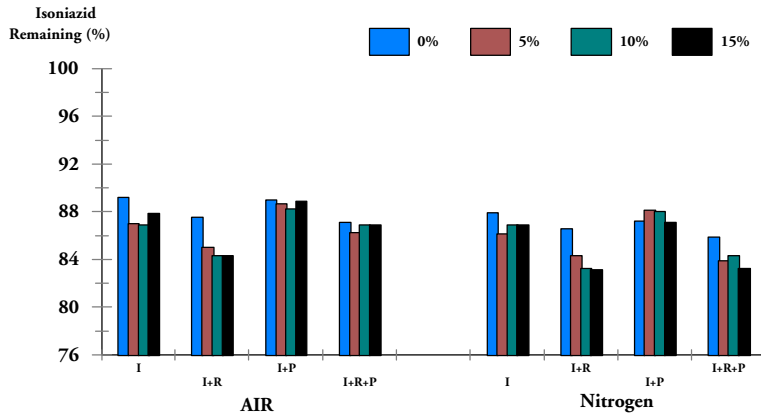


Figure 5.37 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 407 at 25EC

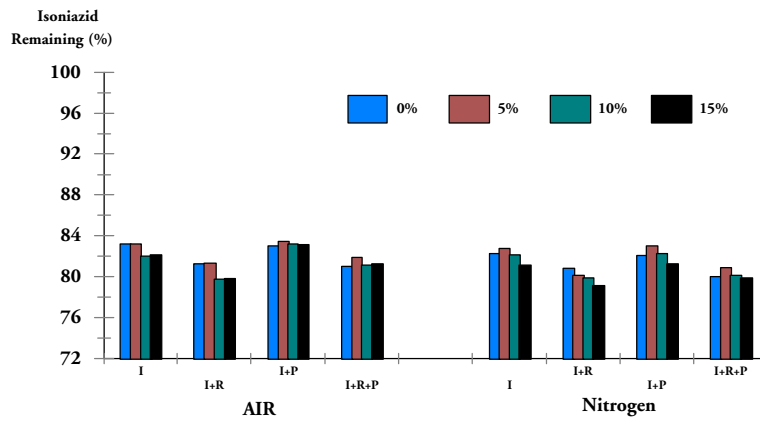


Figure 5.38 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 407 at 40EC

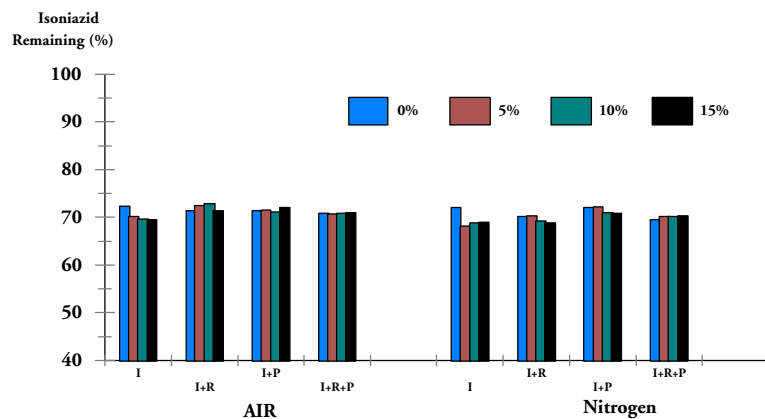


Figure 5.39 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Poloxamer 407 at 60EC

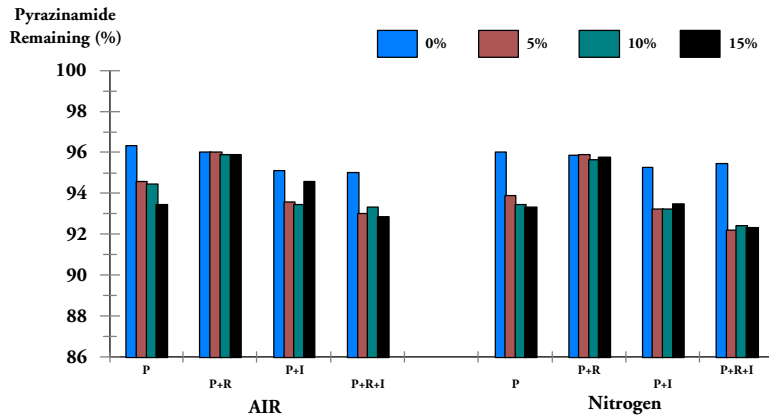


Figure 5.40 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 407 at 25EC

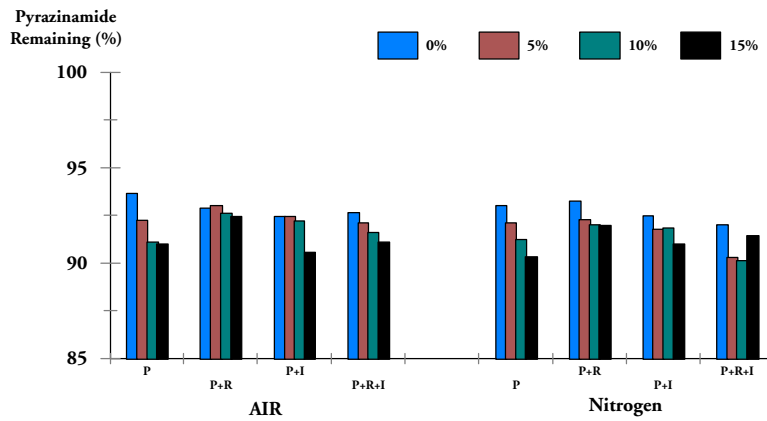


Figure 5.41 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 407 at 40EC

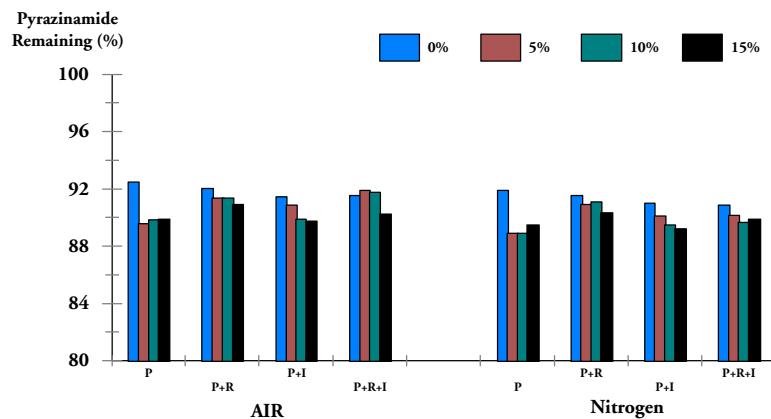


Figure 5.42 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Poloxamer 407 at 60EC

5.2.6 SOLUBILITY STUDIES OF RIF, INH, PZA AND RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE OF SORBITOL

The maximum aqueous solubility of sorbitol is about 70g/100ml and in the pharmaceutical industry, the amount of sorbitol incorporated in formulation is about 20-30g/100ml. The maximum sorbitol allowed per formulation should not exceed 50g/ml since at any concentration above that, sorbitol acts as a laxative [Reynolds et al., 1989]. Five different concentrations of sorbitol solution (7.0, 14.0, 21.0, 35.0 and 49.0% (v/v)) were made up by adding 2.0, 4.0, 6.0, 10.0 or 14.0 ml of the concentrated sorbitol solution (70% v/v) in 20ml water. The results are expressed as the phase-solubility curves in figures 5.43 to 5.45 and the description of the curves is listed in table 5.3.

Table 5.3 Classification of Sorbitol Phase-Solubility Curves

Phase-Solubility Curves			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
<i>Sorbitol only</i>	B or B _s	B _s	B _s
<i>Sorbitol + RIF</i>	B or B _s	B _s	B _s
<i>Sorbitol + INH</i>	B or B _s	B _s	B _s
<i>Sorbitol + PZA</i>	B or B _s	B _s	B _s
<i>Sorbitol + all three Drugs</i>	B or B _s	B _s	B _s

Sorbitol has the solubility reduction effect on rifampicin, isoniazid and pyrazinamide. Nevertheless, the stability studies (section 5.2.7) of these drugs in the presence of sorbitol were conducted.

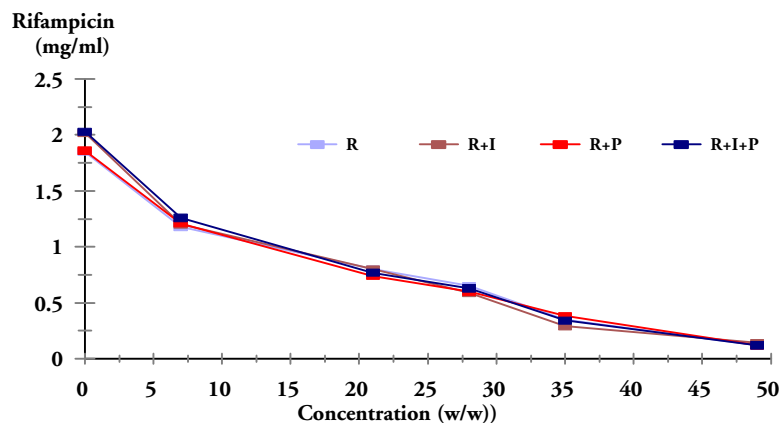


Figure 5.43 Phase-Solubility Curves of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Sorbitol

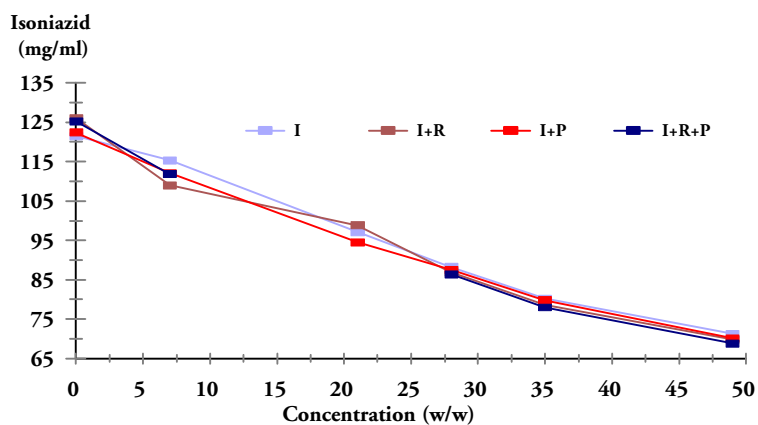


Figure 5.44 Phase-Solubility Curves of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Sorbitol

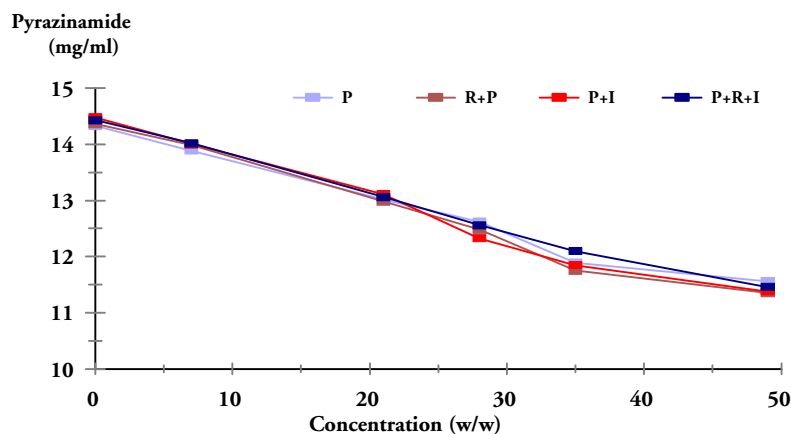


Figure 5.45 Phase-Solubility Curves of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Sorbitol

5.2.7 STABILITY STUDIES OF RIF, INH, PZA, RIF WITH INH, RIF WITH PZA, INH WITH PZA AND THE COMBINATION OF ALL THREE DRUGS IN THE PRESENCE OF SORBITOL

The stability studies were carried out by examining the stability profile of the 21.0 and 49.0% (v/v) sorbitol solution when stored in under nitrogen or air at three different temperatures. The day-to-day stability profiles (figures 5.46 to 5.55) of each individual drugs were plotted. The total degradation of the drugs in various combinations (RIF with INH, RIF with PZA, INH with PZA and RIF with both INH and PZA) is summarized as the final percentage drug remaining under the various conditions (figures 5.56 to 5.63).

The following observations were made

- < Nitrogen enhanced the rifampicin stability, but in the presence of sorbitol, rifampicin degraded substantially. Approximately 16, 24 or 39% reduction in stability caused by sorbitol was observed for rifampicin at 25, 40 or 60EC (figures 5.48 and 5.57). Increasing the sorbitol concentration seemed to increase the rifampicin degradation.
- < Sorbitol had a similar stability reduction effect towards isoniazid and pyrazinamide. Although, the degradation of isoniazid and pyrazinamide were not as dramatic as rifampicin degradation, the total drug remaining after 7 days was reduced at least 4% further for both of these drugs (figures 5.49 to 5.54).
- < The stability reducing effects of sorbitol were also found in every combination of the drugs (figures 5.55 to 5.63).

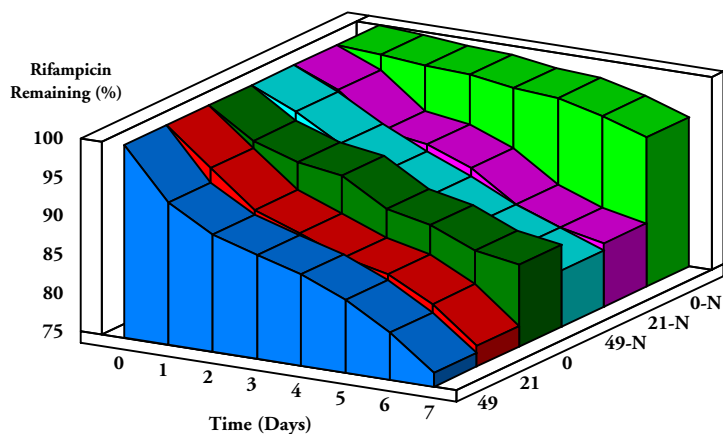


Figure 5.46 Stability Profiles of Rifampicin in the Presence of Sorbitol at 25EC

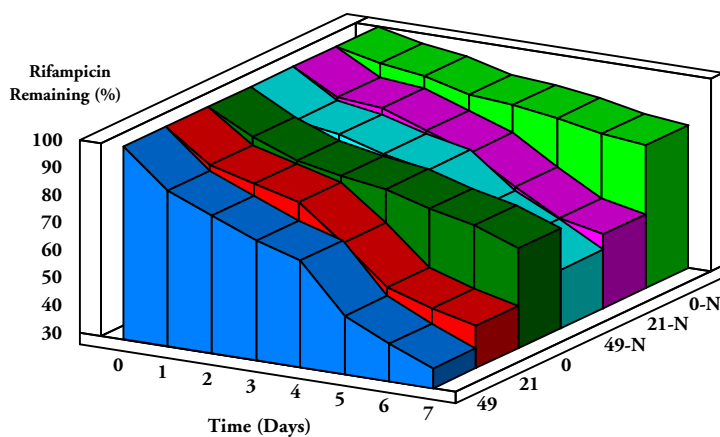


Figure 5.47 Stability Profiles of Rifampicin in the Presence of Sorbitol at 40EC

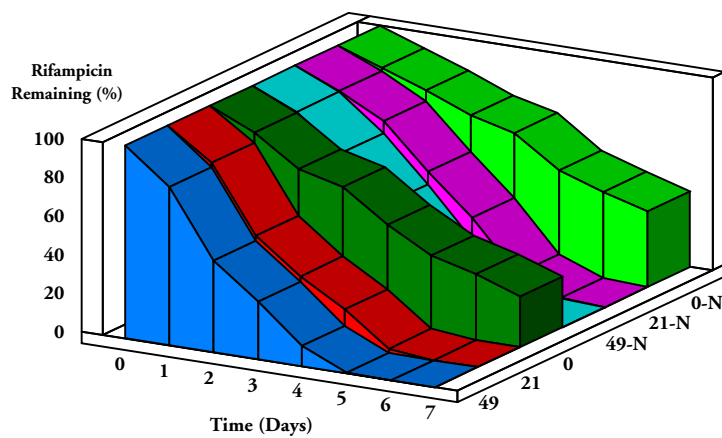


Figure 5.48 Stability Profiles of Rifampicin in the Presence of Sorbitol at 60EC

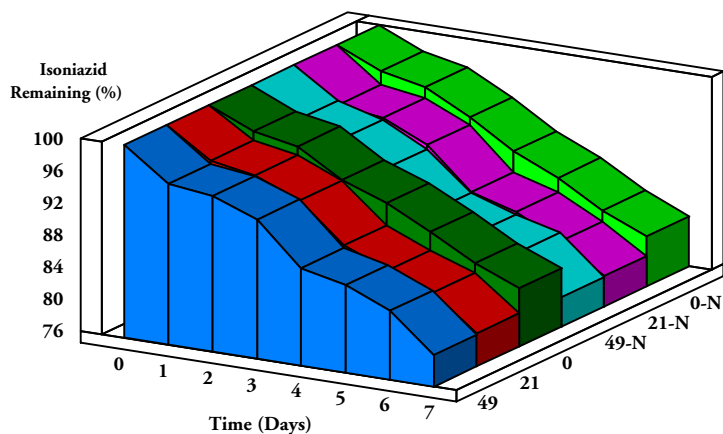


Figure 5.49 Stability Profiles of Isoniazid in the Presence of Sorbitol at 25EC

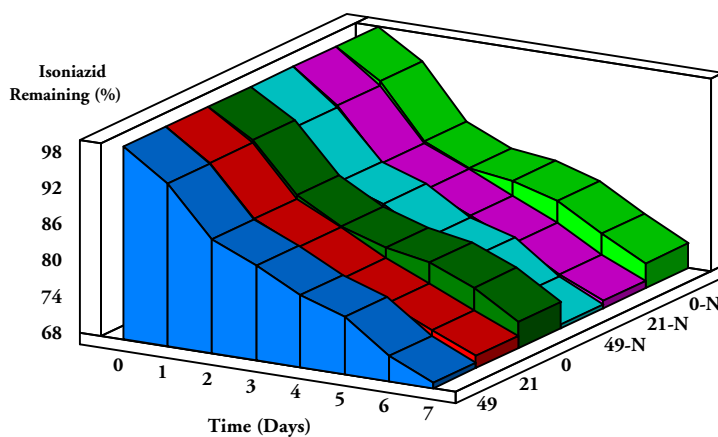


Figure 5.50 Stability Profiles of Isoniazid in the Presence of Sorbitol at 40EC

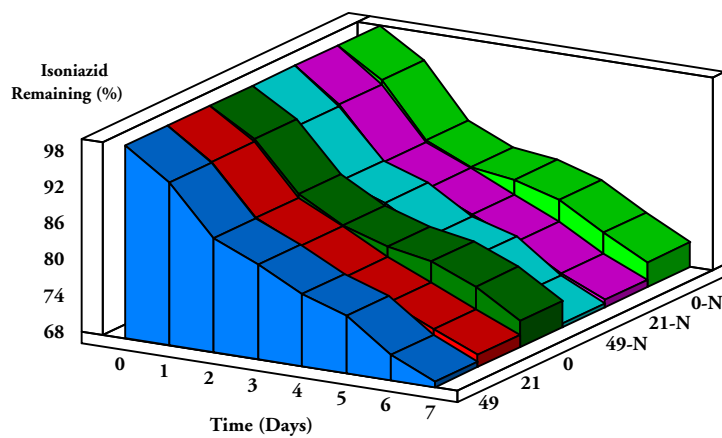


Figure 5.51 Stability Profiles of Isoniazid in the Presence of Sorbitol at 60EC

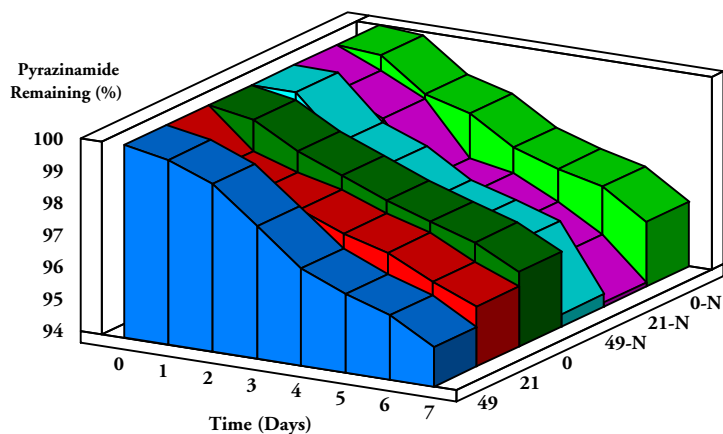


Figure 5.52 Stability Profiles of Pyrazinamide in the Presence of Sorbitol at 25°C

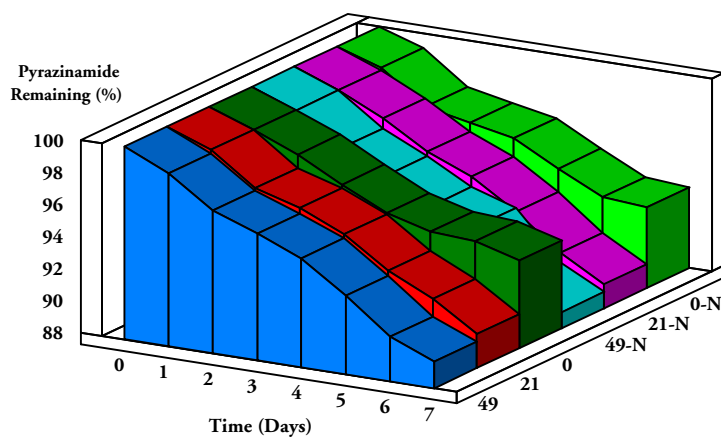


Figure 5.53 Stability Profiles of Pyrazinamide in the Presence of Sorbitol at 40°C

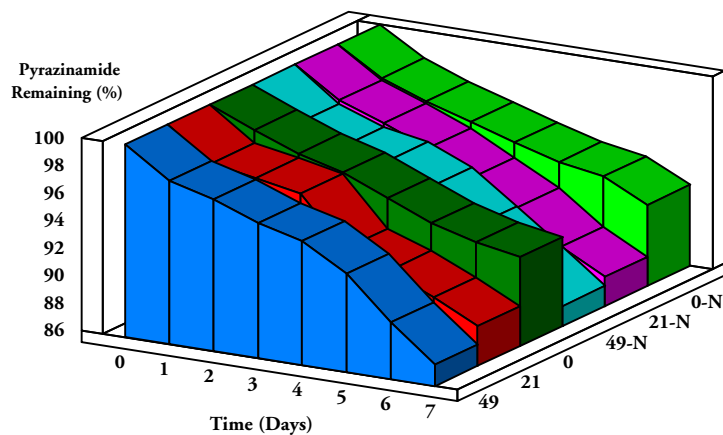


Figure 5.54 Stability Profiles of Pyrazinamide in the Presence of Sorbitol at 60°C

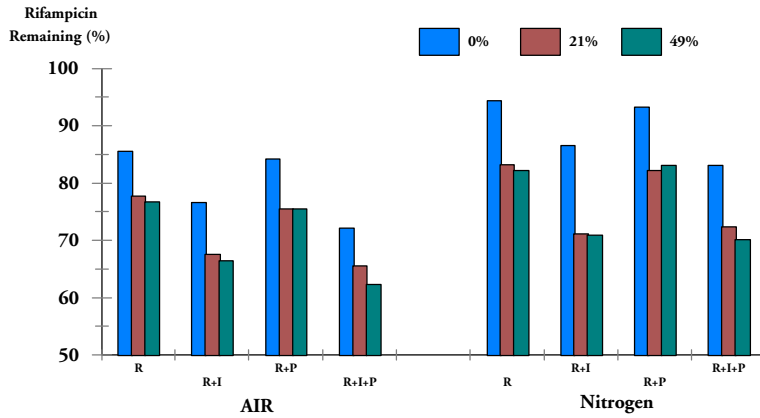


Figure 5.55 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Sorbitol at 25EC

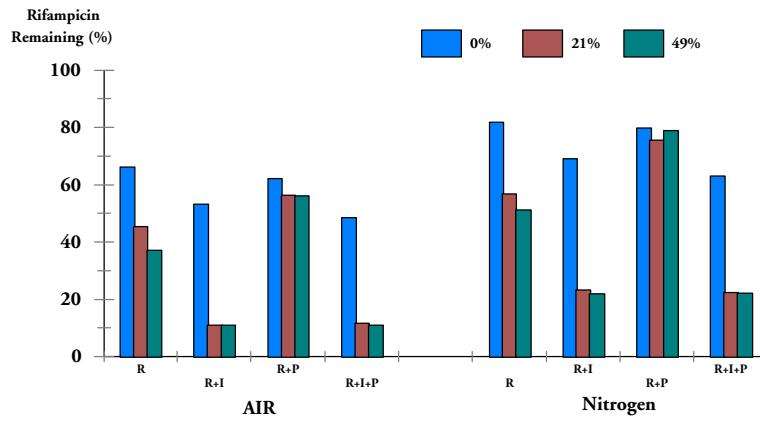


Figure 5.56 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Sorbitol at 40EC

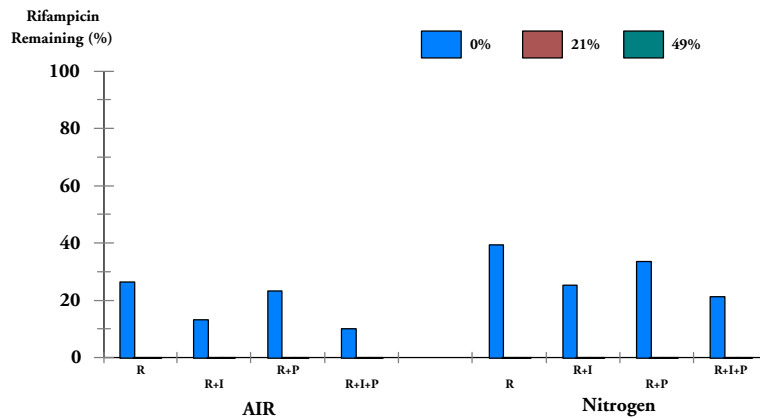


Figure 5.57 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with both INH and PZA) in the Presence of Sorbitol at 60EC

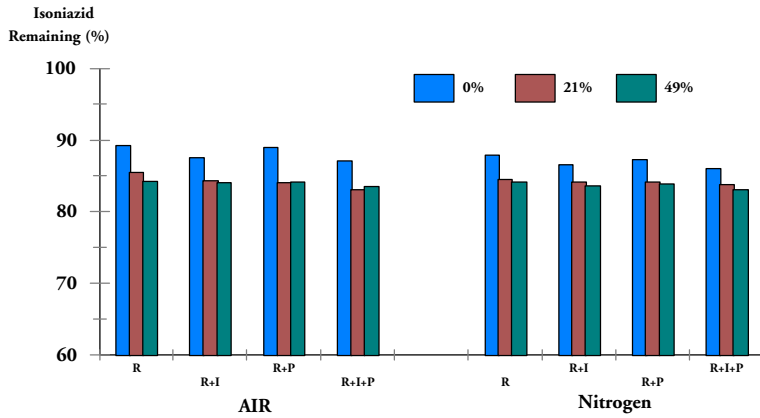


Figure 5.58 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Sorbitol at 25EC

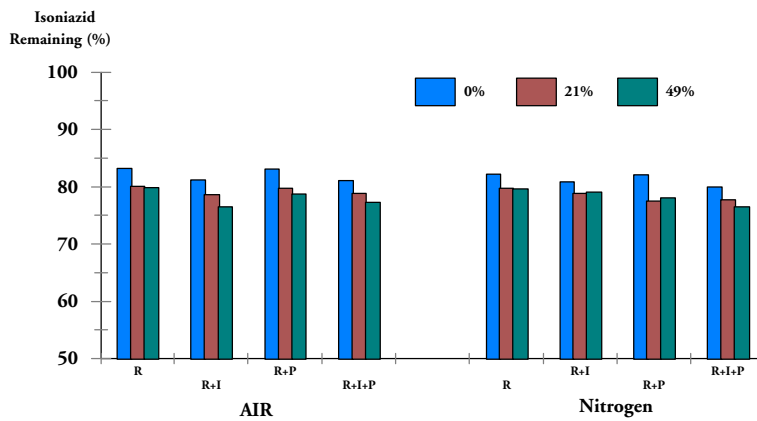


Figure 5.59 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Sorbitol at 40EC

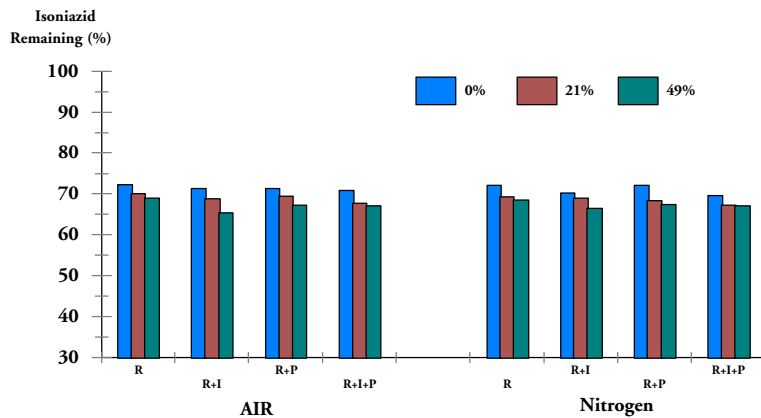


Figure 5.60 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with both RIF and PZA) in the Presence of Sorbitol at 60EC

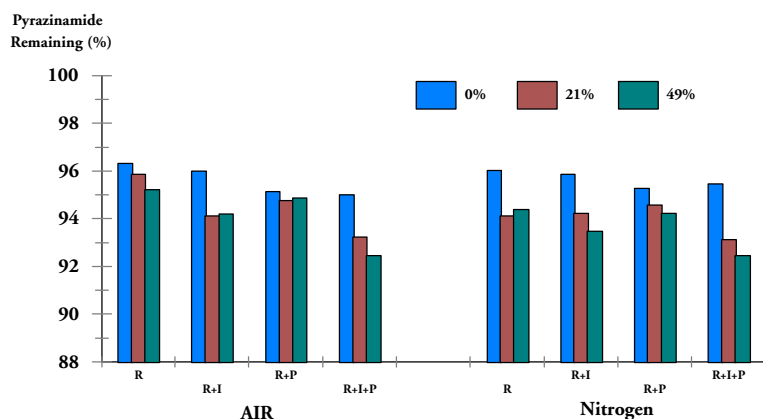


Figure 5.61 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Sorbitol of Drugs at 25EC

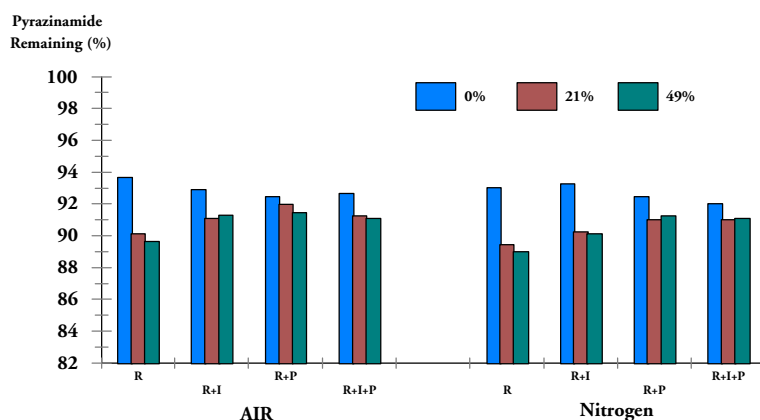


Figure 5.62 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Sorbitol at 40EC

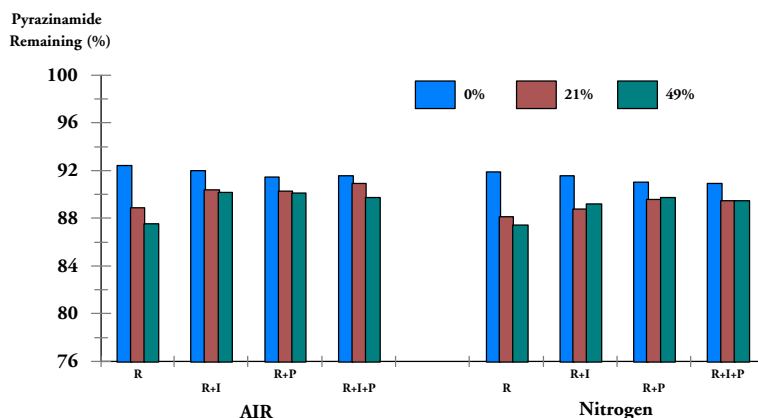


Figure 5.63 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with both RIF and INH) in the Presence of Sorbitol at 60EC

5.3 CONCLUSION

5.3.1 SOLUBILITY STUDIES

Higuchi and Connors stated that “it is the fundamental assumption of solubility behavior of systems of interacting components that can be described by an expression...” and [Higuchi, 1965] and this expression is known as the solubility constant, sometimes referred to as the solubility enhancement constant. The solubility enhancement constant interprets the possibility of complexation formation between two interacting components and indicates the strength of the complexation through the magnitude of the value and it will also show the tendency the solubility alteration which is influenced by the other component.

The simplified version of the equations of a S and Z interaction (section 4.1.1.2) would be:

$$K = (\text{Slope}) / (S_0 \times (1 - \text{Slope}) \dots\dots\dots \text{Eq. 5.1.}$$

Where

K = Solubility constant

S_0 = The initial solubility of component S

In the presence of the poloxamer 188, poloxamer 407 and sorbitol, the solubilities of rifampicin, isoniazid and pyrazinamide were changed in a number of different ways. These changes are listed in tables 5.1. to 5.3.. It may be that both poloxamer 188 and 407 interacted with rifampicin in a ratio higher than one to one (section 4.1.1.2.), thus forming the A_p Curves. The long chain of the poloxamer molecule could well provide multiple affinity sites which enables the high order interaction of the poloxamers with rifampicin. However, the actual interaction between rifampicin and poloxamers was uncertain, with the linearity values of 0.9801 and 0.9789 (for poloxamer 188 - and poloxamer 407- rifampicin phase solubility curves), indicating the tendency for A_s curve formation. Moreover, the molecular modeling software employed in this project was unable to create the “Solvent box”, therefore, the solubility enhancement constant was calculated by assuming the A_s phase-solubility curves which are listed in table 5.4. Nevertheless, a substantial increase of rifampicin solubility was found when employing the non-ionic surfactants such as poloxamer 188

and 407. The solubilities of isoniazid and pyrazinamide was almost unchanged when adding the poloxamer 188, but a minor increase of pyrazinamide solubility was observed (figures 5.2 to 5.3 and 5.23. to 5.24).

Sorbitol had adverse effects on solubilities of all of the drugs, therefore the usage of this surfactant is not advisable.

Table 5.4 The Solubility Constants of Rifampicin, Isoniazid and Pyrazinamide in the Presence of Poloxamer188, Poloxamer 407 and Sorbitol

Solubility Constants			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
<i>Poloxamer 188</i>	496.28 (Enhancement)	1.33 (Enhancement)	9.09 (Enhancement)
<i>Poloxamer 407</i>	518.67 (Enhancement)	1.27 (Enhancement)	41.52 (Enhancement)
<i>Sorbitol</i>	-3.79 (Reduction)	-0.68 (Reduction)	-0.79 (Reduction)

Another important observation was made in the presence of the drugs influence the solubilities each other. The solubility of rifampicin, isoniazid and pyrazinamide increased when in combination. This implies that there could be interactions between the drugs. Although this interaction may be beneficial in the sense of improving the poor aqueous solubility of rifampicin and pyrazinamide, the possibility of an adverse effect must also be considered.

4.2.3.1 STABILITY STUDIES

The poor stability of rifampicin in the aqueous system was very noticeable. It is below the stability acceptance requirement of both ICH and FDA. Rifampicin stability is very temperature dependent. During a 7 day trial period, increasing in the surrounding temperature from 25 to 60EC could cause a 5-fold increase in degradation.

Nitrogen purging increased the aqueous stabilities of rifampicin. This coincided with the scheme II rifampicin degradation prediction, where the atmospheric oxygen readily converts rifampicin to

rifampin quinone where the carbonyl groups on the quinone readily undergo further degradation. Adequate nitrogen flushing could result in a stability enhancement up to an approximation of 16% and is recommended to be incorporated in the formulation stage. However, oxidation did not play any role in the degradation of isoniazid and pyrazinamide. This is deduced from the lack of stability enhancement when the isoniazid and pyrazinamide solutions were flushed with nitrogen.

Both poloxamer 188 and 407 improved rifampicin stability minimally, but the overall stability profiles indicated that these two solubilizing agents did not produce enough of a stabilizing effect towards rifampicin degradation.

In the presence of sorbitol, rifampicin experienced a significant stability reduction. The carboxylic group of sorbitol readily liberates its proton and may initiate the hydrolysis of the azomethine bond on the amino-piperazine side chain [Jindal et al., 1994] which adversely affects the rifampicin stability and produces formylrifamycin SV.

Sorbitol had an adverse effect on both the isoniazid and pyrazinamide stabilities. This could be due to the possible liberation of the proton from sorbitol which causes the hydrolysis of isoniazid and pyrazinamide.

Both isoniazid and pyrazinamide lowered the stability of rifampicin. Especially when combining with isoniazid, rifampicin degradation was accelerated far more than when combining with pyrazinamide. Separation of the drugs may prevent this interaction. For example using a suspending agent could effectively separate the isoniazid which dissolves in the aqueous phase and rifampicin or pyrazinamide which are suspended as solid particles. Moreover, the interactions between two solid particles are generally slow and could result in a reduction in drug degradation.

Surfactants only increased the solubility of the drugs but failed to prevent the drug-drug or drug-solvent interaction and hence are not recommended to be incorporated in a paediatric formulation.

CHAPTER SIX

STABILITY STUDIES OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE IN THE PRESENCE OF CARBOPOL SUSPENDING AGENTS

6.1 INTRODUCTION

In the previous chapter, it was clear, that although the solubility requirement of rifampicin was met by employing the poloxamers, the desired concentration of pyrazinamide was not achieved in the presence of any mentioned surfactants. Moreover, the stabilities of the drugs were found to be below both the ICH and FDA regulatory requirements [Carstensen et al., 1995]. While the interactions between the drugs seem to be a destabilizing factor, hydrolysis still remains as a main obstacle in achieving a satisfactory paediatric formulation. In this chapter, an attempt is made to bypass these problems by making use of the suspension. In consultation with Pharmacare-Lennon[®], two suspending agents were chosen to be part of the study, namely carbopol 934 and 974P.

Suspensions are frequently used in pharmaceutical industries for the formulating of less soluble drugs as a liquid dosage form. A suspension generally employs suspending agents which are described as the compounds which have the property of increasing viscosity of the solution in which they are dissolved or dispersed. The resulting thixotropic effect of the suspending agents contributes to their usefulness [Reynolds, 1989].

Viscosity is the measurement of the force per unit area required to maintain a certain rate of flow. The viscosity of a solution is measured in poise (1 dyne-sec/cm²) or centipoise (100cP = 1 Poise) and can easily be determined by using either an Ostwald or rotating drum viscometer (section 6.2.1). The presence of macromolecular solutes, such as various carbopols, may enhance the viscosity of a solution because the bulky molecules affect the fluid flow within the system [Atkins, 1990].

Carbopol resins also known as carbomers, the commercial name of polyacrylic acid, consist of an acrylic backbone with polyalkenyl ethers or divinyl glycol. The main differences between the various carbopols are related to the presence of monomer and cross-linked density, but all of the resins have a superior thixotropic capability. The carbopol resins are flocculated hygroscopic powders with each particle averaging from 2 to 7 microns. These agglomerates cannot be broken down into particles once produced and these powders have a white and fluffy appearance with a pK_a of 6.0 ± 0.5 and a pH of 2.5-3.0 when dispersed as a 1.0% aqueous solution. The molecular weight is estimated to be in the region of 700,000 to 3 or 4 billion Daltons, however, the actual weight is not able to be determined by any current methods. Linear polymers are very soluble in water and able to swell to 1000 times their original volume when exposed to a pH environment above 4.0-6.0 and result in a viscous gel. This is due to the fact that the resins have an average equivalent weight of 76 per carboxyl group and thus the repulsion between the negatively charged carboxylate groups on the polymer backbone causes the carbopol to swell [BF Goodrich, 1994].

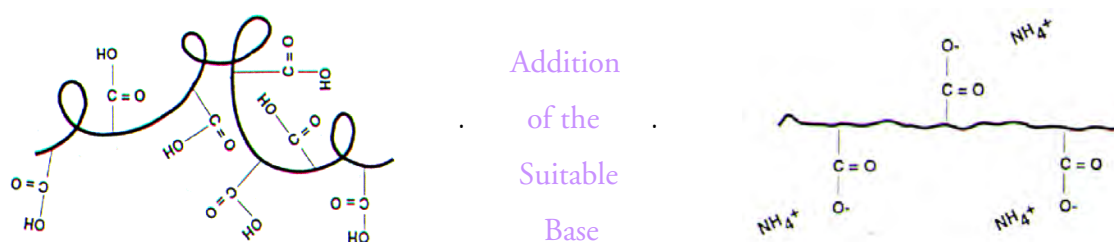


Figure 6.1. Carbopol Chain Uncoiling

Carbopol resins have been used in oral suspensions worldwide and are most suitable for the aqueous based suspension formulation. The excellent abilities of carbopol to thicken, modify flow rate characteristics, suspend insoluble ingredients, and provide bioadhesion have resulted in their use in pharmaceutical research/industry for many years. Carbopol resins swell when hydrated and when neutralized with an adequate base, the resins are soluble in water, most alcohols and glycerol, forming a very viscous colloidal dispersion. The insoluble ingredients in the suspension are trapped in the interstitial spaces between the hydrogel particles permanently. Furthermore, carbopol resin microgels are easily moved by shear forces but reform the macro-gel immediately once the force is removed. This immediate recovery enables the suspension to be easily dispersed or redispersed and

facilitates stirring or pumping. At a viscosity of between 1500 to 2500cPs carbopol resins provide a permanently stable suspension while most of the other suspending agents fails to do so. Other key benefits of using carbopol as suspending agent include [BF Goodrich, 1994]:

- < It yields high viscosity values even at low concentration. At a 0.1 - 1.5% carbopol resin concentration, the suspension is a viscoelastic liquid, but when over 3.0%, the suspension becomes a viscoelastic solid.
- < It masks the unpleasant taste of the drug and this is ideal since rifampicin has a bitter taste.
- < It increases the bioavailability of the drug which increases the effectivity and benefit to the patients.
- < It is versatile over a wide range of pHs and this allows the pH to be set according to the stabilities of the drugs without disturbing the thixotropic effect.
- < It has antifungal and antibacterial properties.
- < It has low toxicity and carbopol is approved by the ICH and FDA. The toxicity studies have been extensively conducted on carbopol 934 resin in rats, mice, guinea pig and dogs. Depending on the specie tested, the studies have shown that an acute toxicity of the oral LD₅₀ has ranged from 2.5g/kg to greater than 40.0g/kg body weight. No significant side effects have been shown except a slight growth suppressing syndrom with a 21-days continuous intake of carbopol higher than 5.0% of per total weight of daily diet. But in general, no related pathological, histopathological, hematological, reproductive, gastro-intestinal and urinary adverse effects have been ever found. Eye and skin irritation can result from a carbopol 934 solution of 1.0% or above this concentration. The toxicity studies of carbopol 974P showed that a 13-week period of oral administration of carbopol 974P at the concentration of 50,000 ppm or above induced significant signs of chronic histopathological inflammation. Weight loss may also increase as the dose concentration of carbopol 974P increases. Carbopol 974P did not induce hazardous eye and dermal irritation if the concentration of the carbopol 974 solution is below 1.0% [BF Goodrich, 1994] and the acute oral LD₅₀ is determined to be greater than 2.0g/kg.

Carbopol 934 and carbopol 974P (BF Goodrich, Cleveland) were kindly donated by Pharmacare-Lennon[®]. The poor aqueous solubilities of rifampicin and pyrazinamide are no longer a problem when they can be suspended within the formulation. The main considerations are in the stabilities which is divided into the physical and chemical aspects. The physical stabilities aspect includes the viscosity and redispersibility whereas the chemical stabilities aspect includes the stabilities of the rifampicin, isoniazid and pyrazinamide.

6.2 EXPERIMENTAL RESULTS AND OBSERVATIONS

6.2.1 KARL FISCHER EXPERIMENTS

Karl Fischer experiments (section 4.1.3.) carried out showed that the water content of carbopol 934 and carbopol 974P were found to be negligible. Thus it was not necessary to take this into consideration in the weights used in further experiments.

6.2.2 VISCOSITY AND REDISPERSIBILITY

Various concentrations of carbopol for suspension formulation were made up by dissolving the required amount of carbopol powders in water. They were adjusted with sodium hydroxide to the a pH range of 7.10 ± 0.01 until a translucent viscous liquid was achieved. The viscosity of the carbopol 934 and 974P at different concentrations was tested and is listed in tables 6.1 to 6.2 and clearly demonstrates that the optimum concentration range of carbopol 934 and carbopol 974P is between 0.01% to 0.02% and 0.005% to 0.01% respectively

All viscosities of all the solutions were found to be constant at 25EC for at least 4 weeks except the 0.005 carbopol 934 solutions and the 0.001 and 0.003% carbopol 974P solutions in which the suspensions deteriorated.

Table 6.1 Viscosity of Different Concentrations of Carbopol 934 Solutions

Viscosity and Redispersibility of Carbopol 934					
<i>Concentration</i> (%)	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>	<i>Concentration</i> (%)	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>
0.005	880	+	0.025	2560	++++
0.010	1290	++	0.030	2800	++++
0.015	1850	++	0.035	3150	++++
0.020	2100	+++	0.040	3700	+++++

Table 6.1 Viscosity of Different Concentrations of Carbopol 974P Solutions

Viscosity and Redispersibility of Carbopol 974P					
<i>Concentration</i>	<i>Viscosity</i> (cPS)	<i>Redispersibility</i>	<i>Concentration</i>	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>
0.001	660	+	0.010	2400	+++
0.003	1050	++	0.015	2850	++++
0.005	1760	+++	0.020	3350	++++
0.007	2110	+++	0.025	3800	+++++

Note:

>+ = Low redispersibility due to the viscosity being too low

#++ = Acceptable redispersibility

\$+++ = Acceptable redispersibility but with a slightly higher viscosity

<+++ = Low redispersibility due to the viscosity is too high

6.2.3 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF CARBOPOL 934P

0.02% is the concentration of the carbopol 934 solution used in the stability studies of rifampicin, isoniazid and pyrazinamide. A 0.01% carbopol 934 solution was also incorporated in order to demonstrate the stabilizing effect of carbopol 934 concentration on the drugs. 100mg of rifampicin

and 500mg of pyrazinamide were suspended in 20ml carbopol 934. Isoniazid was dissolved in 20ml of 0.01 and 0.02% carbopol 934 solutions which is followed by the addition of sodium hydroxide to neutralize the pH. Therefore due to the high solubility of isoniazid, only rifampicin and pyrazinamide were actually suspended.

The methodology for the stability studies adopted the validated HPLC analysis (refer to section 3.2 and 3.3) on a daily basis. The results are presented as the percentage of drug remaining against the time of analyses (figures 6.2 to 6.10) and (figures 6.11 to 6.20).

The results shown in figures 6.2 to 6.10 illustrate the individual stability profiles of rifampicin, isoniazid and pyrazinamide in the presence of carbopol 934 at various constant temperatures. The (N) on the z-axis is the designation for nitrogen flushing and the numerical figure indicates the percentage of the carbopol used. The following observations were made, regarding the figures 6.2 to 6.10 and they were fairly similar to the observations in chapter 5.

- < Carbopol 934 enhanced the stability of rifampicin at 25 and 40EC, but 5% and 14% of rifampicin still undergoes degradation even with the nitrogen flushing process (figures 6.2 to 6.4).
- < Rifampicin degradation was not influenced by the concentration of the carbopol 934 since the no significant pattern was observed (figures 6.2 to 6.4).
- < Under nitrogen flushing, the degradation of rifampicin was reduced upto approximately 12%. This demonstrates that oxidation played a significant role in rifampicin degradation. The oxidized rifampicin solution exhibited a darker orange-red colour with more precipitation (figures 6.2 to 6.4).
- < As the temperature increased, the rifampicin degradation increased approximately 60%. An obvious solution colour change from translucent reddish-orange to opaque black-orange-red was observed as the storage temperature increased (figures 6.2 to 6.4).

- < The carbopol 934 suspension withstood the 7-day trial at both 25 and 40EC but lasted only 3 days at 60EC. In the presence of aqueous solution, isoniazid stability is highly temperature dependant, with approximately a 17.0 ± 2.0 % degradation increment observed as the temperature increased from 25 to 60EC. The solution turned from colourless to light yellow (figures 6.2 to 6.4).

- < After examining the stability profile of isoniazid, the presence of carbopol 934P did not alter the degradation of isoniazid as the profiles were very similar to each other (figures 6.5 to 6.7).

- < Nitrogen flushing did not contribute any significance in either enhancing or reducing the stability of isoniazid. However, an approximation of upto 1.4 ± 0.5 % instability was expected from the isoniazid solution with nitrogen flushing (figures 6.5 to 6.7).

- < Carbopol 934 suspension enhanced the pyrazinamide stability at 25 and 40EC but failed to do so at 60EC, but pyrazinamide stability is not as temperature dependant as rifampicin and isoniazid (figures 6.8 to 6.10).

- < Nitrogen flushing failed to provide any stabilizing influence on pyrazinamide degradation. Only a 0.5 ± 0.2 % difference was observed between storing in the presence or the absence of oxygen (figures 6.5 to 6.10).

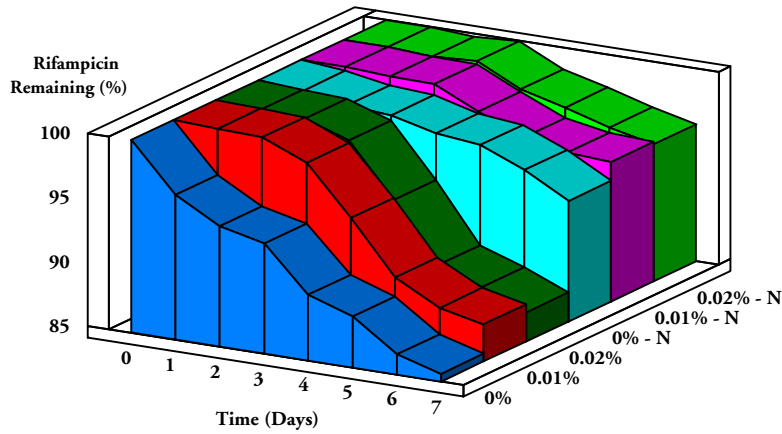


Figure 6.2 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 25EC

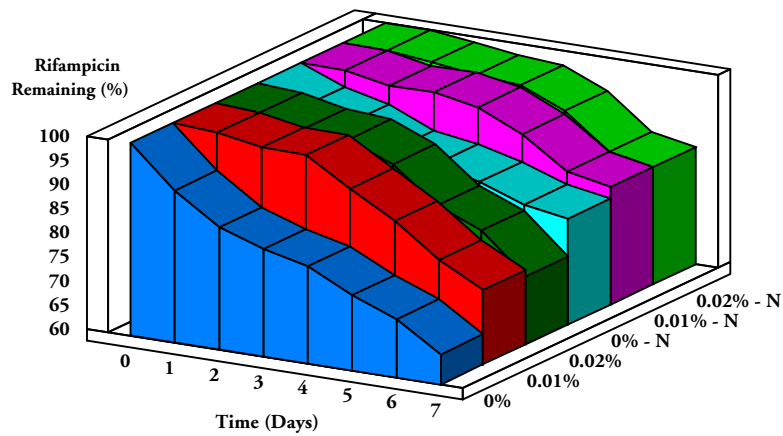


Figure 6.3 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 40EC

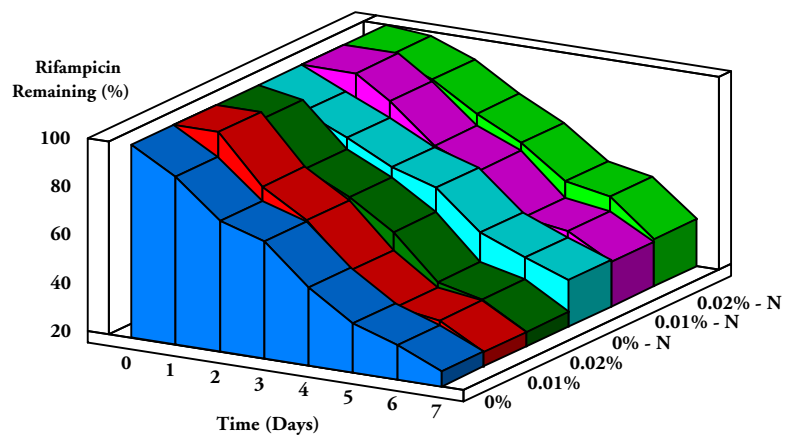


Figure 6.4 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 60EC

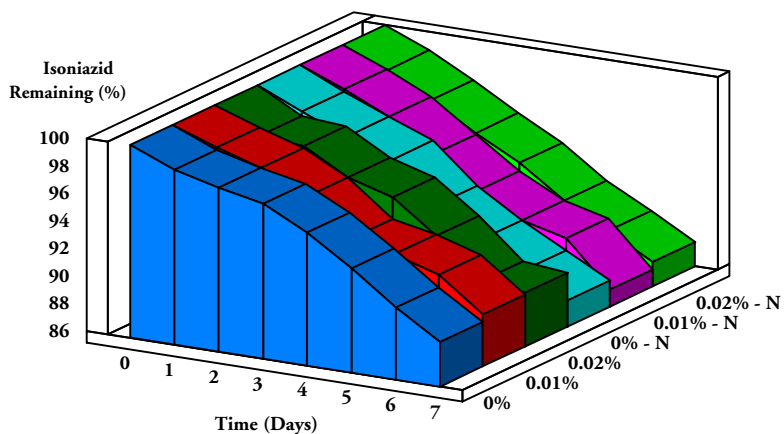


Figure 6.5 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 25EC

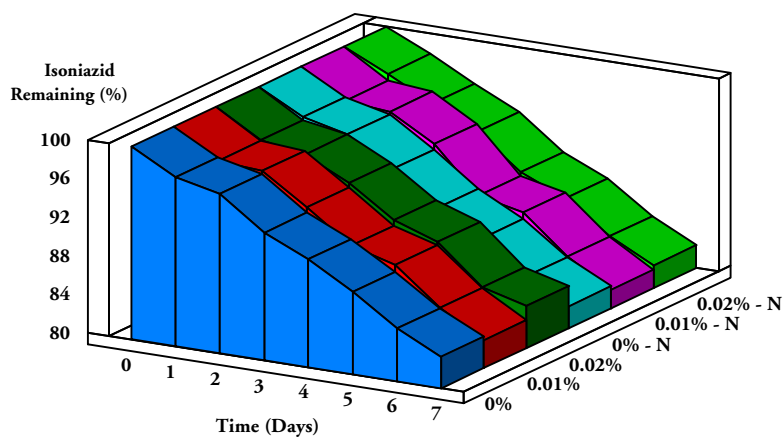


Figure 6.6 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 40EC

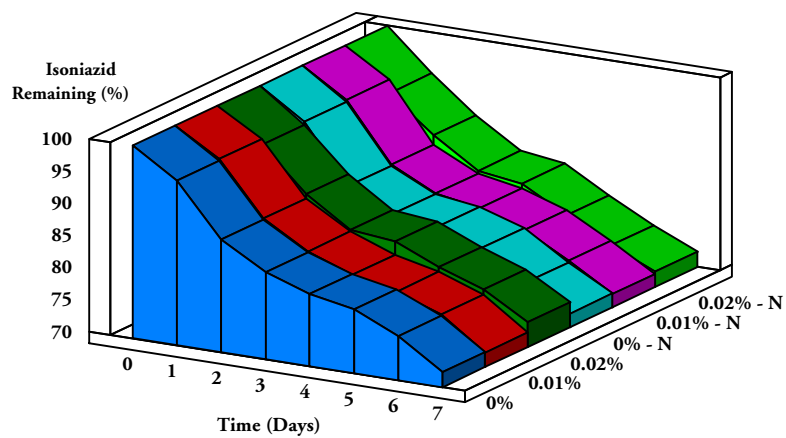


Figure 6.7 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 60EC

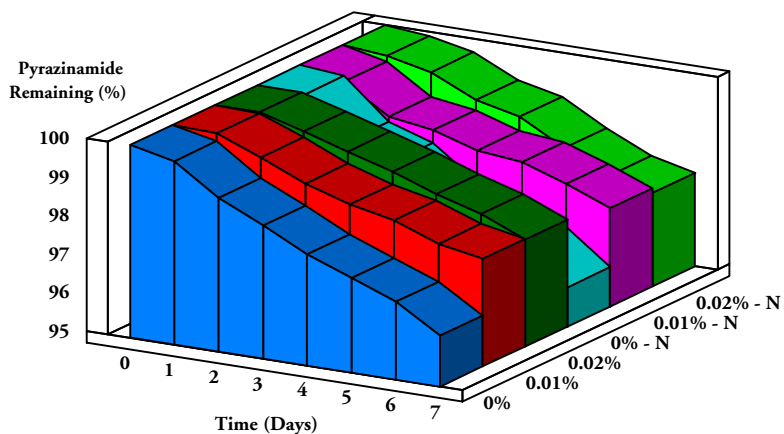


Figure 6.8 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 25°C

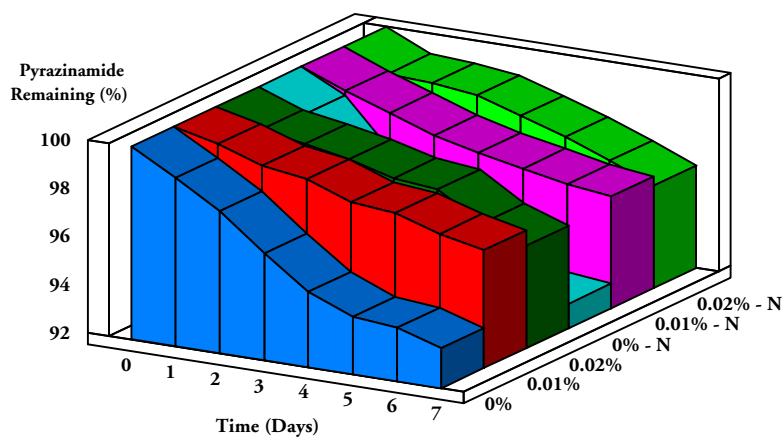


Figure 6.9 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 40°C

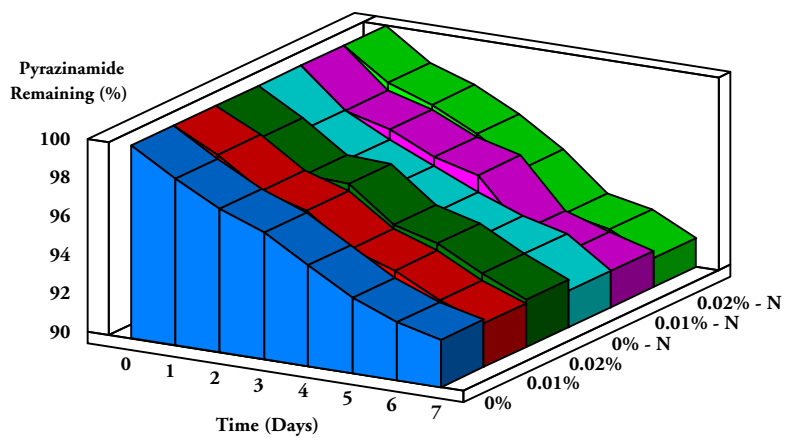


Figure 6.10 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 60°C

The observations regarding the overall degradation profiles of different combinations of drugs (figures 6.11 to 6.19) are as follows:

- < As the concentration of the carbopol 934 increased, the stability of rifampicin in the presence of pyrazinamide was found to improve by 3.0%.
- < The isoniazid and rifampicin interaction decreased as the amount of carbopol 934 increased. The phase segregation of these two drugs enhanced the overall stability of rifampicin by about 8.0% and isoniazid by 1.2%..
- < Although isoniazid stability was not influenced by the suspending agents (as isoniazid was dissolved in water and the hydrolysis could still take place), the interaction between isoniazid and rifampicin or/and pyrazinamide was reduced, as these isoniazid degradation profiles were unaltered regardless of the presence of rifampicin or/and pyrazinamide.
- < Pyrazinamide stability was enhanced by about 7.6% in the form of a suspension and about 1.3 and 1.1% stability enhancement was achieved when combining with rifampicin or isoniazid.

The above behavior was only observed from the samples stored at 25 and 40EC, whereas the suspensions did not withstand the 60EC after 4-days.

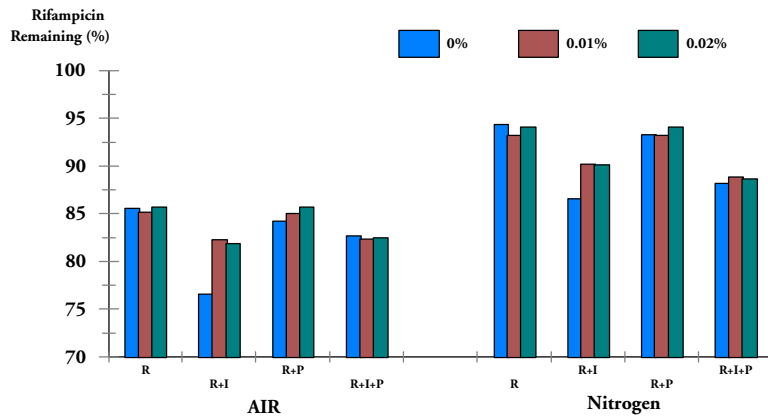


Figure 6.11 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 25EC

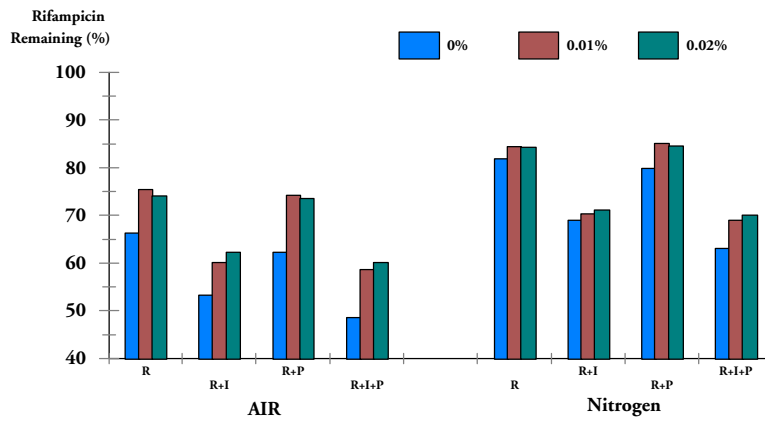


Figure 6.12 Stability Profiles of Rifampicin, (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 40EC

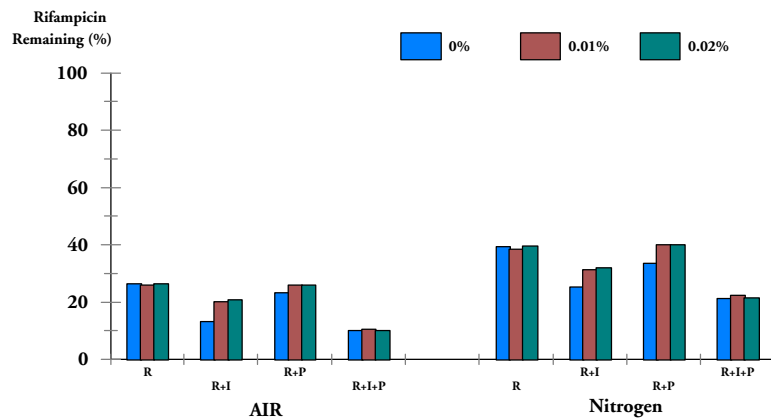


Figure 6.13 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 60EC

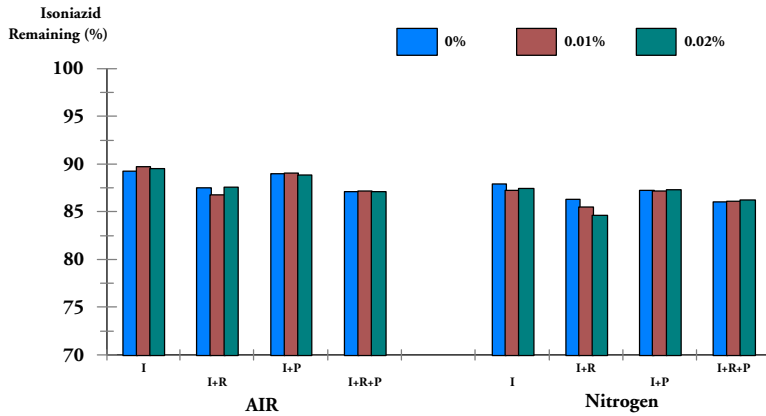


Figure 6.14 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 25°C

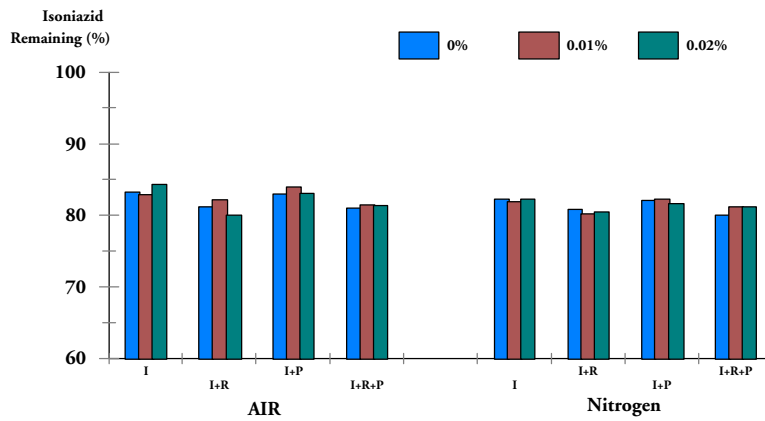


Figure 6.15 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 40°C

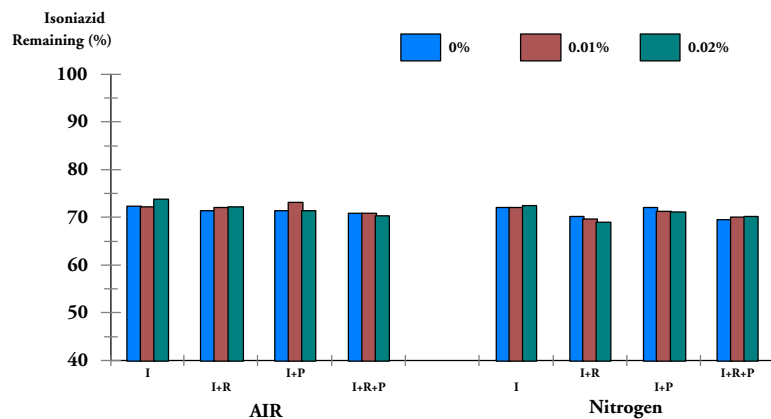


Figure 6.16 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 60°C

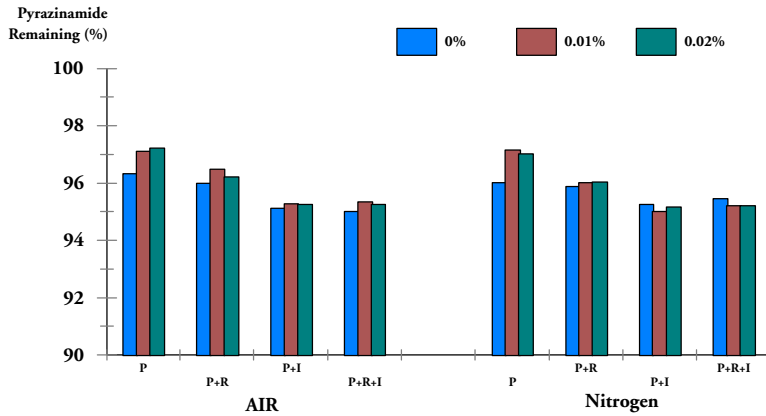


Figure 6.17 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 25EC

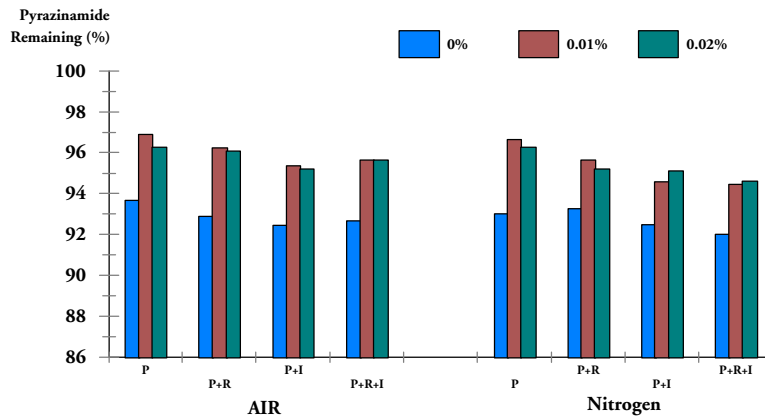


Figure 6.18 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 40EC

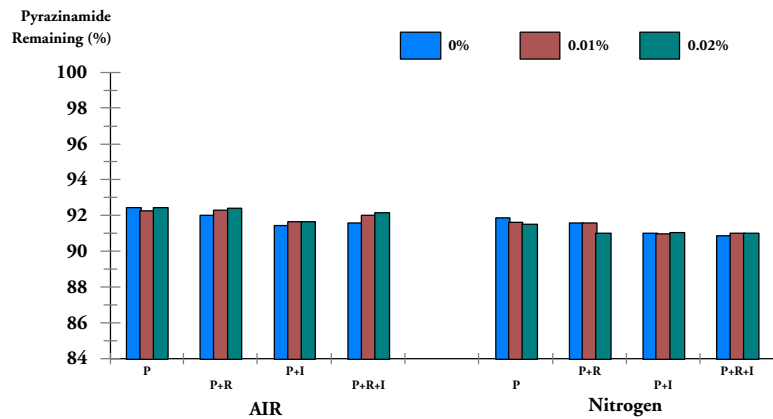


Figure 6.19 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 60EC

6.2.4 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF CARBOPOL 974 P

Two concentrations of carbopol 974P suspension, 0.005% and 0.01%, were prepared. 100mg of rifampicin and 500mg of pyrazinamide were suspended in 20ml carbopol 974P. Isoniazid was dissolved in 20ml of 0.01 and 0.02% carbopol 974P solution which was followed by the addition of sodium hydroxide to neutralize the pH. Therefore due to the high solubility of isoniazid, only rifampicin and pyrazinamide were actually suspended.

The following observations were made:

- < Carbopol 934 and carbopol 974P have a similar stabilizing effect on the degradation of rifampicin, isoniazid and pyrazinamide. The stability profiles of the drugs in the presence of carbopol 974P resembles the stability profiles of the drugs in the presence of carbopol 934 (figure 6.20 to 6.25).
- < As rifampicin and pyrazinamide were suspended as the solid particles and only a small amount of the drug was dissolved in water with subsequent hydrolytic cleavage, thus the stability of these drugs were increased. These enhancements were clearly observed especially when the suspensions were stored at 40EC with approximately 8.9 and 7.5% enhancements observed in the rifampicin and pyrazinamide suspension. Therefore, this correlation clearly indicates that hydrolysis has played a dominant role in the stabilities of these two drugs. However, the stability of isoniazid was not altered as it was already dissolved in the water and therefore hydrolysis still occurred (figures 6.20 to 6.25).
- < The interactions between the drugs were effectively reduced when they are segregated into different phases. This is clearly demonstrated by fact that the stability of the drugs in combination increased up to 12.1% (figure 6.30).

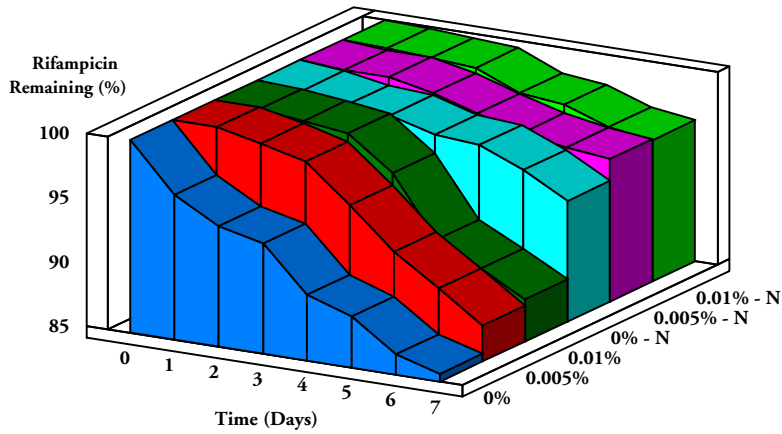


Figure 6.20 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 25EC

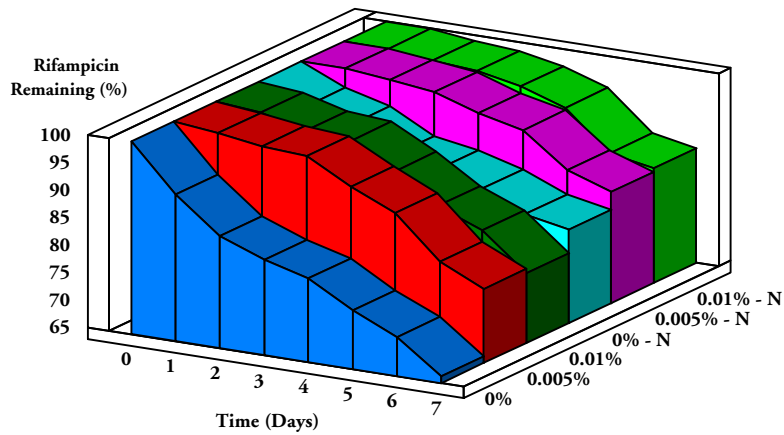


Figure 6.21 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 40EC

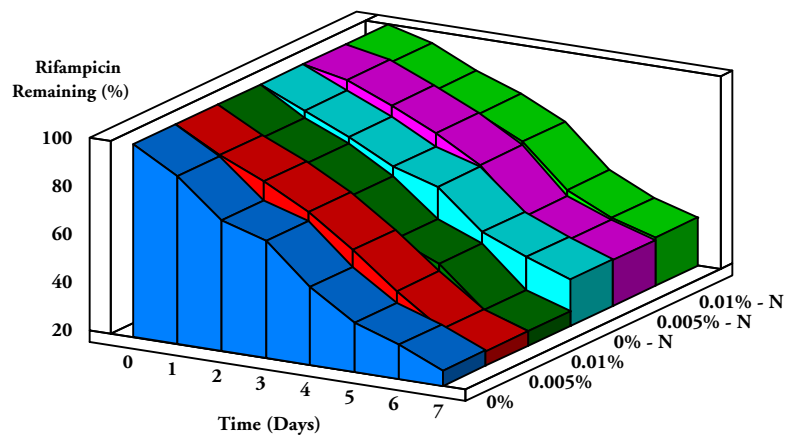


Figure 6.22 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 60EC

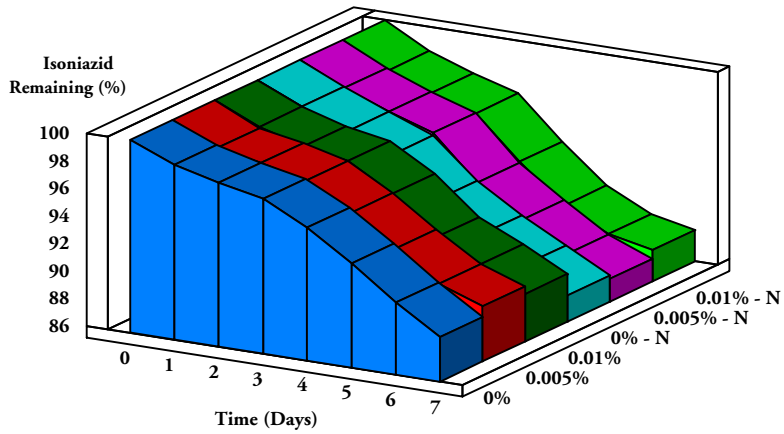


Figure 6.23 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 25EC

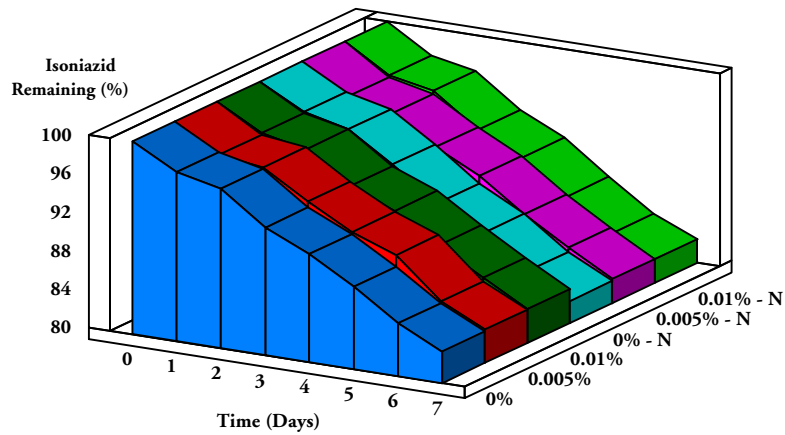


Figure 6.24 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 40EC

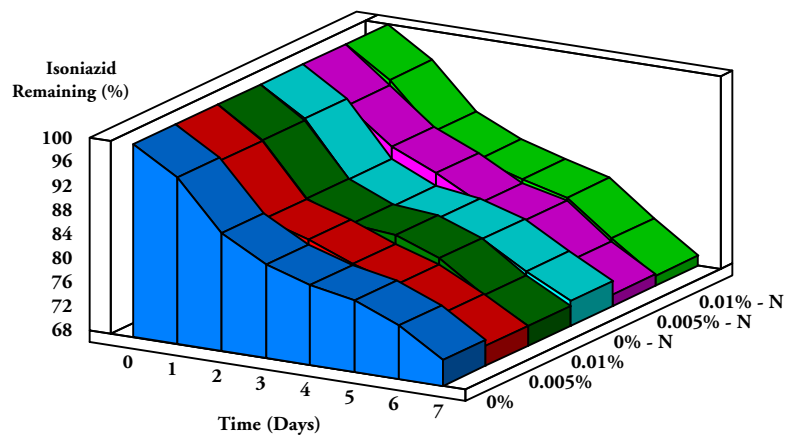


Figure 6.25 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 60EC

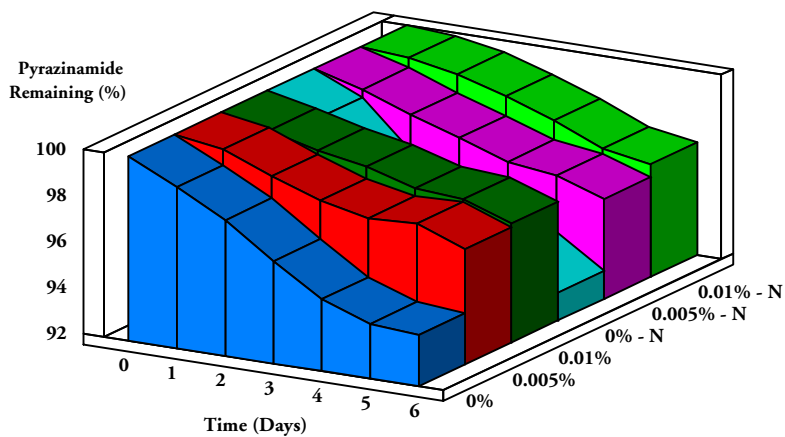


Figure 6.26 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 25EC

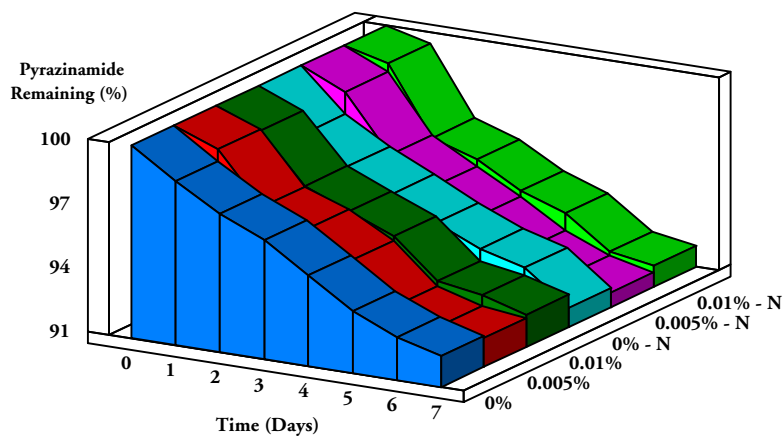


Figure 6.27 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 40EC

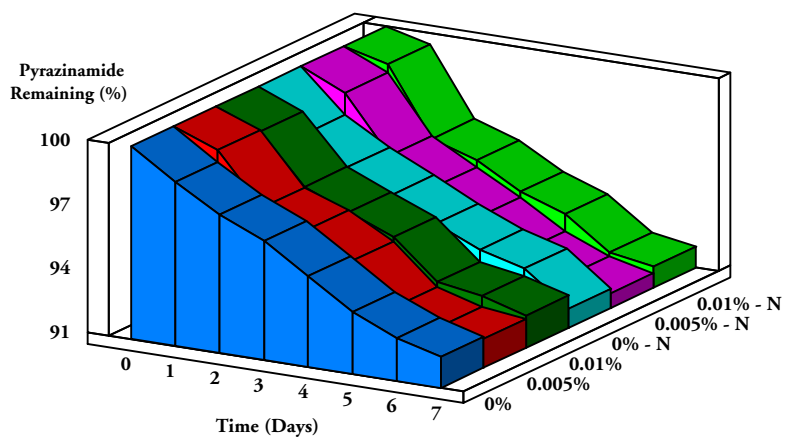


Figure 6.28 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 60EC

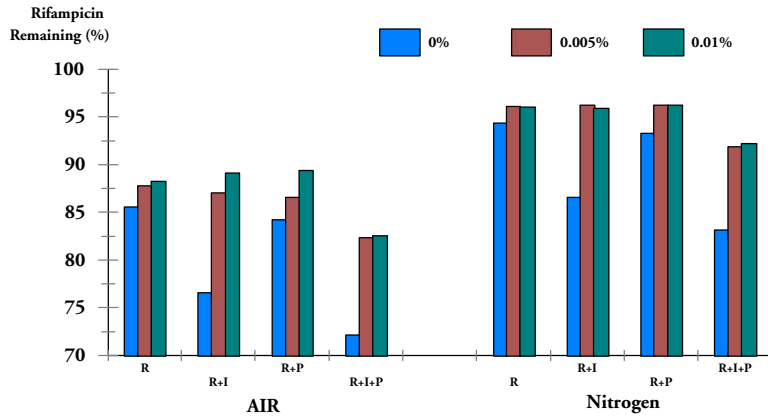


Figure 6.29 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 25EC

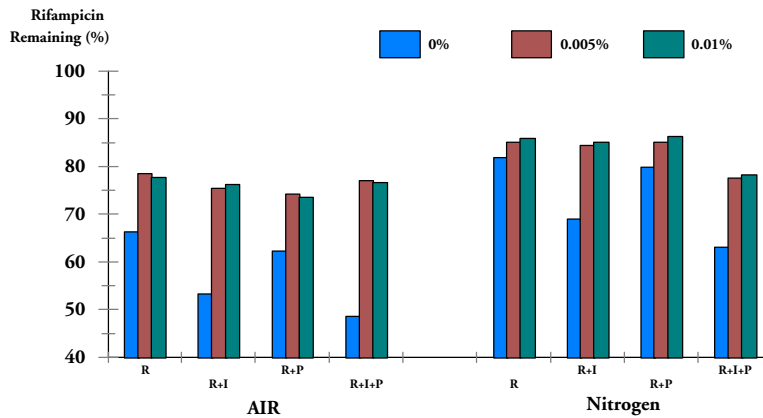


Figure 6.30 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 40EC

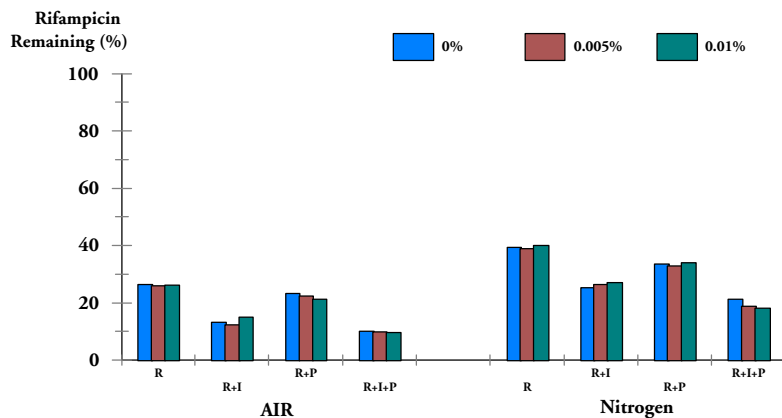


Figure 6.31 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 60EC

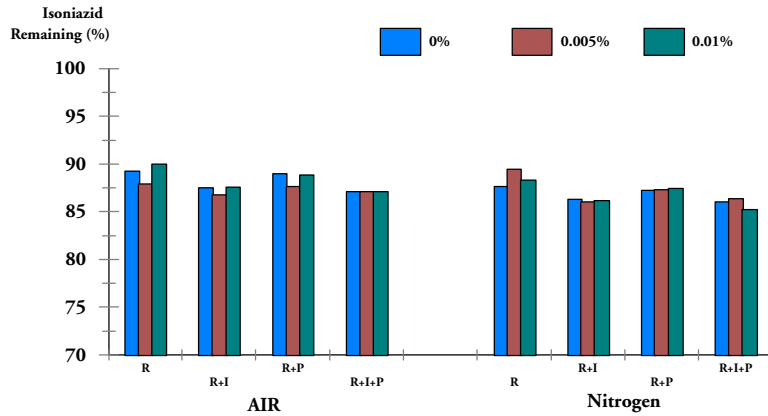


Figure 6.32 Stability Profiles of Isoniazid, (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 25EC

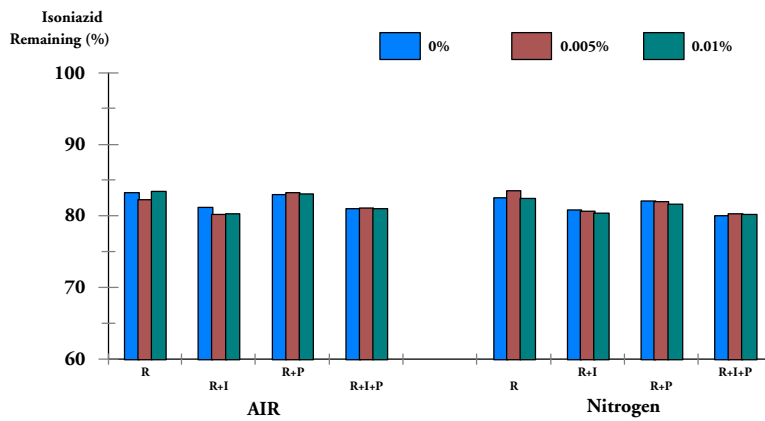


Figure 6.33 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 40EC

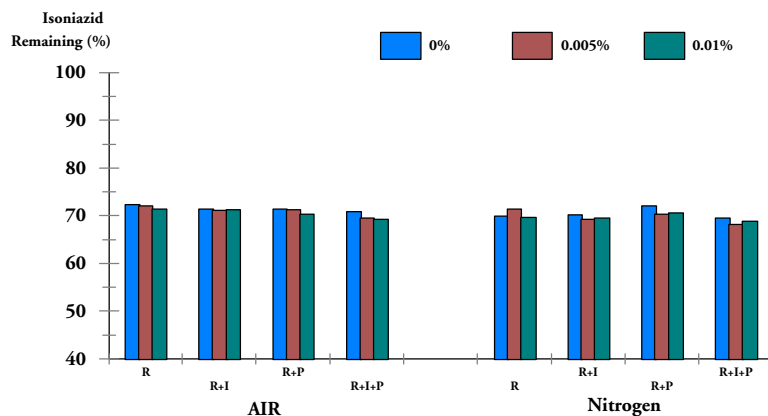


Figure 6.34 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 60EC

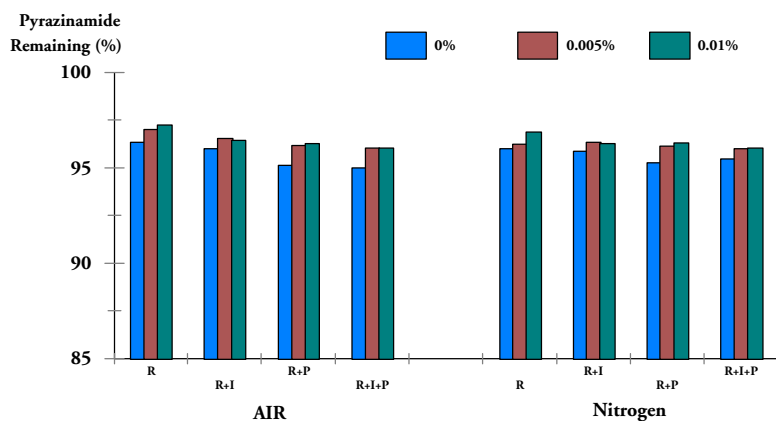


Figure 6.35 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934P at 25EC

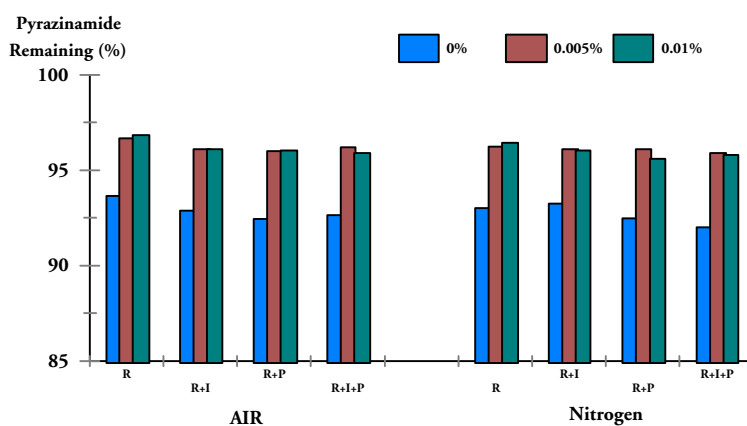


Figure 6.36 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 974P at 40EC

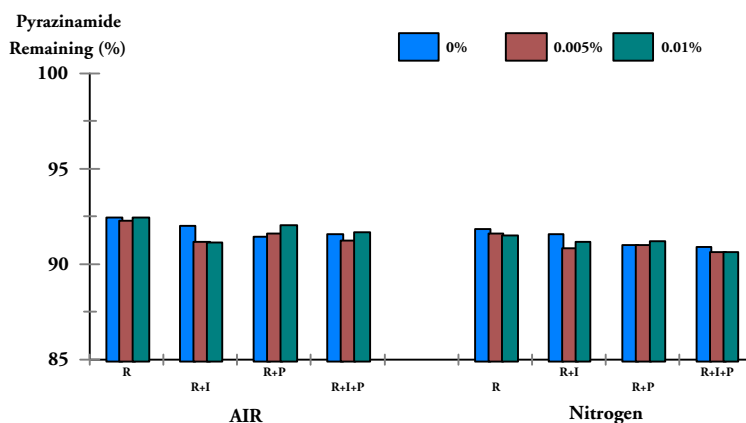


Figure 6.37 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 974P at 60EC

6.3 CONCLUSION

The suspension is frequently used in pharmaceutical industries for formulating the less soluble drugs making use of the thixotropic effect of the suspending agents in order to suspend the drug particles in the formulation [Reynolds et al., 1989]. Since most of the drug particles are presented in the solid phase, the adverse destabilizing effect caused by hydrolysis is reduced.

Carbopol 934 and 974P are excellent suspending agents. Following the neutralization by a suitable base, the repulsion between the negatively charged carboxylate groups on the polymer backbone occurs and induces the carbopols to swell up to 1000 times their original volume. This swelling causes a change in viscosity within the solutions. It was found that the most suitable concentration for the use of suspension lies within carbopol 934 solution of 0.005 to 0.010% and carbopol 974P solutions of 0.001 to 0.003%. These solutions are fairly stable as the viscosity remains within 1.5% range of the original viscosity and this is used as the stability assessment of rifampicin, isoniazid and pyrazinamide.

The carbopol 934 and 974P have very similar stability enhancement effect on rifampicin and pyrazinamide but failed to cause any significant changes to isoniazid as it was present in the aqueous phase. The rifampicin-isoniazid and pyrazinamide-isoniazid interactions were reduced due to the segregation of the drugs into two phases. Although stability of rifampicin and pyrazinamide did not reach the ICH and FDA requirements, these stability assessments indicate that in order to achieve a more stable formulation in the aqueous media, the potential degradation sites of the drug molecules need to be protected.

CHAPTER SIX

STABILITY STUDIES OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE IN THE PRESENCE OF CARBOPOL SUSPENDING AGENTS

6.1 INTRODUCTION

In the previous chapter, it was clear, that although the solubility requirement of rifampicin was met by employing the poloxamers, the desired concentration of pyrazinamide was not achieved in the presence of any mentioned surfactants. Moreover, the stabilities of the drugs were found to be below both the ICH and FDA regulatory requirements [Carstensen et al., 1995]. While the interactions between the drugs seem to be a destabilizing factor, hydrolysis still remains as a main obstacle in achieving a satisfactory paediatric formulation. In this chapter, an attempt is made to bypass these problems by making use of the suspension. In consultation with Pharmacare-Lennon[®], two suspending agents were chosen to be part of the study, namely carbopol 934 and 974P.

Suspensions are frequently used in pharmaceutical industries for the formulating of less soluble drugs as a liquid dosage form. A suspension generally employs suspending agents which are described as the compounds which have the property of increasing viscosity of the solution in which they are dissolved or dispersed. The resulting thixotropic effect of the suspending agents contributes to their usefulness [Reynolds, 1989].

Viscosity is the measurement of the force per unit area required to maintain a certain rate of flow. The viscosity of a solution is measured in poise (1 dyne-sec/cm²) or centipoise (100cP = 1 Poise) and can easily be determined by using either an Ostwald or rotating drum viscometer (section 6.2.1). The presence of macromolecular solutes, such as various carbopols, may enhance the viscosity of a solution because the bulky molecules affect the fluid flow within the system [Atkins, 1990].

Carbopol resins also known as carbomers, the commercial name of polyacrylic acid, consist of an acrylic backbone with polyalkenyl ethers or divinyl glycol. The main differences between the various carbopols are related to the presence of monomer and cross-linked density, but all of the resins have a superior thixotropic capability. The carbopol resins are flocculated hygroscopic powders with each particle averaging from 2 to 7 microns. These agglomerates cannot be broken down into particles once produced and these powders have a white and fluffy appearance with a pK_a of 6.0 ± 0.5 and a pH of 2.5-3.0 when dispersed as a 1.0% aqueous solution. The molecular weight is estimated to be in the region of 700,000 to 3 or 4 billion Daltons, however, the actual weight is not able to be determined by any current methods. Linear polymers are very soluble in water and able to swell to 1000 times their original volume when exposed to a pH environment above 4.0-6.0 and result in a viscous gel. This is due to the fact that the resins have an average equivalent weight of 76 per carboxyl group and thus the repulsion between the negatively charged carboxylate groups on the polymer backbone causes the carbopol to swell [BF Goodrich, 1994].

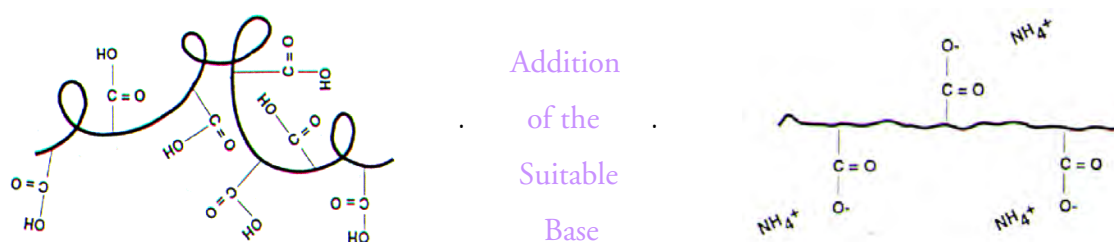


Figure 6.1. Carbopol Chain Uncoiling

Carbopol resins have been used in oral suspensions worldwide and are most suitable for the aqueous based suspension formulation. The excellent abilities of carbopol to thicken, modify flow rate characteristics, suspend insoluble ingredients, and provide bioadhesion have resulted in their use in pharmaceutical research/industry for many years. Carbopol resins swell when hydrated and when neutralized with an adequate base, the resins are soluble in water, most alcohols and glycerol, forming a very viscous colloidal dispersion. The insoluble ingredients in the suspension are trapped in the interstitial spaces between the hydrogel particles permanently. Furthermore, carbopol resin microgels are easily moved by shear forces but reform the macro-gel immediately once the force is removed. This immediate recovery enables the suspension to be easily dispersed or redispersed and

facilitates stirring or pumping. At a viscosity of between 1500 to 2500cPs carbopol resins provide a permanently stable suspension while most of the other suspending agents fails to do so. Other key benefits of using carbopol as suspending agent include [BF Goodrich, 1994]:

- < It yields high viscosity values even at low concentration. At a 0.1 - 1.5% carbopol resin concentration, the suspension is a viscoelastic liquid, but when over 3.0%, the suspension becomes a viscoelastic solid.
- < It masks the unpleasant taste of the drug and this is ideal since rifampicin has a bitter taste.
- < It increases the bioavailability of the drug which increases the effectivity and benefit to the patients.
- < It is versatile over a wide range of pHs and this allows the pH to be set according to the stabilities of the drugs without disturbing the thixotropic effect.
- < It has antifungal and antibacterial properties.
- < It has low toxicity and carbopol is approved by the ICH and FDA. The toxicity studies have been extensively conducted on carbopol 934 resin in rats, mice, guinea pig and dogs. Depending on the specie tested, the studies have shown that an acute toxicity of the oral LD₅₀ has ranged from 2.5g/kg to greater than 40.0g/kg body weight. No significant side effects have been shown except a slight growth suppressing syndrom with a 21-days continuous intake of carbopol higher than 5.0% of per total weight of daily diet. But in general, no related pathological, histopathological, hematological, reproductive, gastro-intestinal and urinary adverse effects have been ever found. Eye and skin irritation can result from a carbopol 934 solution of 1.0% or above this concentration. The toxicity studies of carbopol 974P showed that a 13-week period of oral administration of carbopol 974P at the concentration of 50,000 ppm or above induced significant signs of chronic histopathological inflammation. Weight loss may also increase as the dose concentration of carbopol 974P increases. Carbopol 974P did not induce hazardous eye and dermal irritation if the concentration of the carbopol 974 solution is below 1.0% [BF Goodrich, 1994] and the acute oral LD₅₀ is determined to be greater than 2.0g/kg.

Carbopol 934 and carbopol 974P (BF Goodrich, Cleveland) were kindly donated by Pharmacare-Lennon®. The poor aqueous solubilities of rifampicin and pyrazinamide are no longer a problem when they can be suspended within the formulation. The main considerations are in the stabilities which is divided into the physical and chemical aspects. The physical stabilities aspect includes the viscosity and redispersibility whereas the chemical stabilities aspect includes the stabilities of the rifampicin, isoniazid and pyrazinamide.

6.2 EXPERIMENTAL RESULTS AND OBSERVATIONS

6.2.1 KARL FISCHER EXPERIMENTS

Karl Fischer experiments (section 4.1.3.) carried out showed that the water content of carbopol 934 and carbopol 974P were found to be negligible. Thus it was not necessary to take this into consideration in the weights used in further experiments.

6.2.2 VISCOSITY AND REDISPERSIBILITY

Various concentrations of carbopol for suspension formulation were made up by dissolving the required amount of carbopol powders in water. They were adjusted with sodium hydroxide to the a pH range of 7.10 ± 0.01 until a translucent viscous liquid was achieved. The viscosity of the carbopol 934 and 974P at different concentrations was tested and is listed in tables 6.1 to 6.2 and clearly demonstrates that the optimum concentration range of carbopol 934 and carbopol 974P is between 0.01% to 0.02% and 0.005% to 0.01% respectively

All viscosities of all the solutions were found to be constant at 25EC for at least 4 weeks except the 0.005 carbopol 934 solutions and the 0.001 and 0.003% carbopol 974P solutions in which the suspensions deteriorated.

Table 6.1 Viscosity of Different Concentrations of Carbopol 934 Solutions

Viscosity and Redispersibility of Carbopol 934					
<i>Concentration</i> (%)	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>	<i>Concentration</i> (%)	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>
0.005	880	+	0.025	2560	++++
0.010	1290	++	0.030	2800	++++
0.015	1850	++	0.035	3150	++++
0.020	2100	+++	0.040	3700	+++++

Table 6.1 Viscosity of Different Concentrations of Carbopol 974P Solutions

Viscosity and Redispersibility of Carbopol 974P					
<i>Concentration</i>	<i>Viscosity</i> (cPS)	<i>Redispersibility</i>	<i>Concentration</i>	<i>Viscosity</i> (cPs)	<i>Redispersibility</i>
0.001	660	+	0.010	2400	+++
0.003	1050	++	0.015	2850	++++
0.005	1760	+++	0.020	3350	++++
0.007	2110	+++	0.025	3800	+++++

Note:

>+ = Low redispersibility due to the viscosity being too low

#++ = Acceptable redispersibility

\$+++ = Acceptable redispersibility but with a slightly higher viscosity

<+++ = Low redispersibility due to the viscosity is too high

6.2.3 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF CARBOPOL 934P

0.02% is the concentration of the carbopol 934 solution used in the stability studies of rifampicin, isoniazid and pyrazinamide. A 0.01% carbopol 934 solution was also incorporated in order to demonstrate the stabilizing effect of carbopol 934 concentration on the drugs. 100mg of rifampicin

and 500mg of pyrazinamide were suspended in 20ml carbopol 934. Isoniazid was dissolved in 20ml of 0.01 and 0.02% carbopol 934 solutions which is followed by the addition of sodium hydroxide to neutralize the pH. Therefore due to the high solubility of isoniazid, only rifampicin and pyrazinamide were actually suspended.

The methodology for the stability studies adopted the validated HPLC analysis (refer to section 3.2 and 3.3) on a daily basis. The results are presented as the percentage of drug remaining against the time of analyses (figures 6.2 to 6.10) and (figures 6.11 to 6.20).

The results shown in figures 6.2 to 6.10 illustrate the individual stability profiles of rifampicin, isoniazid and pyrazinamide in the presence of carbopol 934 at various constant temperatures. The (N) on the z-axis is the designation for nitrogen flushing and the numerical figure indicates the percentage of the carbopol used. The following observations were made, regarding the figures 6.2 to 6.10 and they were fairly similar to the observations in chapter 5.

- < Carbopol 934 enhanced the stability of rifampicin at 25 and 40EC, but 5% and 14% of rifampicin still undergoes degradation even with the nitrogen flushing process (figures 6.2 to 6.4).
- < Rifampicin degradation was not influenced by the concentration of the carbopol 934 since the no significant pattern was observed (figures 6.2 to 6.4).
- < Under nitrogen flushing, the degradation of rifampicin was reduced upto approximately 12%. This demonstrates that oxidation played a significant role in rifampicin degradation. The oxidized rifampicin solution exhibited a darker orange-red colour with more precipitation (figures 6.2 to 6.4).
- < As the temperature increased, the rifampicin degradation increased approximately 60%. An obvious solution colour change from translucent reddish-orange to opaque black-orange-red was observed as the storage temperature increased (figures 6.2 to 6.4).

- < The carbopol 934 suspension withstood the 7-day trial at both 25 and 40EC but lasted only 3 days at 60EC. In the presence of aqueous solution, isoniazid stability is highly temperature dependant, with approximately a 17.0 ± 2.0 % degradation increment observed as the temperature increased from 25 to 60EC. The solution turned from colourless to light yellow (figures 6.2 to 6.4).

- < After examining the stability profile of isoniazid, the presence of carbopol 934P did not alter the degradation of isoniazid as the profiles were very similar to each other (figures 6.5 to 6.7).

- < Nitrogen flushing did not contribute any significance in either enhancing or reducing the stability of isoniazid. However, an approximation of upto 1.4 ± 0.5 % instability was expected from the isoniazid solution with nitrogen flushing (figures 6.5 to 6.7).

- < Carbopol 934 suspension enhanced the pyrazinamide stability at 25 and 40EC but failed to do so at 60EC, but pyrazinamide stability is not as temperature dependant as rifampicin and isoniazid (figures 6.8 to 6.10).

- < Nitrogen flushing failed to provide any stabilizing influence on pyrazinamide degradation. Only a 0.5 ± 0.2 % difference was observed between storing in the presence or the absence of oxygen (figures 6.5 to 6.10).

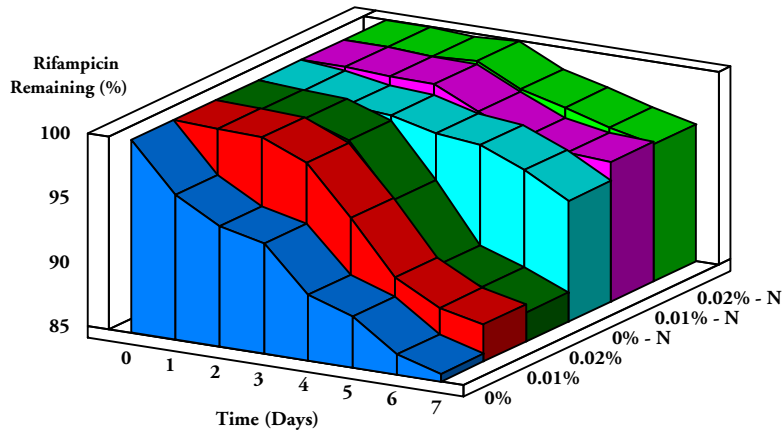


Figure 6.2 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 25EC

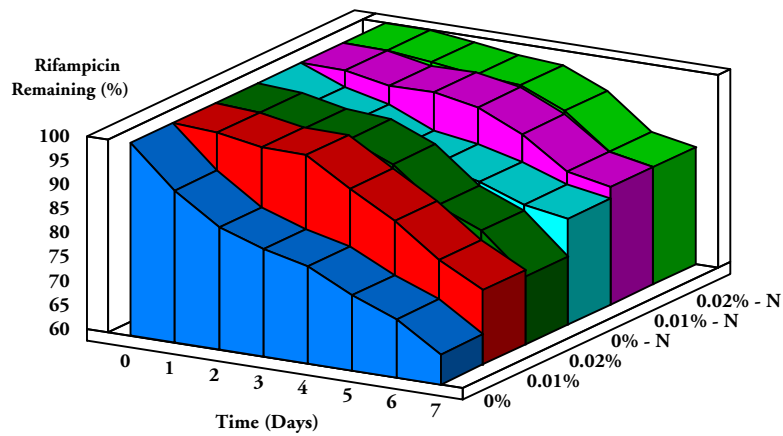


Figure 6.3 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 40EC

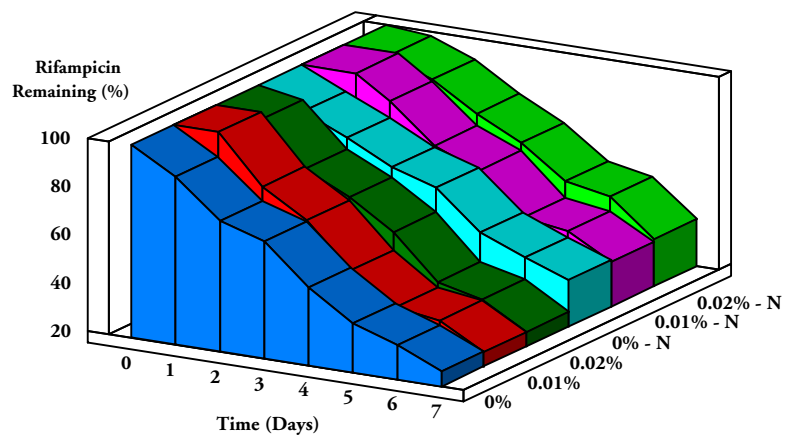


Figure 6.4 Stability Profiles of Rifampicin in the Presence of Carbopol 934 at 60EC

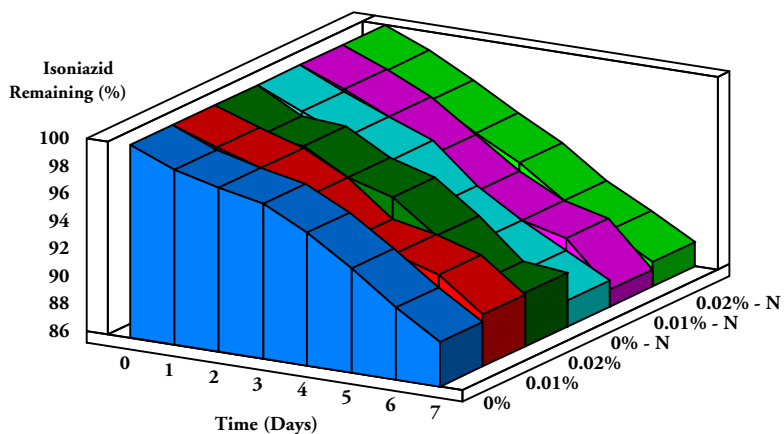


Figure 6.5 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 25EC

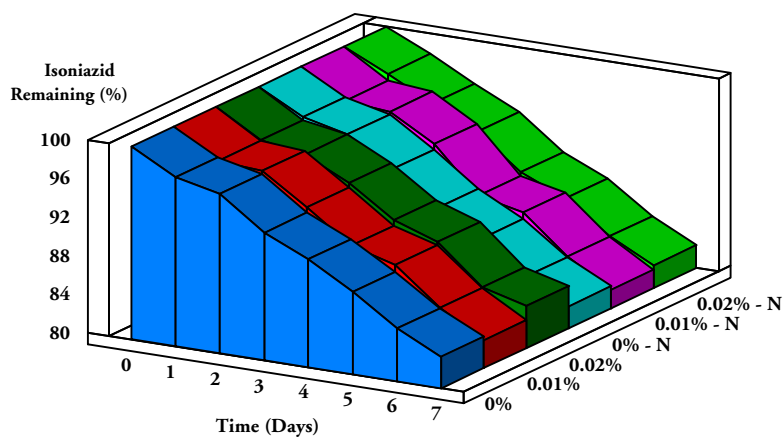


Figure 6.6 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 40EC

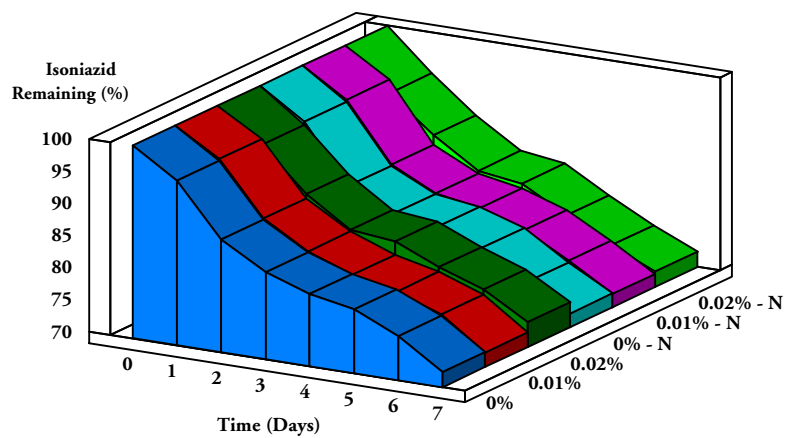


Figure 6.7 Stability Profiles of Isoniazid in the Presence of Carbopol 934 at 60EC

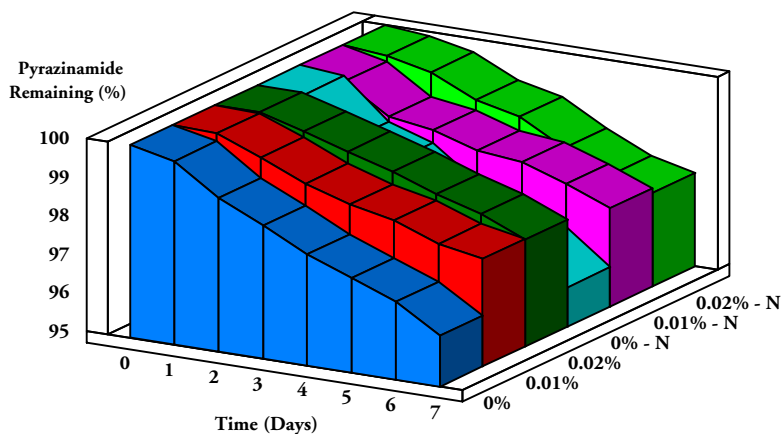


Figure 6.8 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 25°C

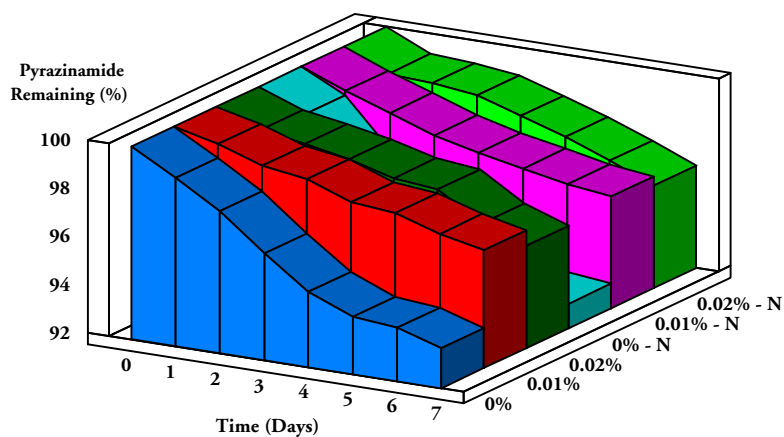


Figure 6.9 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 40°C

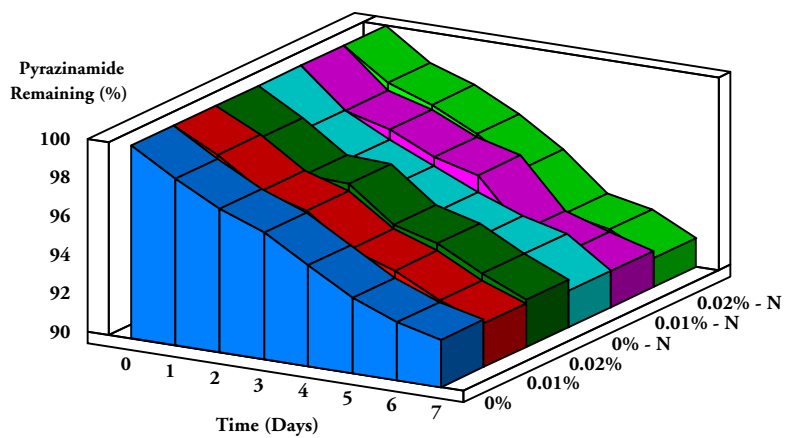


Figure 6.10 Stability Profiles of Pyrazinamide in the Presence of Carbopol 934 at 60°C

The observations regarding the overall degradation profiles of different combinations of drugs (figures 6.11 to 6.19) are as follows:

- < As the concentration of the carbopol 934 increased, the stability of rifampicin in the presence of pyrazinamide was found to improve by 3.0%.
- < The isoniazid and rifampicin interaction decreased as the amount of carbopol 934 increased. The phase segregation of these two drugs enhanced the overall stability of rifampicin by about 8.0% and isoniazid by 1.2%..
- < Although isoniazid stability was not influenced by the suspending agents (as isoniazid was dissolved in water and the hydrolysis could still take place), the interaction between isoniazid and rifampicin or/and pyrazinamide was reduced, as these isoniazid degradation profiles were unaltered regardless of the presence of rifampicin or/and pyrazinamide.
- < Pyrazinamide stability was enhanced by about 7.6% in the form of a suspension and about 1.3 and 1.1% stability enhancement was achieved when combining with rifampicin or isoniazid.

The above behavior was only observed from the samples stored at 25 and 40EC, whereas the suspensions did not withstand the 60EC after 4-days.

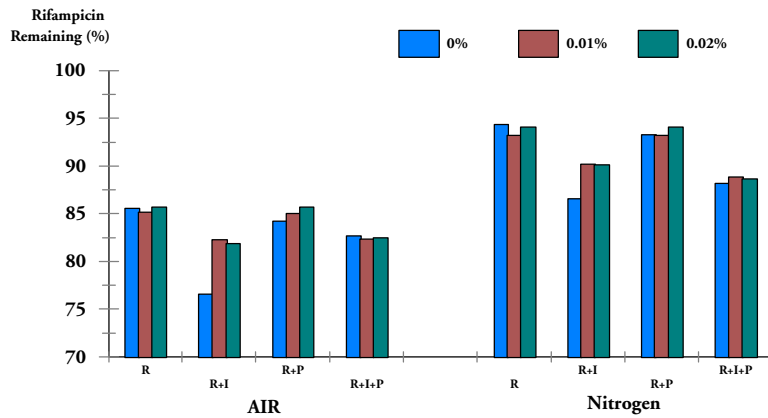


Figure 6.11 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 25EC

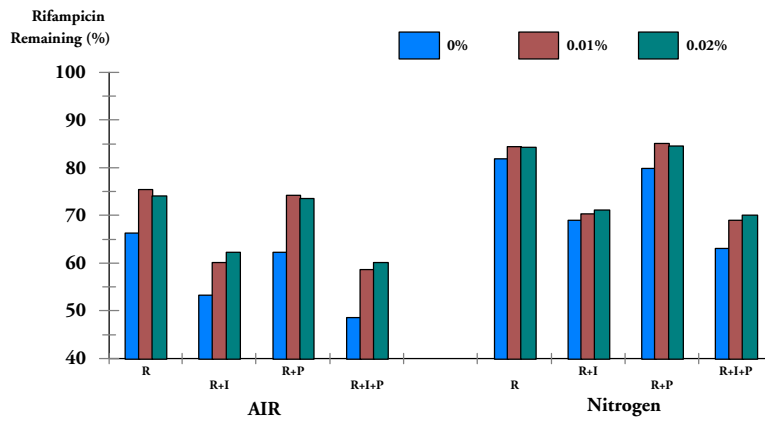


Figure 6.12 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 40EC

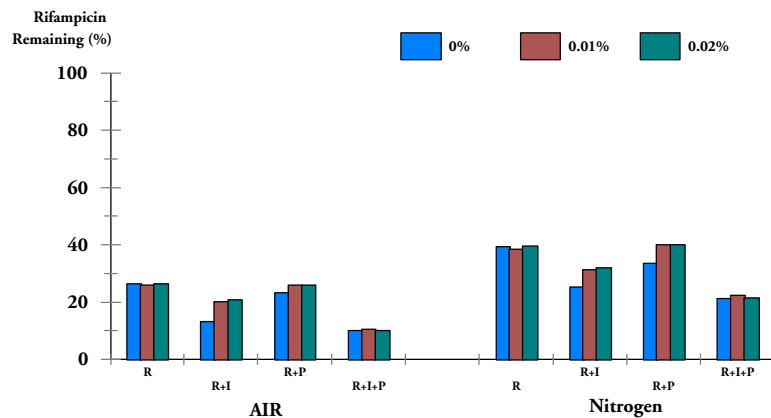


Figure 6.13 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 934 at 60EC

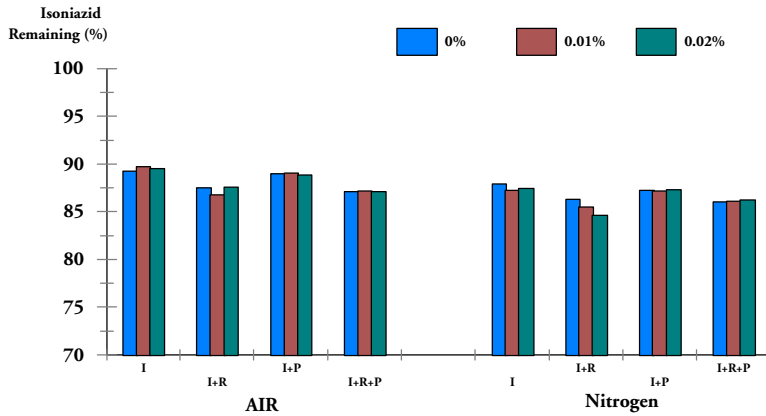


Figure 6.14 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 25°C

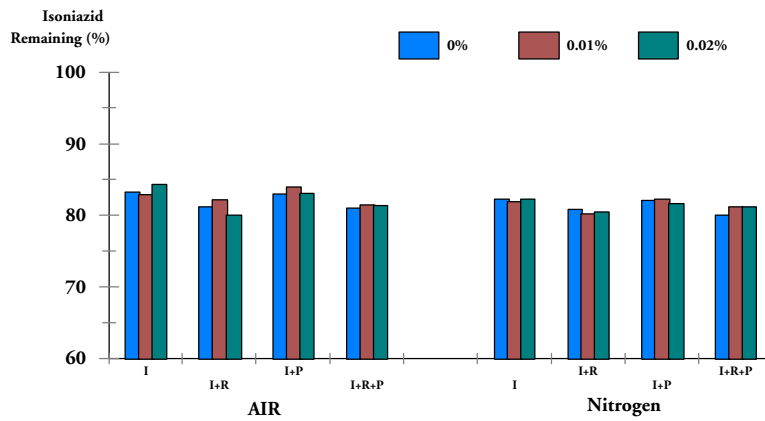


Figure 6.15 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 40°C

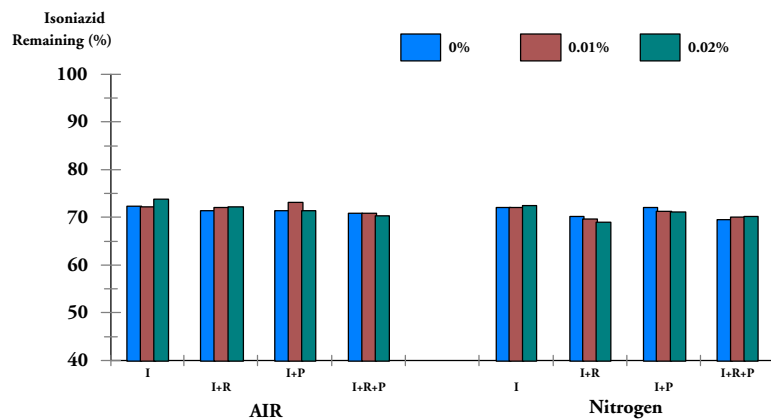


Figure 6.16 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 934 at 60°C

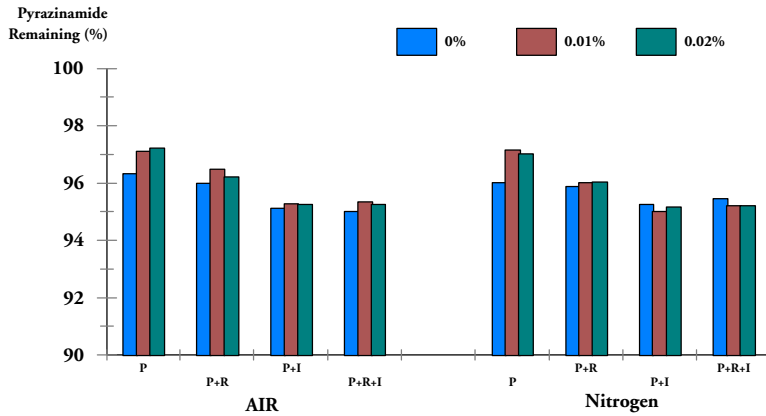


Figure 6.17 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 25EC

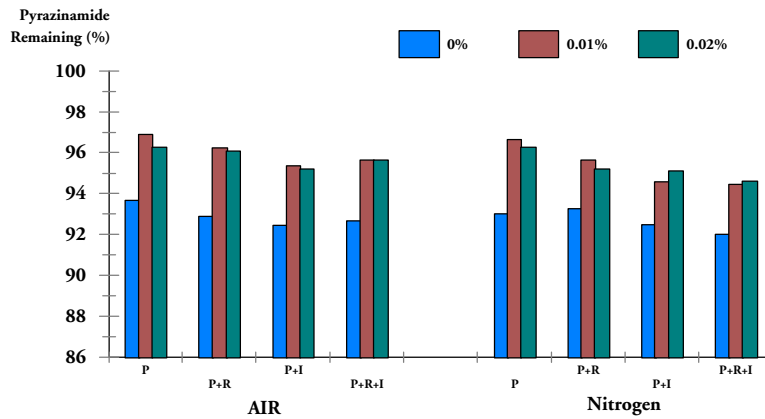


Figure 6.18 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 40EC

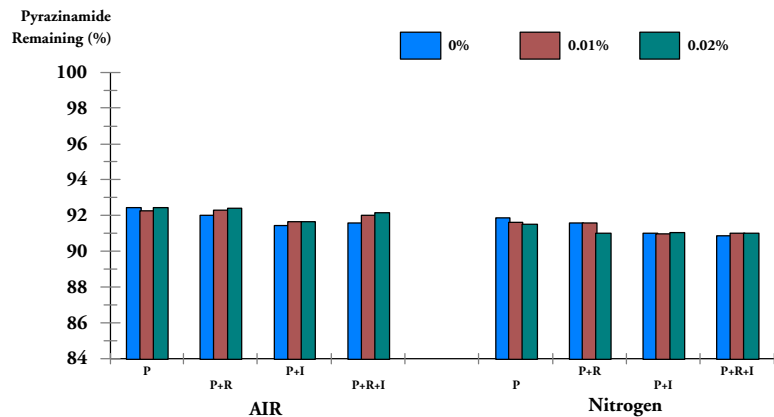


Figure 6.19 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934 at 60EC

6.2.4 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF CARBOPOL 974 P

Two concentrations of carbopol 974P suspension, 0.005% and 0.01%, were prepared. 100mg of rifampicin and 500mg of pyrazinamide were suspended in 20ml carbopol 974P. Isoniazid was dissolved in 20ml of 0.01 and 0.02% carbopol 974P solution which was followed by the addition of sodium hydroxide to neutralize the pH. Therefore due to the high solubility of isoniazid, only rifampicin and pyrazinamide were actually suspended.

The following observations were made:

- < Carbopol 934 and carbopol 974P have a similar stabilizing effect on the degradation of rifampicin, isoniazid and pyrazinamide. The stability profiles of the drugs in the presence of carbopol 974P resembles the stability profiles of the drugs in the presence of carbopol 934 (figure 6.20 to 6.25).
- < As rifampicin and pyrazinamide were suspended as the solid particles and only a small amount of the drug was dissolved in water with subsequent hydrolytic cleavage, thus the stability of these drugs were increased. These enhancements were clearly observed especially when the suspensions were stored at 40EC with approximately 8.9 and 7.5% enhancements observed in the rifampicin and pyrazinamide suspension. Therefore, this correlation clearly indicates that hydrolysis has played a dominant role in the stabilities of these two drugs. However, the stability of isoniazid was not altered as it was already dissolved in the water and therefore hydrolysis still occurred (figures 6.20 to 6.25).
- < The interactions between the drugs were effectively reduced when they are segregated into different phases. This is clearly demonstrated by fact that the stability of the drugs in combination increased up to 12.1% (figure 6.30).

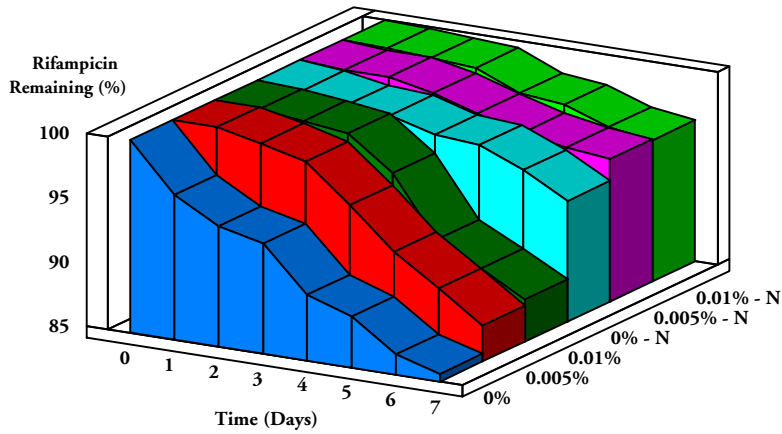


Figure 6.20 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 25EC

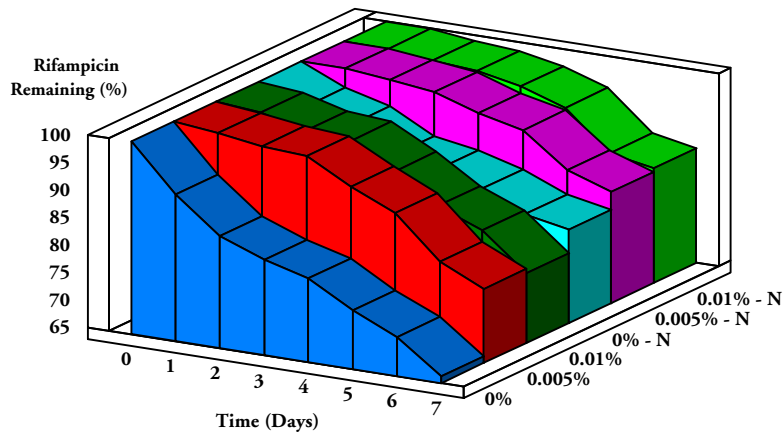


Figure 6.21 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 40EC

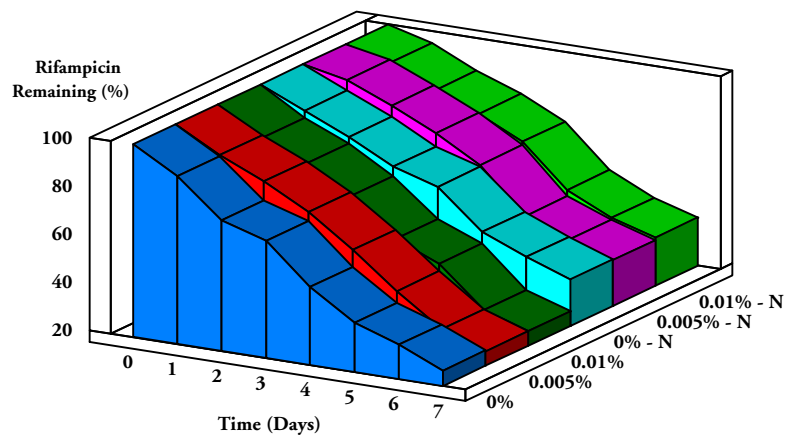


Figure 6.22 Stability Profiles of Rifampicin in the Presence of Carbopol 974P at 60EC

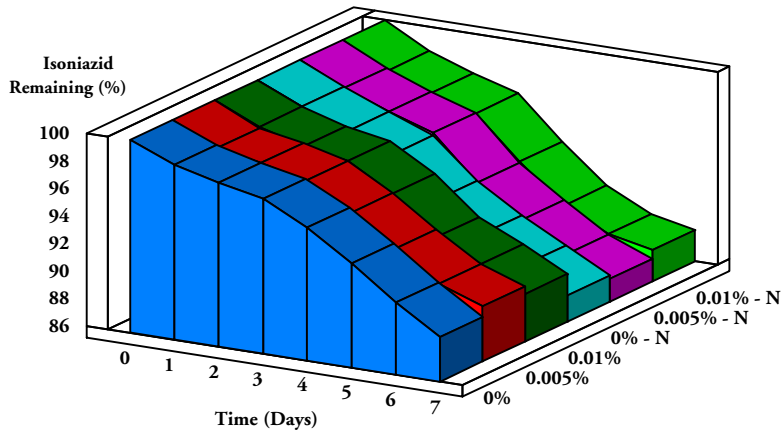


Figure 6.23 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 25EC

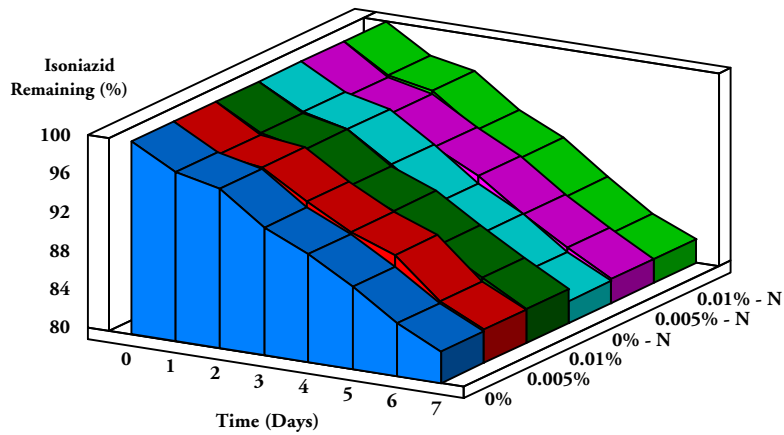


Figure 6.24 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 40EC

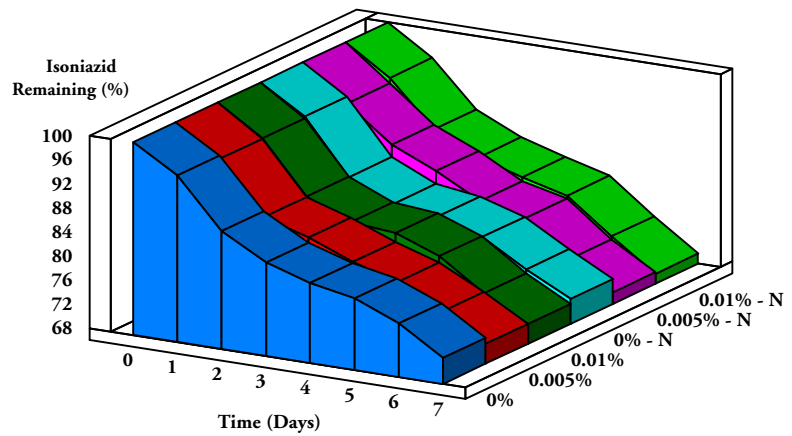


Figure 6.25 Stability Profiles of Isoniazid in the Presence of Carbopol 974P at 60EC

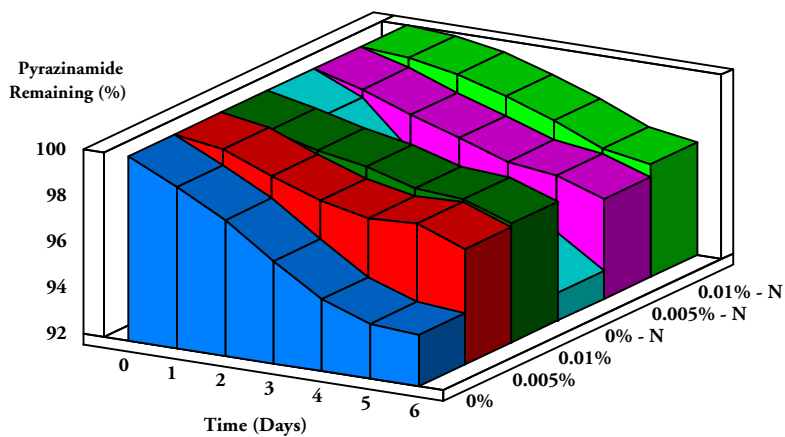


Figure 6.26 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 25EC

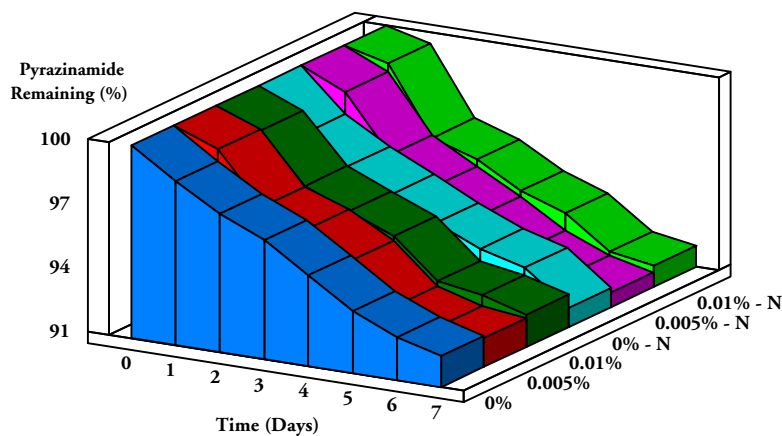


Figure 6.27 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 40EC

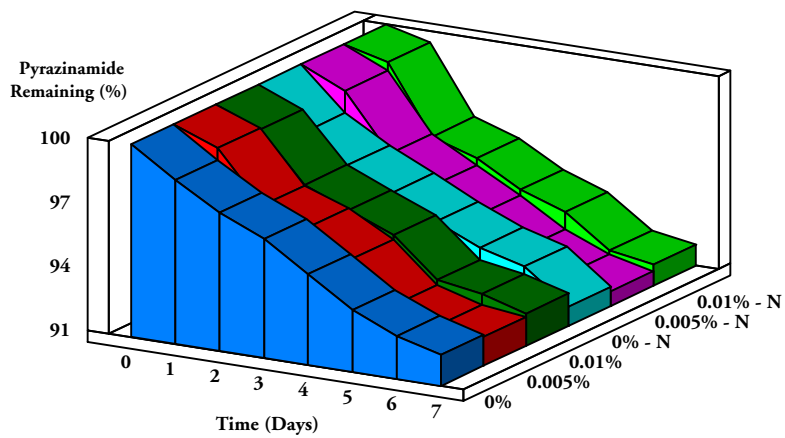


Figure 6.28 Stability Profiles of Pyrazinamide in the Presence of Carbopol 974P at 60EC

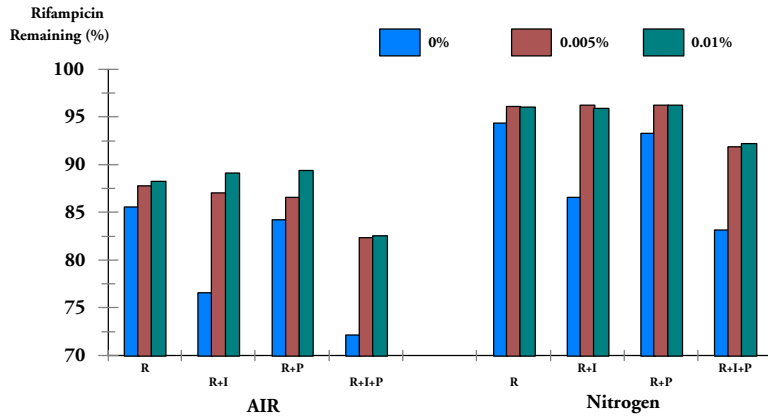


Figure 6.29 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 25EC

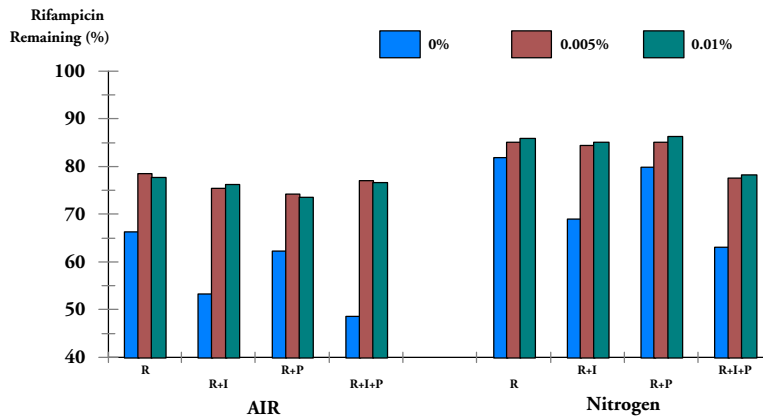


Figure 6.30 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 40EC

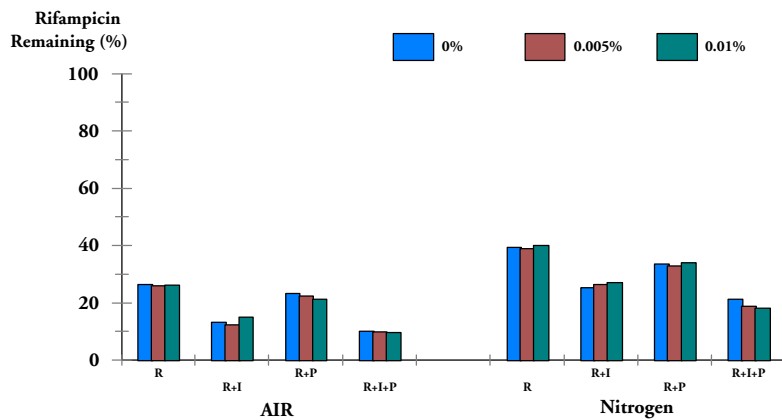


Figure 6.31 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Carbopol 974P at 60EC

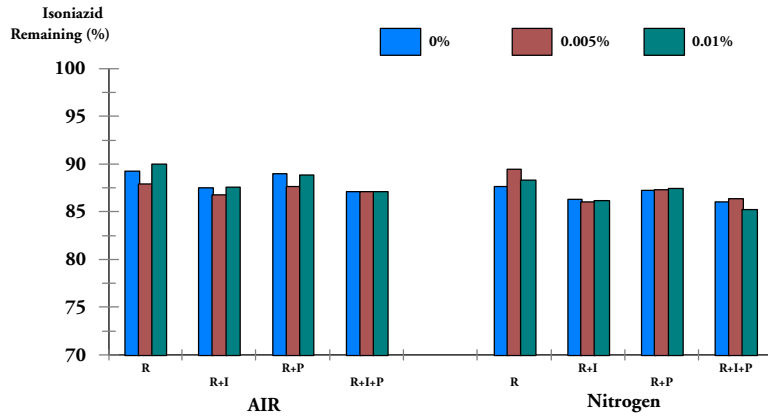


Figure 6.32 Stability Profiles of Isoniazid, (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 25EC

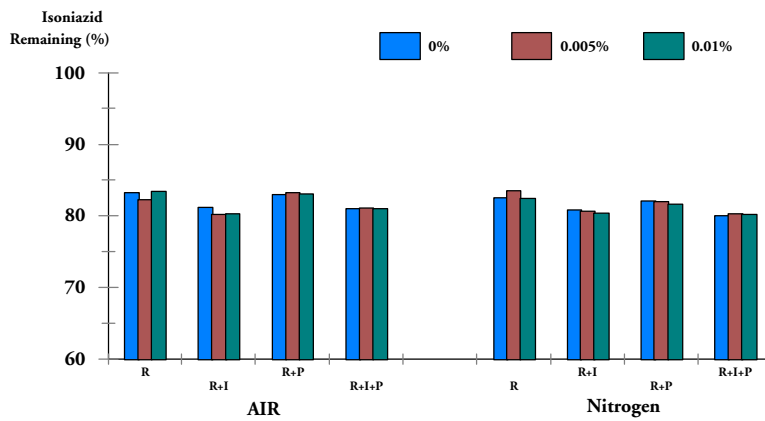


Figure 6.33 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 40EC

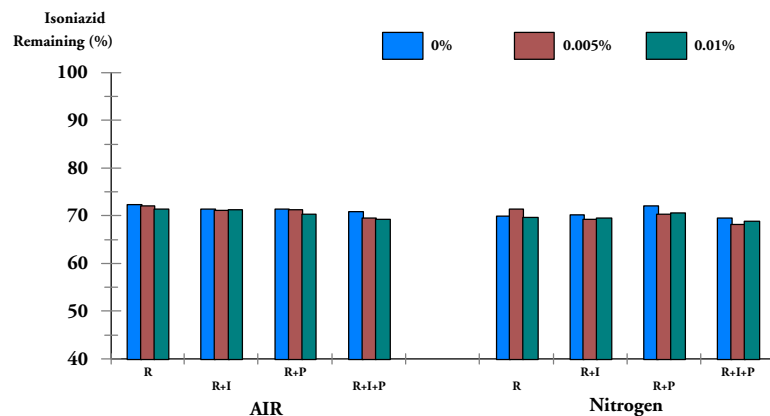


Figure 6.34 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Carbopol 974P at 60EC

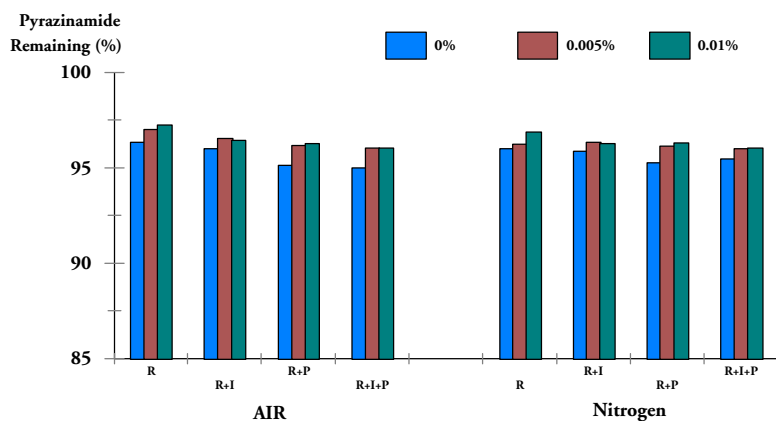


Figure 6.35 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 934P at 25EC

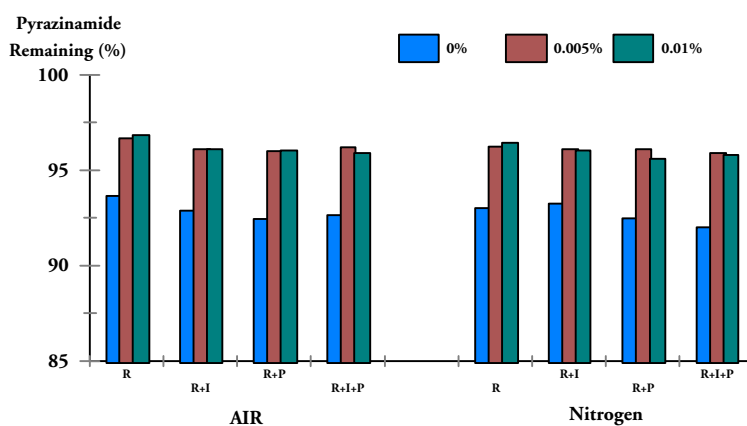


Figure 6.36 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 974P at 40EC

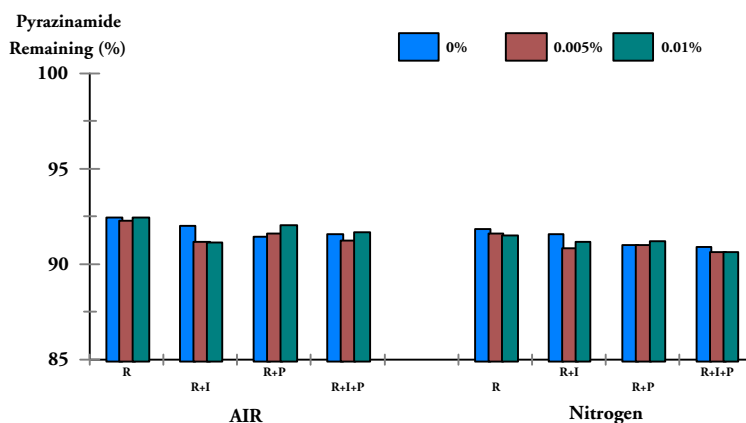


Figure 6.37 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Carbopol 974P at 60EC

6.3 CONCLUSION

The suspension is frequently used in pharmaceutical industries for formulating the less soluble drugs making use of the thixotropic effect of the suspending agents in order to suspend the drug particles in the formulation [Reynolds et al., 1989]. Since most of the drug particles are presented in the solid phase, the adverse destabilizing effect caused by hydrolysis is reduced.

Carbopol 934 and 974P are excellent suspending agents. Following the neutralization by a suitable base, the repulsion between the negatively charged carboxylate groups on the polymer backbone occurs and induces the carbopols to swell up to 1000 times their original volume. This swelling causes a change in viscosity within the solutions. It was found that the most suitable concentration for the use of suspension lies within carbopol 934 solution of 0.005 to 0.010% and carbopol 974P solutions of 0.001 to 0.003%. These solutions are fairly stable as the viscosity remains within 1.5% range of the original viscosity and this is used as the stability assessment of rifampicin, isoniazid and pyrazinamide.

The carbopol 934 and 974P have very similar stability enhancement effect on rifampicin and pyrazinamide but failed to cause any significant changes to isoniazid as it was present in the aqueous phase. The rifampicin-isoniazid and pyrazinamide-isoniazid interactions were reduced due to the segregation of the drugs into two phases. Although stability of rifampicin and pyrazinamide did not reach the ICH and FDA requirements, these stability assessments indicate that in order to achieve a more stable formulation in the aqueous media, the potential degradation sites of the drug molecules need to be protected.

CHAPTER SEVEN

SOLUBILITY AND STABILITY STUDIES OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE IN THE PRESENCE OF VARIOUS CYCLODEXTRINS

7.1 INTRODUCTION

In chapters 5 and 6, the aqueous stability of the rifampicin and isoniazid were found to be poor. Unless the degradation sites of rifampicin and isoniazid are protected, the stability of these drugs in the presence of any protic solvent would most likely be poor. In this chapter, a different approach involves the use of a soluble complexing agent to provide protection of the degradation site and hopefully enhance both the solubility and the stability of rifampicin, isoniazid and pyrazinamide. Cyclodextrins have the remarkable characteristic of being able to provide a non-bonding complexation with other guest compounds and change the physicochemical properties of the guest compound [Frijlink, 1990]. This complexation characteristic is known as inclusion which allows cyclodextrins to be extensively used in pharmaceutical applications. This chapter reports the use of cyclodextrins to overcome the poor aqueous solubility and the stability of the drug and the combinations of the drugs.

7.1.1 CYCLODEXTRIN STRUCTURE AND PHYSICAL PROPERTIES

Cyclodextrins (CyDs) are the cyclic oligosaccharides containing different numbers of D-(+)-glucopyranose units as the basic monomer. Under the action of the cyclodextrin-trans-glycosidase enzyme, the starch is naturally converted into either a six, seven or eight cyclic glucopyranose units which are attached by α -1,4-linkages. These cyclic glucopyranoses are designated as alpha-, beta-, and gamma- cyclodextrin respectively with a cone-shaped structure. They feature a macro ring with

hydrophilic outer surface which makes them fairly soluble in an aqueous medium and a large internal hydrophobic cavity which allows the inclusion to take place [Bekers et al., 1991; Duchêne et al., 1990; Menard et al., 1988; Szejtli et al., (June) 1991].

The structure and the numbering of the atoms of a cyclodextrin is shown in figure 7.1 and the three dimensional computer model of cyclodextrin is represented in chapter 8.

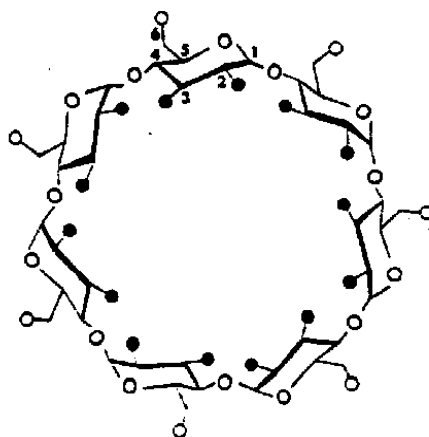


Figure 7.1 The Structure And Numbering of The Atoms of a Cyclodextrin.

The lack of free rotation around the glycosidic bonds of the C-1 conformation of the D-(+)-glycopyranosyl residues is responsible for the cone-shape of the cyclodextrin. Many intramolecular hydrogen bonds exist between the secondary hydroxyl groups. The C-2 hydroxyl groups of one glycopyranose unit can form a hydrogen bond with the C-3 hydroxyl groups of the adjacent glycopyranose unit. These two factors cause the rigidity of the cyclodextrin and govern the orientation of the glycopyranose units: the hydroxyl groups situated on the edge of the cyclodextrin ring which contribute the exterior hydrophilicity and the C-3 and C-5 hydrogen atoms which are directed into the cavity result in the hydrophobicity of the cavity. The dimension of the cyclodextrins alter with the number of glucose units and due to the difference in the internal diameters each parent cyclodextrin shows a different capability of inclusion complex formation with different guests. Hydroxypropylated derivatives such as hydroxypropyl- β -cyclodextrin (HP- β -CyD) are commonly used commercially in the pharmaceutical field due to its high aqueous solubility [Bakers et al., 1991; Duchêne et al., 1990; Frijlink et al., 1990; Menard et al., 1988; Szejtli, June 1991]. The cavity diameters of the cyclodextrins and the hydroxypropylated derivatives are listed in table 7.1.

Table 7.1 Physical Properties of the Parent Cyclodextrins and Hydroxypropylated Derivative

Cyclodextrins Physical Properties				
	α -CyD	β -CyD	HP- β -CyD	γ -CyD
Glucose Units	6	7	7	8
Cavity Diameter	5D	6D	6D	8D
Cavity Height	7.9D	7.9D	7.9D	7.9D
Molecular Mass	973	1135	1297	\pm 1300
Aqueous Solubility (g/100ml, m/v)	14.5	1.85	>50	23.2

7.1.2 CYCLODEXTRIN INCLUSION COMPLEXATION

Cyclodextrins are capable of forming non covalent bonds with a large variety of molecules. Although the binding force of complexation remains controversial, the basic interactions are based on the factors listed below [Bekers et al., 1991]:

- < Van der Waals interactions occurs between the hydrophobic guest molecule and the hydrogen atoms found in the cavity of the cyclodextrin. The Van der Waals interactions include both the induced-dipole-dipole interactions and London dispersion forces.
- < The release of high energy water molecules occurs on complex formation. In aqueous solution such as water, the internal cavity of cyclodextrins is occupied by the water molecules. However, the polarity difference between the water molecule and the cyclodextrin cavity make them energetically unfavourable as a host-guest inclusion. Therefore, the high enthalpy water molecules will be replaced spontaneously by the less polar compound with an appropriate size [Szejtli, June 1991].
- < The release of high energy occurs in the macromolecular cyclodextrin ring. The energy change occurs from the high energy conformation of cyclodextrin-water complex to the lower energy conformation of cyclodextrin guest complex. The inclusion tendency is also dependent on the size and the polarity of the guest molecule.

The complex generally occurs in the ratio of one guest molecule per one host cyclodextrin, however, 2:1 or 1:2 complexation has also occurred. From the pharmaceutical application point of view, the cavity β -cyclodextrin is generally too small to include the majority of the active ingredients, and α -cyclodextrin or γ -cyclodextrin are more suitable. From the stability and the solubility enhancement point of view, it has been reported that the effectiveness is in the order of Hydroxypropyl- α -cyclodextrin > α -cyclodextrin > γ -cyclodextrin > β -cyclodextrin [Jones et al., 1984].

7.1.3 TOXICITY AND BIOAVAILABILITY

Unlike starch, cyclodextrins are completely resistant to α -amylases [Duchéne, 1990]. Since cyclodextrin-glycosyltransferase is not consumed by humans, the use of the cyclodextrin in food or medicinal products has to be approved by the relevant health authorities [Szejtli et al., (Aug) 1991]. The pharmacokinetics of cyclodextrins in the rat gastrointestinal tract served as a model for cyclodextrin pharmacokinetics. It was found that after 24 hours of oral administration less than 1.5% of the cyclodextrin was absorbed. This could explain the low toxicity of cyclodextrins (oral LD₅₀ of the rat is more than 12.5g of cyclodextrin per every Kg of rats' body weight.) [Frijlink et al., 1990]. In humans, when a cyclodextrin is administered orally, most of the cyclodextrin is not absorbed through the intestinal tract. Small amount of cyclodextrins are metabolized by the microflora in the colon into acyclic maltodextrin, maltose and glucose which are further metabolized and absorbed in the same way as starch [Szejtli, Aug 1991]. Long term oral administration of cyclodextrin leads to significant toxicological side effects but intramuscular administration does induce nephrotoxicity and haemolytic effects [Duchéne, 1990]. By the late 80s to the early 90s, most of the cyclodextrins were patented and approved by most regulatory authorities worldwide [Strattan et al., Jan 1992]. The *in vitro* cytotoxic effect decreases in the order of γ -cyclodextrin < α -cyclodextrin < β -cyclodextrin < hydroxypropyl- α -cyclodextrin [Leroy-Lechat et al., 1994].

The bioavailability of the orally administered drug depends on the dissolution rate, solubility of the drug alone and the rate of intestinal absorption. The poor bioavailability is mainly the consequence of low dissolution and solubility and not low absorption rate. Cyclodextrins will thus enhance the bioavailability of the guest drug by enhancing the drug's solubility [Duchéne et al., 1990; Bekers et al., 1991].

β -cyclodextrin is widely used in pharmaceutical applications due to its ready availability. β -cyclodextrin was incorporated in this chapter as a solubility enhancing agent. However, due to the low aqueous solubility of β -cyclodextrin itself, hydroxypropyl- β -cyclodextrin was included. The solubility and the stability of rifampicin, isoniazid and pyrazinamide in the aqueous solution in the presence of these cyclodextrins is assessed. Furthermore, α -cyclodextrin with the larger cavity was also included in the abovementioned studies. The results are shown in sections 7.2.1 to 7.2.4.

7.2 EXPERIMENTAL RESULTS AND OBSERVATIONS

7.2.1 KARL FISCHER EXPERIMENT

The water content of each cyclodextrin was analysed (4.1.3) with the mean values of water content of each cyclodextrin used listed in table 7.2. All the experiments related to these cyclodextrins are adjusted accordingly.

Table 7.2 Water Content of Various Cyclodextrin

Water Content of Various Cyclodextrins			
<i>CyD</i>	<i>Total % Water Content</i>	<i>CyD : Water Ratio (Calculated Values)</i>	<i>CyD : Water Ratio (Assumed Values)</i>
β -CyD	4.81	1 : 3.029	1 : 3
HP- β -CyD	5.23	1 : 4.108	1 : 4
α -CyD	5.52	1 : 3.977	1 : 4

7.2.2 SOLUBILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF β -CYCLODEXTRIN

The following amount of β -cyclodextrin (0.04, 0.08, 0.12, 0.14 and 0.18g) was accurately weighed and dissolved in 10ml water to form the 0.4, 0.8, 1.2, 1.4 and 1.8% m/v β -cyclodextrin solutions. These solutions were saturated with the rifampicin, isoniazid, pyrazinamide, rifampicin with isoniazid, rifampicin with pyrazinamide, isoniazid with pyrazinamide and rifampicin with both isoniazid and pyrazinamide. The supernatants were analysed by HPLC after 12 hours of constant shaking at $25 \pm 2^\circ\text{C}$. Figures 7.2 to 7.4 represent the solubility changes of rifampicin, isoniazid and pyrazinamide (y-axis) in the presence of various concentration of β -cyclodextrin (x-axis) and the data are listed in tables 7.3 to 7.5. The intrinsic solubility of the drugs had been taken into account when attempting to calculate the interaction ratio between the drugs and the cyclodextrin.

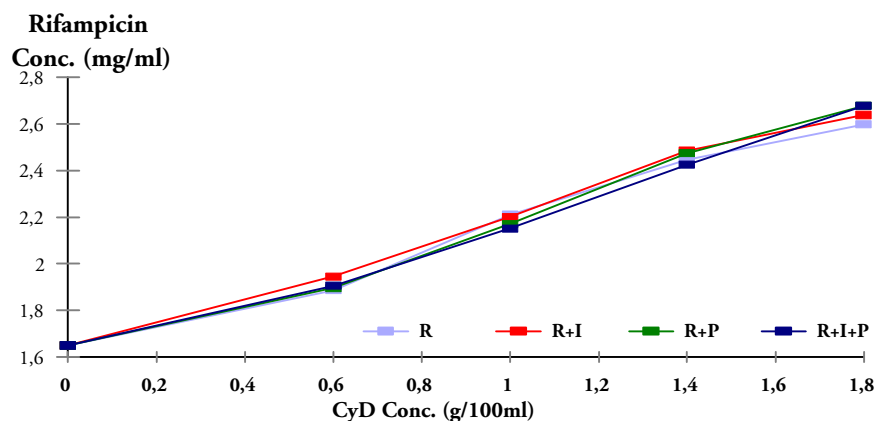


Figure 7.2 Solubility Curves of Rifampicin (RIF, RIF With INH, RIF With PZA or RIF With Both INH or PZA) in the Presence of β -Cyclodextrin

The above figure clearly demonstrates that the solubility of rifampicin is enhanced in the presence of β -cyclodextrin from 1.65 to 2.62mg/ml in a linear fashion ($y = 0.5674x + 1.6232$) with a correlation factor of 0.9856. The presence of isoniazid and pyrazinamide did not significantly influence the solubility of rifampicin. The solubility ratio is about 0.7910 mole of rifampicin per one mole molecule of β -cyclodextrin. This indicates that there could be multiple binding sites within the rifampicin molecule itself which enables the rifampicin molecule to complex with two β -cyclodextrins. Regardless of the type of complexation, the desired rifampicin concentration for formulation (75mg per 10ml) was not achieved while using 1.8% m/v of β -cyclodextrin solution.

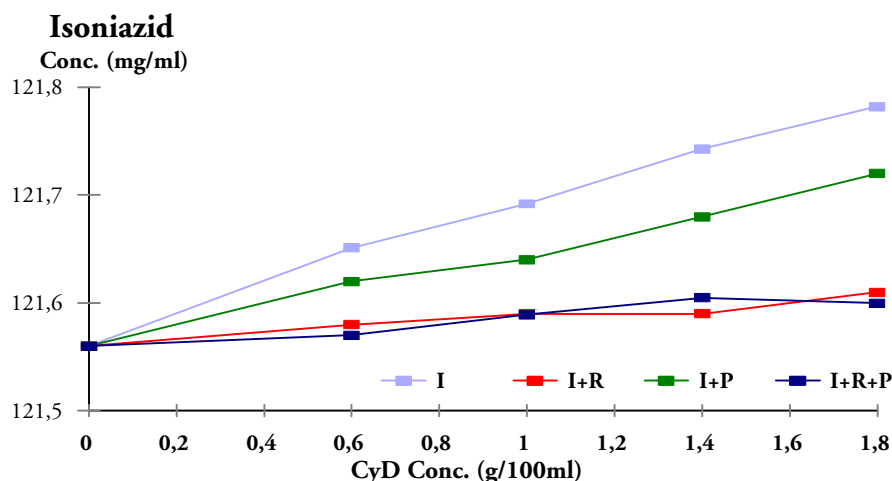


Figure 7.3 Solubility Curves of Isoniazid (INH Alone, INH With RIF, INH, With PZA or INH With Both RIF And PZA)in the Presence of β -Cyclodextrin

The isoniazid- β -cyclodextrin phase-solubility indicates that the solubility of isoniazid is enhanced in the presence of β -cyclodextrin in the ratio of 0.977mole of isoniazid per one mole of β -cyclodextrin, suggesting a 1 to 1 guest-host complexation. However, when rifampicin or/and pyrazinamide was/were present, the solubility curve of isoniazid differed. The presence of the other drugs seem to compete with isoniazid in complexing with β -cyclodextrin. After examining the ratio list in table 7.3, it can be predicted that in complexing with β -cyclodextrin, rifampicin competes with isoniazid more than pyrazinamide. The solubility constants can be refer to table 7.10 in page 210.

Table 7.3 Isoniazid Solubility Curves in the Presence of β -Cyclodextrin

Isoniazid Solubility Curve Data				
Analyte	Additional Factors	Equation	Linear Correlation Factor	Interaction Ratio INH : β -CyD (mole : mole)
INH	β -CyD only	$y = 0.1227x + 121.568$	0.9929	0.977 : 1
INH	RIF + β -CyD	$y = 0.0252x + 121.567$	0.9494	0.218 : 1
INH	PZA + β -CyD	$y = 0.0865x + 121.561$	0.9917	0.699 : 1
INH	RIF + PZA + β -CYD	$y = 0.0236x + 121.563$	0.9528	0.175 : 1

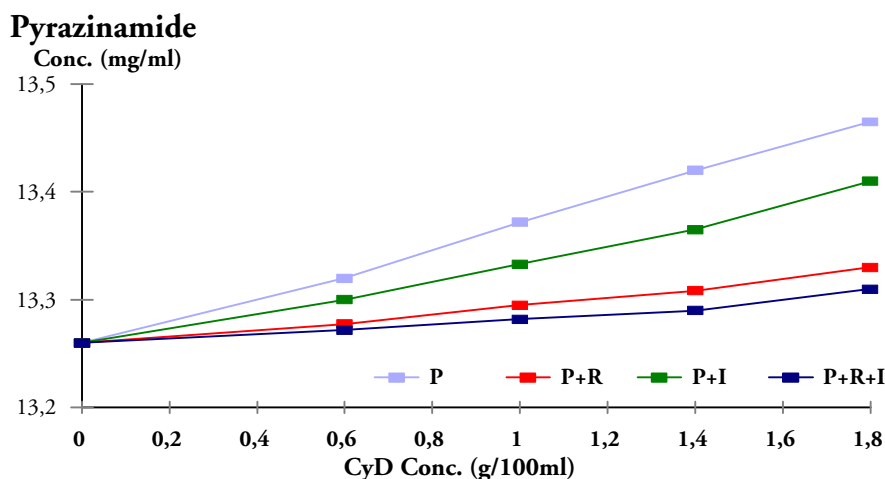


Figure 7.4 Solubility Curves of Pyrazinamide (PZA Alone, PZA With RIF, PZA With INH, or PZA With Both RIF And INH)in the Presence of β -Cyclodextrin

β -cyclodextrin enhanced the solubility of pyrazinamide by only 0.2051mg/ml. 13.465mg/ml of pyrazinamide in the presence of 1.8% m/v of β -cyclodextrin is far less than the desired 100mg/ml target. Furthermore, with the addition of rifampicin or isoniazid, the solubility enhancement effect of β -cyclodextrin was further reduced. Figure 7.4 clearly demonstrates that the drugs are competing with each other for the complexation site. The classification of these curves is listed in table 7.5.

Table 7.4 Pyrazinamide Solubility Curves in the Presence of β -Cyclodextrin

Pyrazinamide Solubility Curve Data				
<i>Analyte</i>	<i>Additional Factors</i>	<i>Equation</i>	<i>Linear Correlation Factor</i>	<i>Interaction Ratio INH : β-CyD (mole : mole)</i>
PZA	β -CyD only	$y = 0.1155x + 13.257$	0.9981	0.984 : 1
PZA	RIF + β -CyD	$y = 0.0385x + 13.257$	0.9885	0.320 : 1
PZA	PZA + β -CyD	$y = 0.0903x + 13.255$	0.9898	0.687 : 1
PZA	RIF + INH + β -CYD	$y = 0.0265x + 13.257$	0.9606	0.229 : 1

Table 7.5 Classification of β -Cyclodextrin Phase-Solubility Curves

β-Cyclodextrin Phase-Solubility Curves			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
β -Cyclodextrin only	A _S	A _P	A _P
β -Cyclodextrin + RIF	A _S	Minor A _P	Minor A _P
β -Cyclodextrin + INH	Minor A _N	A _P	A _P
β -Cyclodextrin + PZA	A _S	A _P	A _P
β -Cyclodextrin + Combination of all three	A _S	Minor A _P	Minor A _P

7.2.3 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF β -CYCLODEXTRIN

The 7-day stability assessments of rifampicin, isoniazid and pyrazinamide in the presence of β -cyclodextrin were performed by saturating the drugs in either 0.5, 1.0, 1.5 or 1.8g/100ml β -cyclodextrin solution. Each solution was further divided into 42 ampoules of which 24 were stored under nitrogen (designated (N)) and rest contained normal air in the head space. They were further evenly distributed into three ovens with temperature of 25, 40 and 60EC and then analysed by HPLC daily. The results are reflected in figures 7.5 to 7.22 as the percentage of drug remaining *versus* time of storage.

In addition, the following aspects were observed:

- < β -cyclodextrin caused a stability enhancement of isoniazid and pyrazinamide by approximately 4.9 and 2.1 % but substantially reduced the stability of rifampicin up to 14.5%. This implies that the degradation site of rifampicin could be situated outside the β -cyclodextrin cavity, whereas isoniazid and pyrazinamide are minimally protected.
- < Nitrogen improves the stability of rifampicin but does not cause any significant effect on isoniazid and pyrazinamide stability. Rifampicin, isoniazid and pyrazinamide interact with each other and thus reduce the general stability.
- < The stability influence of β -cyclodextrin is concentration dependent. An increase in β -cyclodextrin resulted in further destabilization of rifampicin and the stability enhancement of isoniazid and pyrazinamide. These effects were shown at 25EC.
- < Although β -cyclodextrin caused a destabilizing effect on rifampicin, the general stability of rifampicin combining with isoniazid and/or pyrazinamide was enhanced in the presence of β -cyclodextrin. This could be due to isoniazid and pyrazinamide being protected by β -cyclodextrin, but most likely this implies that the interaction of β -cyclodextrin and rifampicin protected the certain parts of rifampicin which are essential for interaction with isoniazid or pyrazinamide.
- < In the presence of β -cyclodextrin, the stability of isoniazid or pyrazinamide is also enhanced when in combination with each other and/or with rifampicin over the temperature ranges studied.

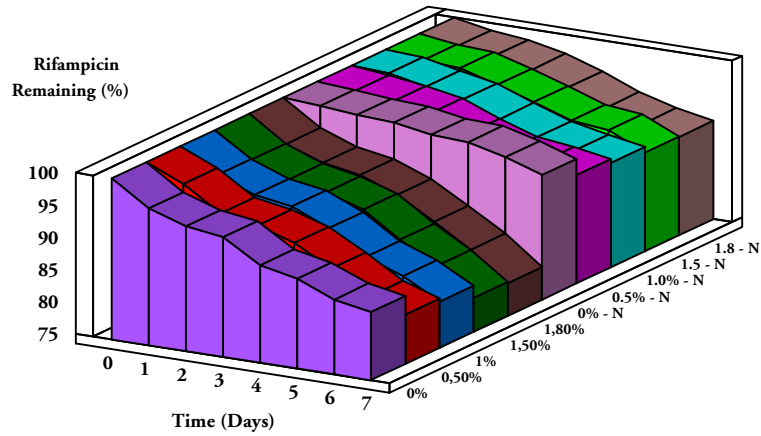


Figure 7.5 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 25°C

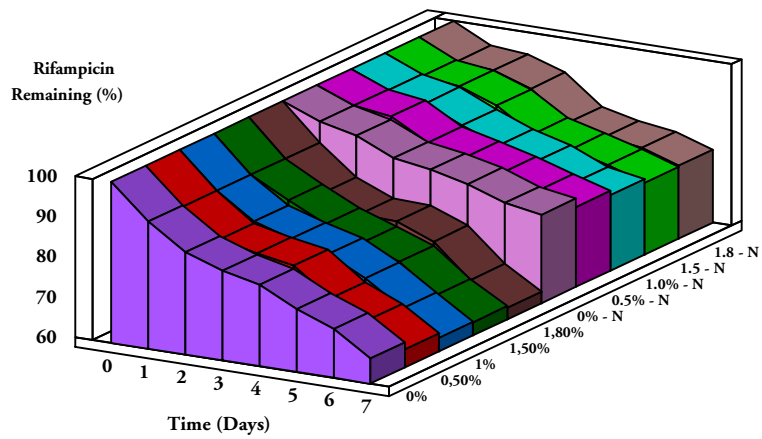


Figure 7.6 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 40°C

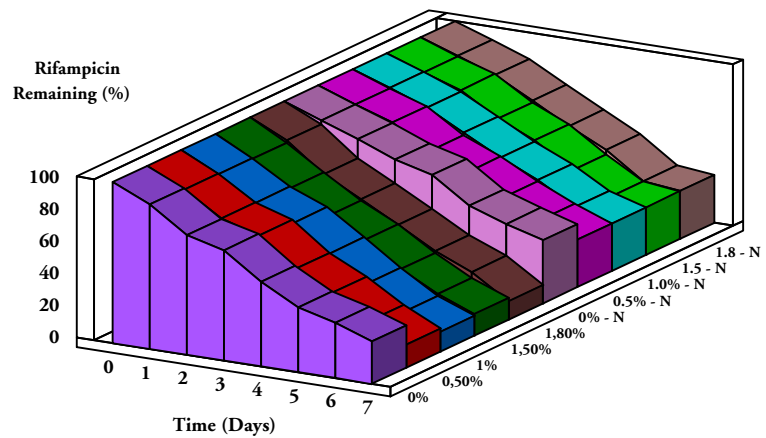


Figure 7.7 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 60°C

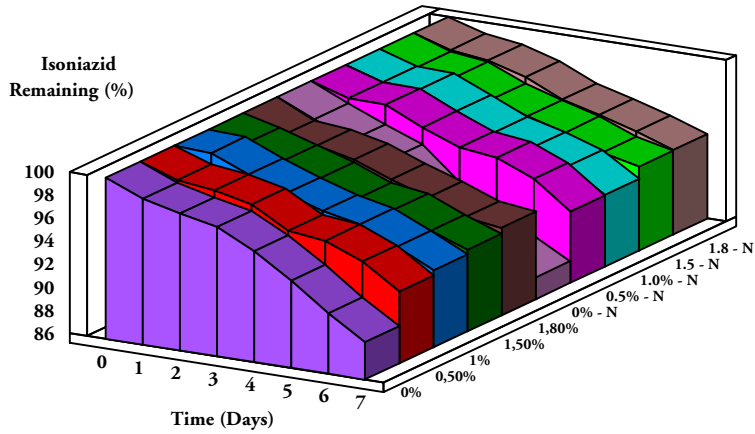


Figure 7.8 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 25EC

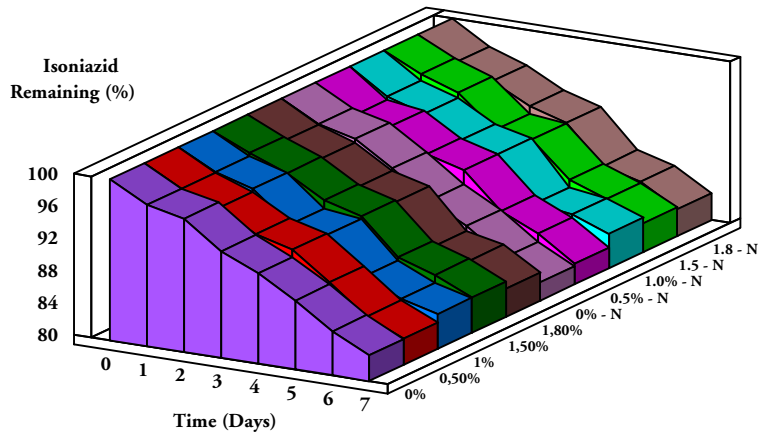


Figure 7.9 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 40EC

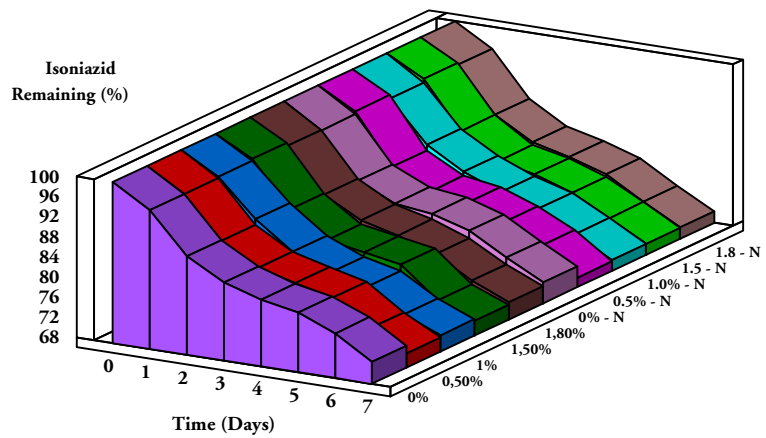


Figure 7.10 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 60EC

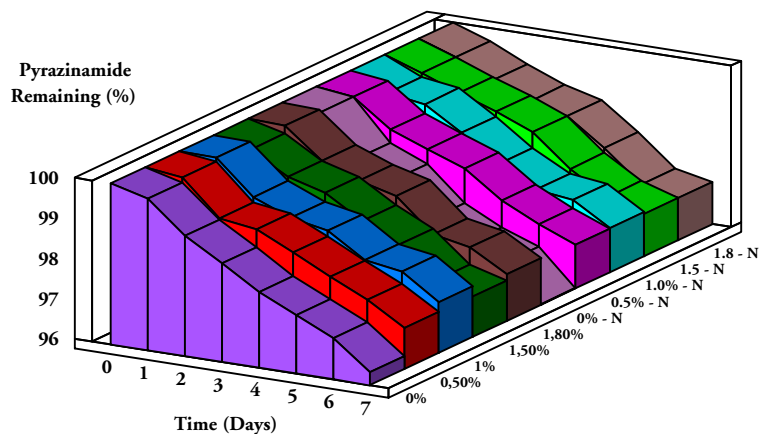


Figure 7.11 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 25°C

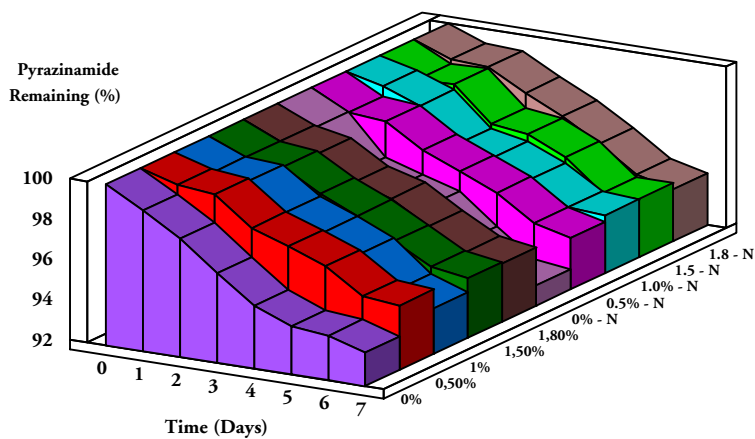


Figure 7.12 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 40°C

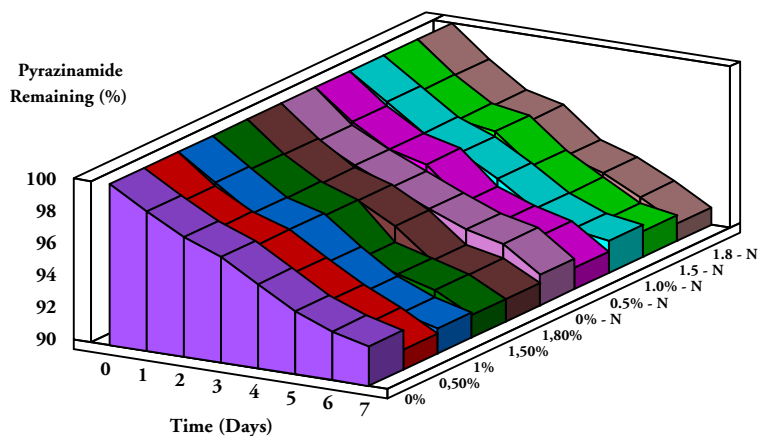


Figure 7.13 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 60°C

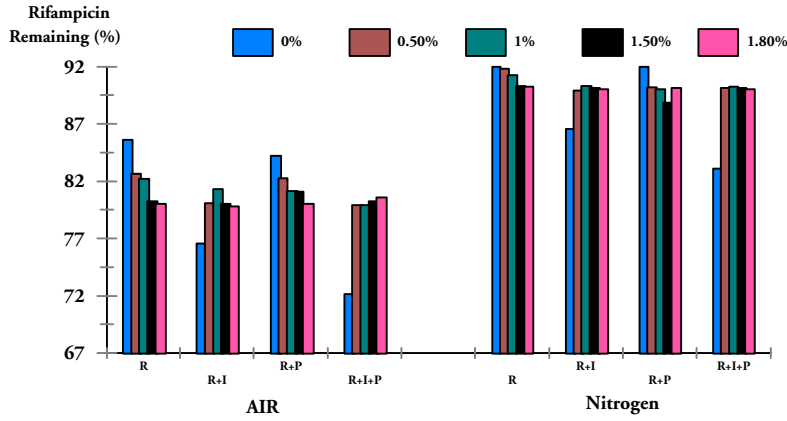


Figure 7.14 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 25°C

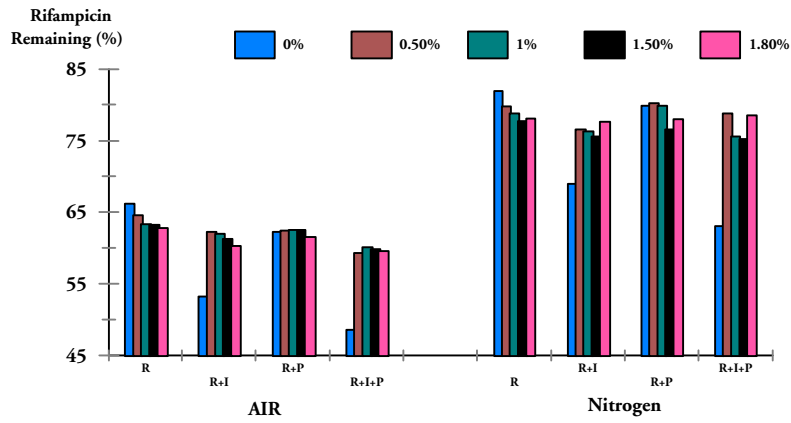


Figure 7.15 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 40°C

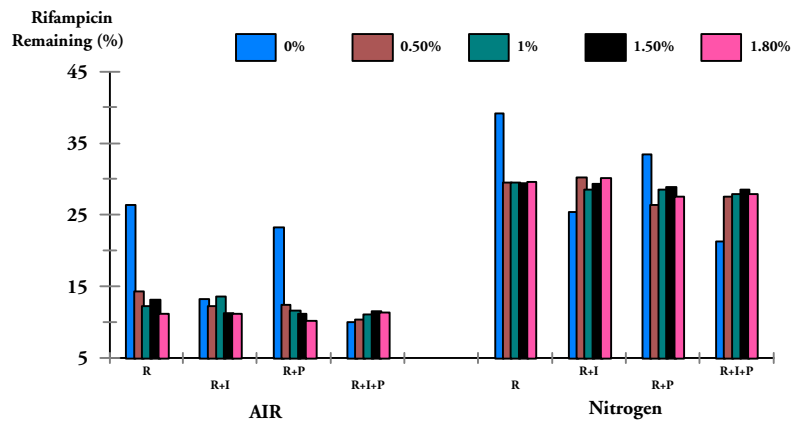


Figure 7.16 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 60°C

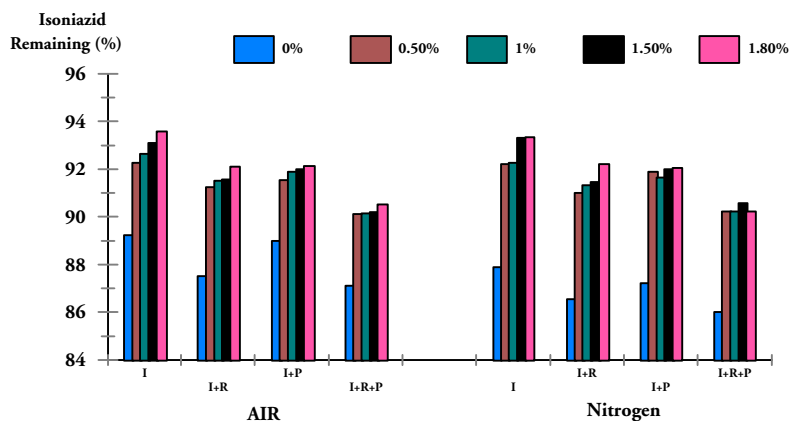


Figure 7.17 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 25EC

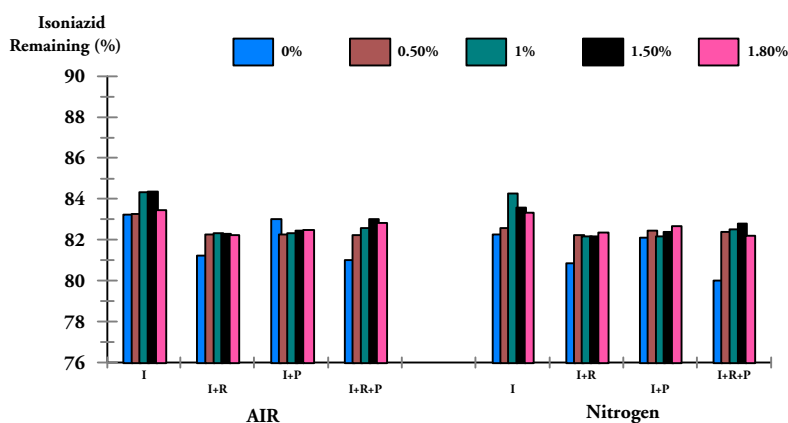


Figure 7.18 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 40EC

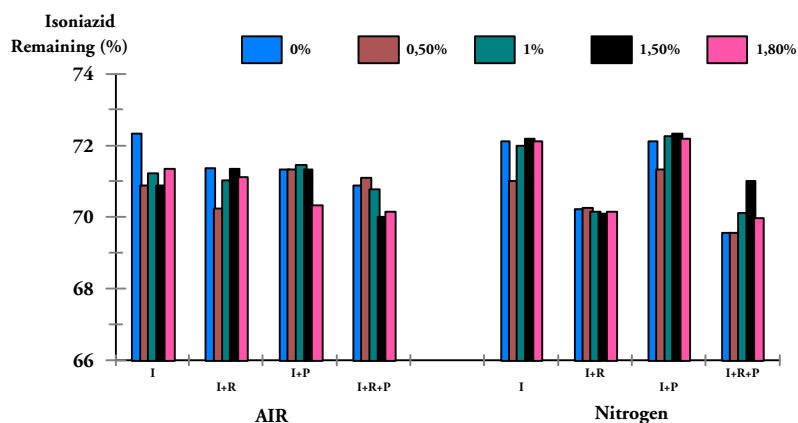


Figure 7.19 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 60EC

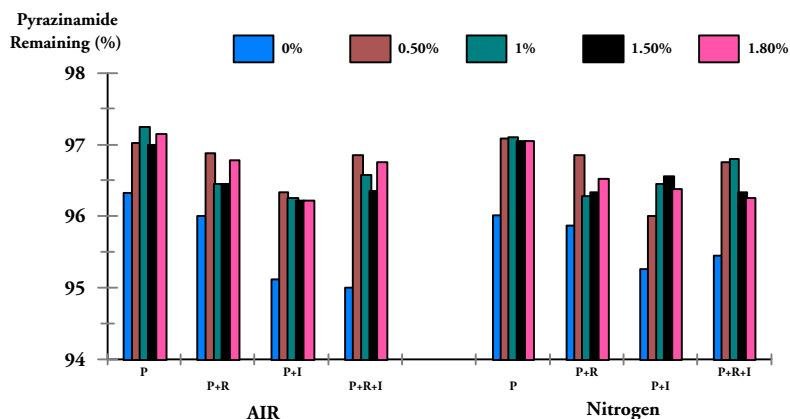


Figure 7.20 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 25EC

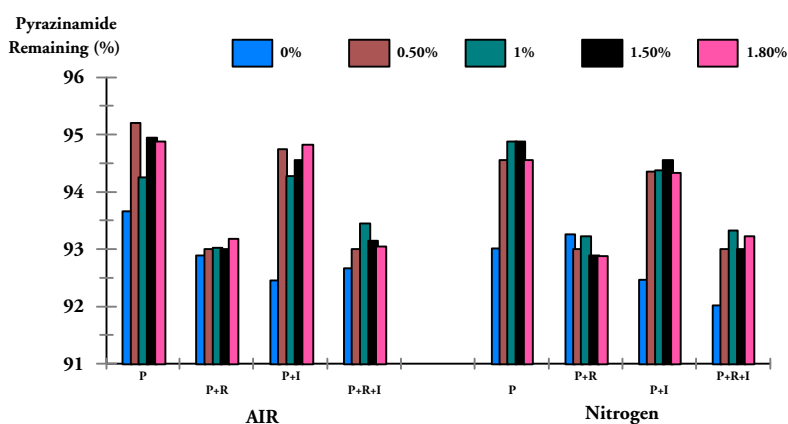


Figure 7.21 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 40EC

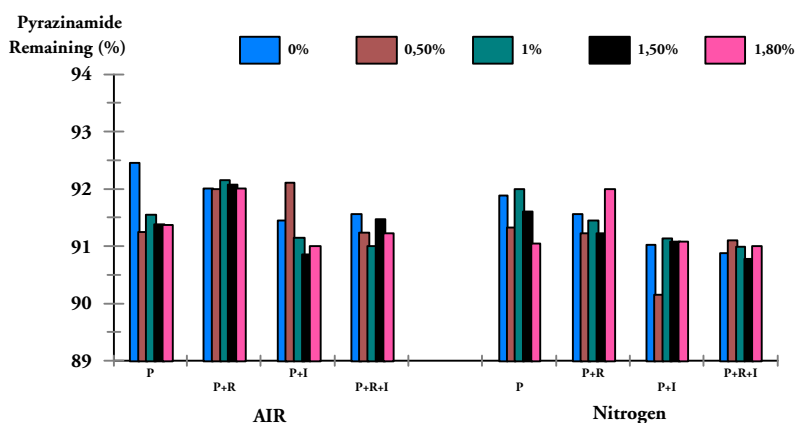


Figure 7.22 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 60EC

7.2.4 SOLUBILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF HYDROXYPROPYL- β -CYCLODEXTRIN

On the request from Pharmacare-Lennon[®], 0.1, 0.2, 0.3, 0.4 and 0.5g of hydroxypropyl- β -cyclodextrin were accurately weighed and dissolved in 10ml water to form 1.0, 2.0, 3.0, 4.0 and 5.0% m/v hydroxypropyl- β -cyclodextrin solutions. These solutions were saturated with the rifampicin, isoniazid, pyrazinamide, rifampicin with isoniazid, rifampicin with pyrazinamide, isoniazid with pyrazinamide and rifampicin with both isoniazid and pyrazinamide and shaken for 12 hours in a 25 \pm 2EC waterbath shaker. The results of the HPLC analyses are represented in figures 7.23 to 7.25.

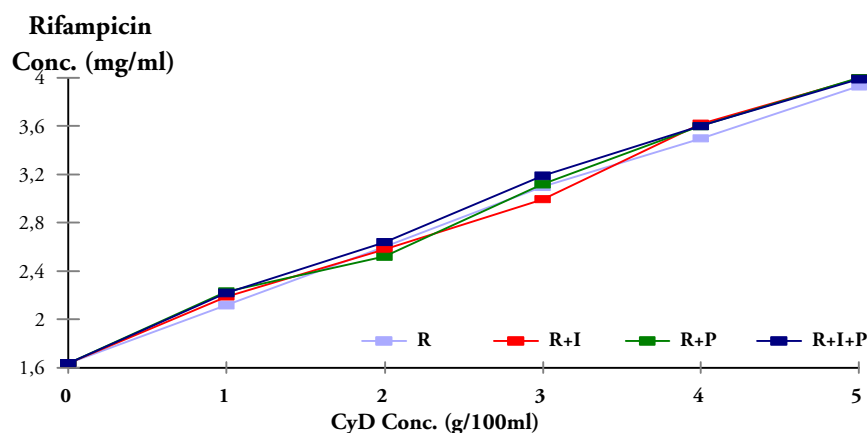


Figure 7.23 Solubility Curves of Rifampicin (RIF , RIF With INH, RIF With PZA or RIF With Both INH or PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin

The curves indicate that the complexation between hydroxypropyl- β -cyclodextrin and rifampicin was not significantly affected by the presence of isoniazid and/or pyrazinamide. The curves follow the equation of $y = (0.460 \pm 0.002)x + (1.665 \pm 0.001)$ with the correlation value ranging from 0.9986 to 0.9775. After the calculating the molar ratio between the drug and the cyclodextrin, the ratio appears to be approximately 0.775 mole of rifampicin per mole of hydroxypropyl- β -cyclodextrin. This could possibly imply that there should be multiple binding sites if rifampicin binds with two hydroxypropyl- β -cyclodextrins (table 7.6) although it is impossible to deduce a conclusive statement base on the results obtained from the phase solubility curve .

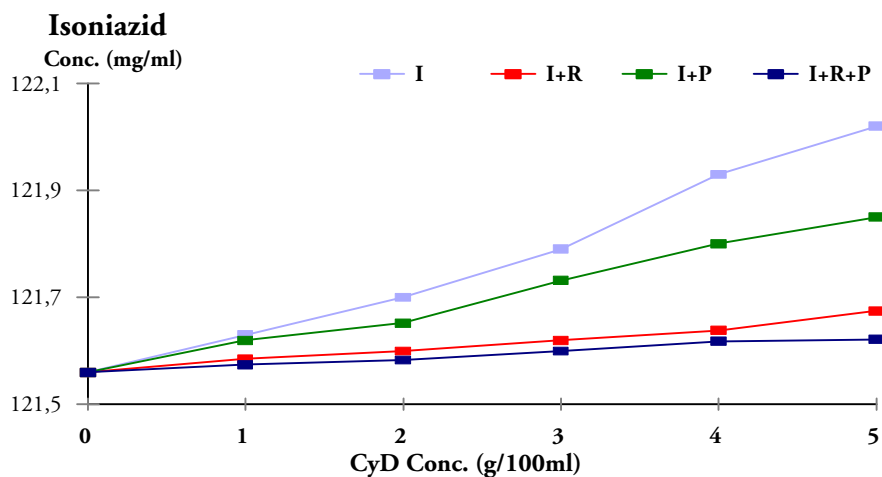


Figure 7.24 Solubility Curves of Isoniazid (INH Alone, INH With RIF, INH, With PZA or INH With Both RIF And PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin

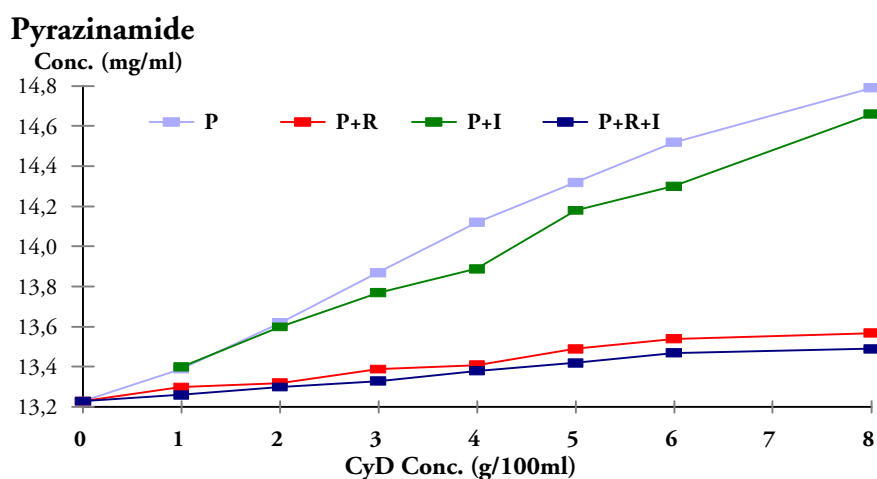


Figure 7.25 Solubility Curves of Pyrazinamide (PZA Alone, PZA With RIF, PZA With INH, or PZA With Both RIF And INH) in the Presence of Hydroxypropyl- β -Cyclodextrin

The interaction between hydroxypropyl- β -cyclodextrin and isoniazid or pyrazinamide is substantially decreased when rifampicin is present. The solubility of isoniazid or pyrazinamide in the combination of I(P) + R and I + P + R shows only marginal increases. Therefore, it is predicted that the rifampicin has a more affinity for hydroxypropyl- β -cyclodextrin. Furthermore, there seems to be competition between isoniazid and pyrazinamide in interacting with hydroxypropyl- β -cyclodextrin as they decrease each others solubility when in the presence of hydroxypropyl- β -cyclodextrin. The corresponding data are listed in tables 7.6 to 7.7.

Table 7.6 Rifampicin, Isoniazid and Pyrazinamide Solubility Curves in the Presence of Hydroxypropyl- β -Cyclodextrin

Drug- HP- β -CyD Solubility Curve Data				
<i>Drug</i>	<i>Cyclodextrin [plus other drug(s)]</i>	<i>Equation</i>	<i>Linear Correlation Factor</i>	<i>Interaction Ratio Drug : HP- β-CyD (mole : mole)</i>
RIF	HP- β -CYD	$y = 0.460x + 1.665$	0.9976	0.786 : 1
INH	HP- β -CYD	$y = 0.250x + 121.487$	0.9894	1.067 : 1
PZA	HP- β -CYD	$y = 0.206 + 13.236$	0.9990	0.968 : 1
RIF	INH +HP- β -CYD	$y = 0.460x + 1.665$	0.9775	0.781 : 1
INH	RIF +HP- β -CYD	$y = 0.044x + 121.513$	0.9616	0.237 : 1
RIF	PZA + HP- β -CYD	$y = 0.459x + 1.664$	0.9977	0.739 : 1
PZA	RIF + HP- β -CYD	$y = 0.031 + 13.253$	0.9866	0.316 : 1
INH	PZA + HP- β -CYD	$y = 0.180x + 121.546$	0.9961	0.472 : 1
PZA	INH + HP- β -CYD	$y = 0.073x + 13.258$	0.9960	0.688 : 1
RIF	INH + PZA + HP- β -CYD	$y = 0.461x + 1.665$	0.9986	0.767 : 1
INH	RIF + PZA +HP- β -CYD	$y = 0.035 + 121.564$	0.9777	0.125 : 1
PZA	RIF + INH +HP- β -CYD	$y = 0.037x + 13.25$	0.9753	0.243 : 1

The drug to hydroxypropyl- β -cyclodextrin ratios listed in table 7.5 suggests the following:

- < When rifampicin is combined with isoniazid in the presence of hydroxypropyl- β -cyclodextrin, the ratio is approximately 8 : 2 : 10 (RIF : INH : HP- β -CyD; mol. : mol. : mol.).
- < When rifampicin is combined with pyrazinamide in the presence of hydroxypropyl- β -cyclodextrin, the ratio is approximately 7 : 3 : 10 (RIF : PZA : HP- β -CyD; mol. : mol. : mol.).
- < When isoniazid is combined with pyrazinamide in the presence of hydroxypropyl- β -cyclodextrin, the ratio is approximately 5 : 7 : 12 (INH : PZA : HP- β -CyD; mol. : mol. : mol.).

- < When rifampicin, isoniazid and pyrazinamide are combined in the presence of hydroxypropyl- β -cyclodextrin, the ratio is approximately 8 : 1 : 2 : 11 (RIF : INH : PZA : HP- β -CyD; mol. : mol. : mol.).
- < The drug-hydroxypropyl- β -cyclodextrin interaction affinity is in the order of RIF > PZA > INH.

Table 7.7 Classification of Hydroxypropyl- β -Cyclodextrin Phase-Solubility Curves

Hydroxypropyl- β-Cyclodextrin Phase-Solubility Curves			
<i>Additional Factors</i>	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
HP- β -CyD only	A _p	A _p	A _p
HP- β -CyD + RIF	A _p	Minor A _p	Minor A _p
HP- β -CyD + INH	A _p	A _p	A _p
HP- β -CyD + PZA	A _p	A _p	A _p
HP- β -CyD + Combination of all Three Drugs	A _p	Minor A _p	Minor A _p

7.2.5 STABILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF HYDROXYPROPYL- β -CYCLODEXTRIN

1.0, 3.0, 5.0 and 7.0 % m/v of hydroxypropyl- β -cyclodextrin solutions were made up by dissolving 0.1, 0.3, 0.5 or 0.7g of hydroxypropyl- β -cyclodextrin in 10 ml of water. The stability assessments of rifampicin, isoniazid and pyrazinamide in the presence of hydroxypropyl- β -cyclodextrin were conducted similarly to section 7.2.3.

The stability profiles below indicate that hydroxypropyl- β -cyclodextrin has a similar stability reduction effect towards rifampicin and stability enhancement effect towards isoniazid and pyrazinamide (figures 7.26. to 7.43).

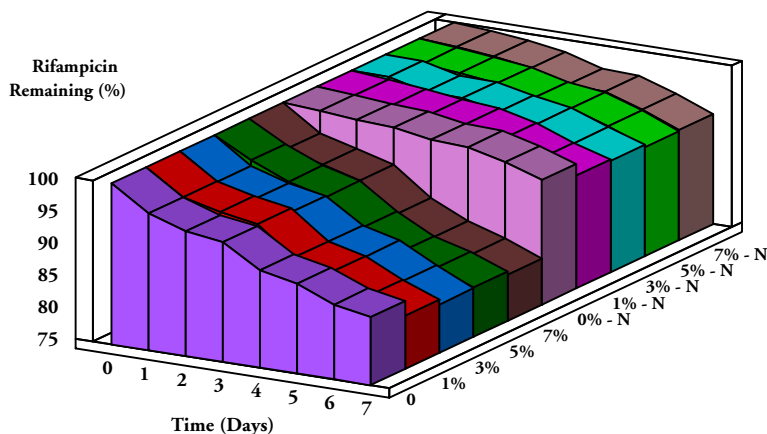


Figure 7.26 Stability Profiles of Rifampicin in the Presence of Hydroxypropyl- β -Cyclodextrin at 25EC

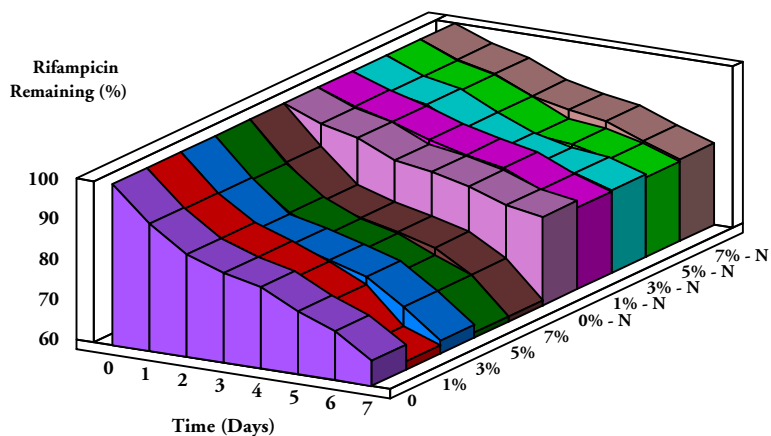


Figure 7.27 Stability Profiles of Rifampicin in the Presence of Hydroxypropyl- β -Cyclodextrin at 40EC

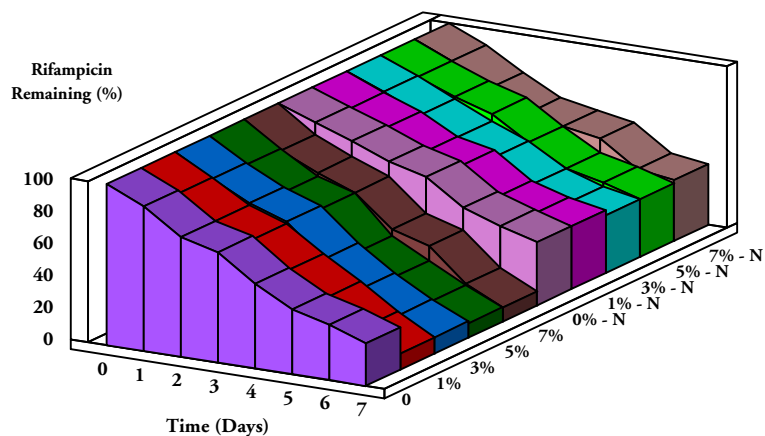


Figure 7.28 Stability Profiles of Rifampicin in the Presence of Hydroxypropyl- β -Cyclodextrin at 60EC

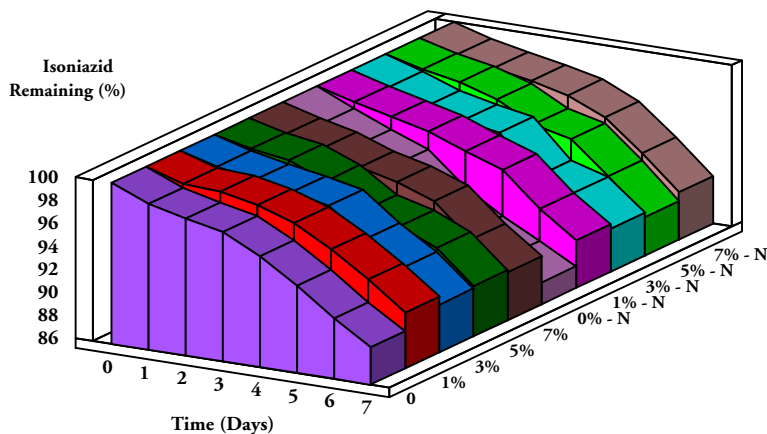


Figure 7.29 Stability Profiles of Isoniazid in the Presence of Hydroxypropyl- β -Cyclodextrin at 25EC

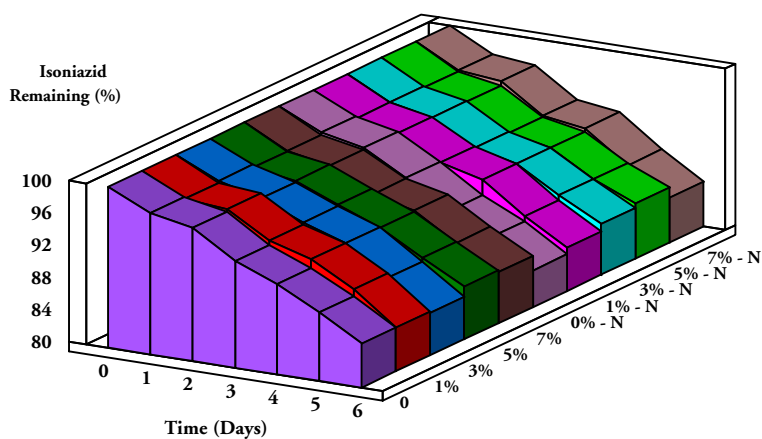


Figure 7.30 Stability Profiles of Isoniazid in the Presence of Hydroxypropyl- β -Cyclodextrin at 40EC

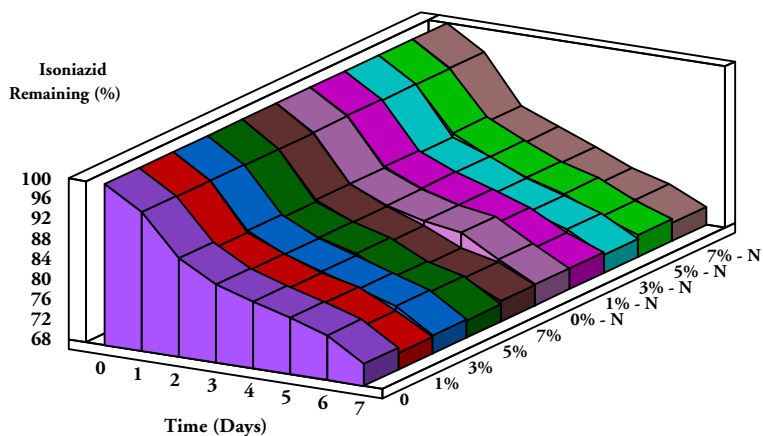


Figure 7.31 Stability Profiles of Isoniazid in the Presence of Hydroxypropyl- β -Cyclodextrin at 60EC

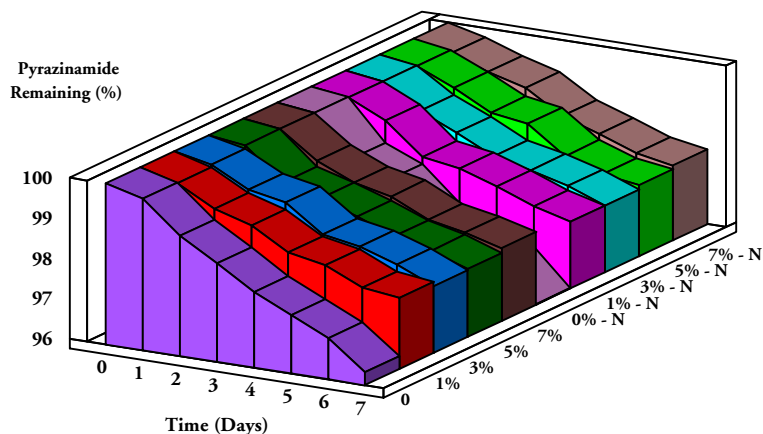


Figure 7.32 Stability Profiles of Pyrazinamide in the Presence of Hydroxypropyl- β -Cyclodextrin at 25EC

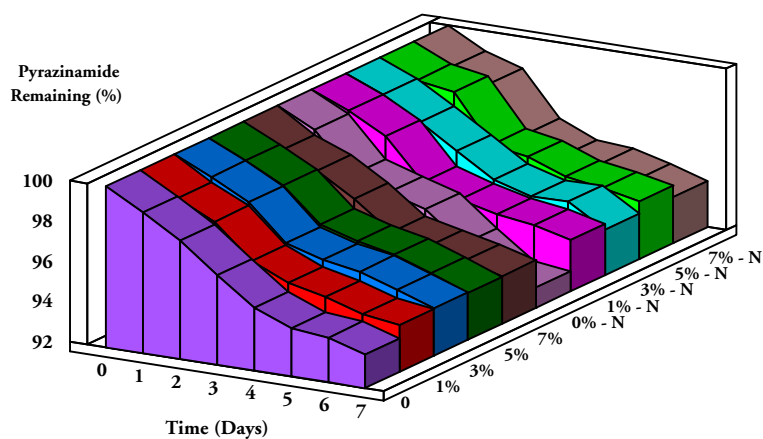


Figure 7.33 Stability Profiles of Pyrazinamide in the Presence of Hydroxypropyl- β -Cyclodextrin at 40EC

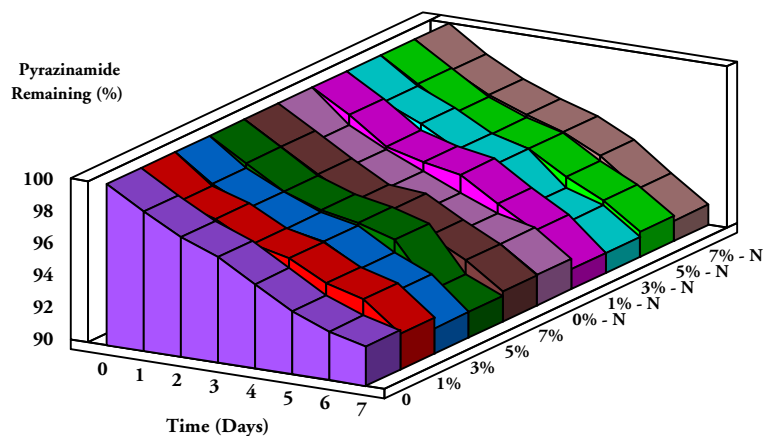


Figure 7.34 Stability Profiles of Pyrazinamide in the Presence of Hydroxypropyl- β -Cyclodextrin at 60EC

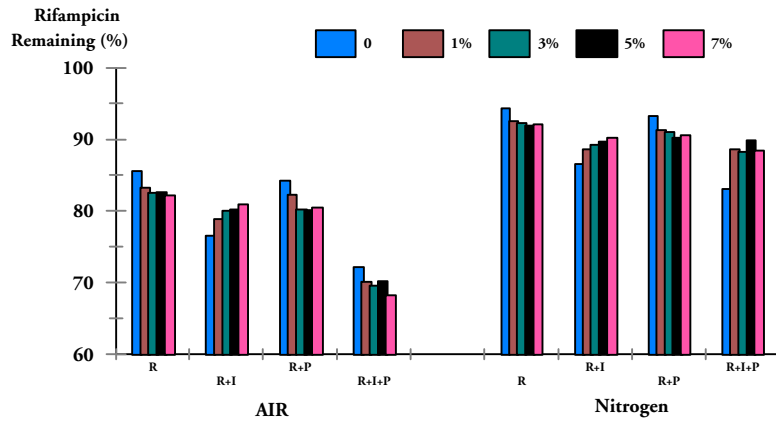


Figure 7.35 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 25°C

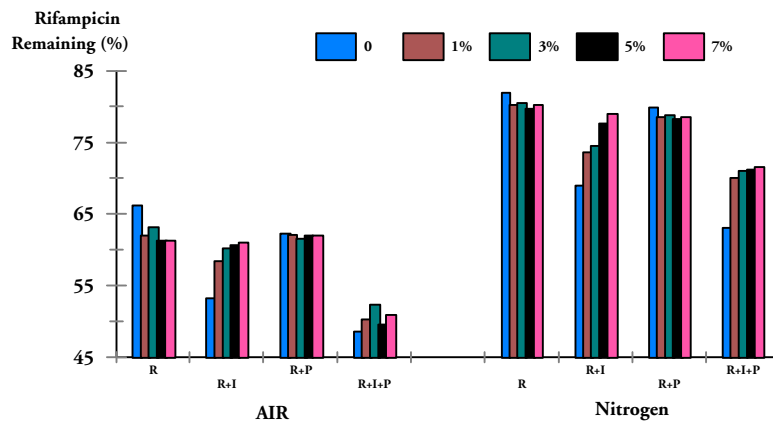


Figure 7.36 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 40°C

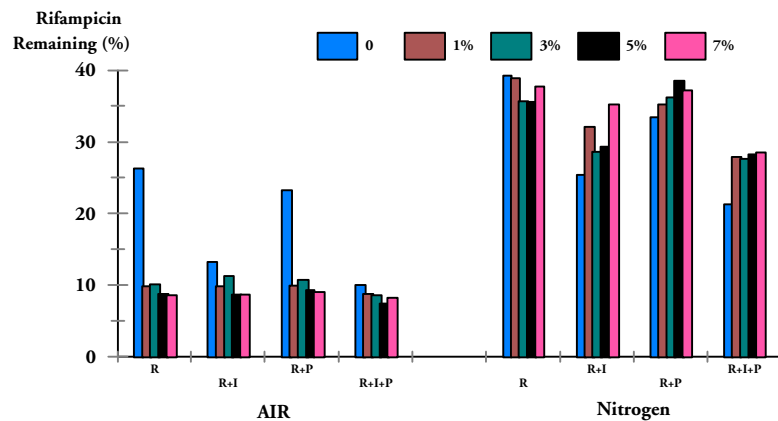


Figure 7.37 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 60°C

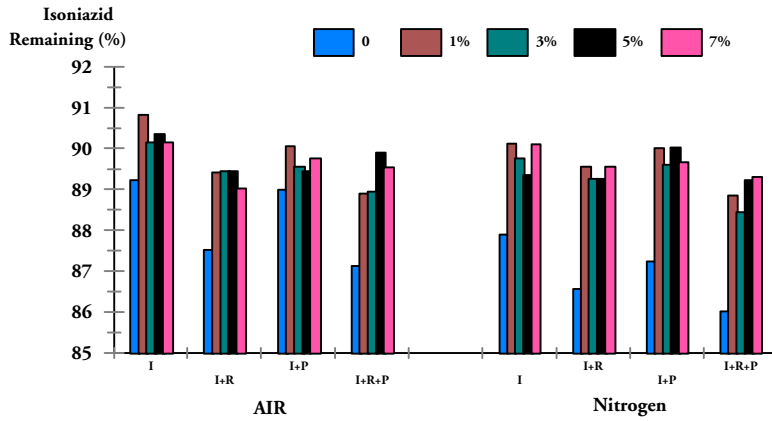


Figure 7.38 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 25°C

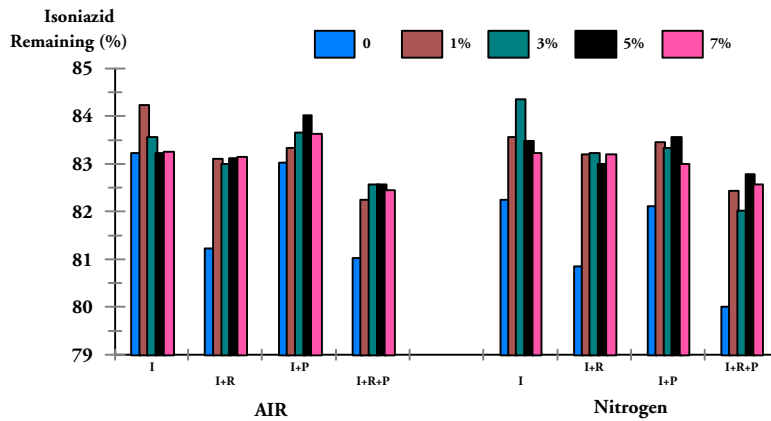


Figure 7.39 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 40°C

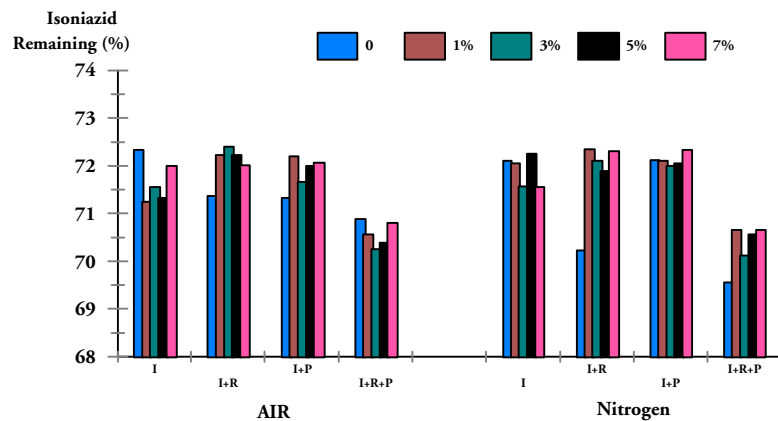


Figure 7.40 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of Hydroxypropyl- β -Cyclodextrin at 60°C

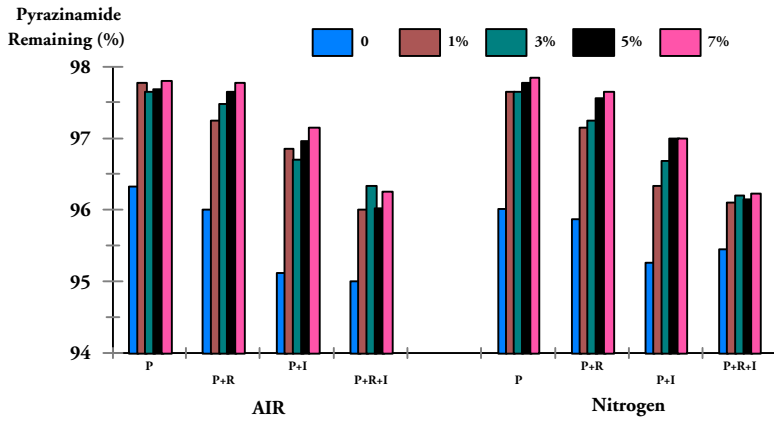


Figure 7.41 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Hydroxypropyl-β-Cyclodextrin at 25EC

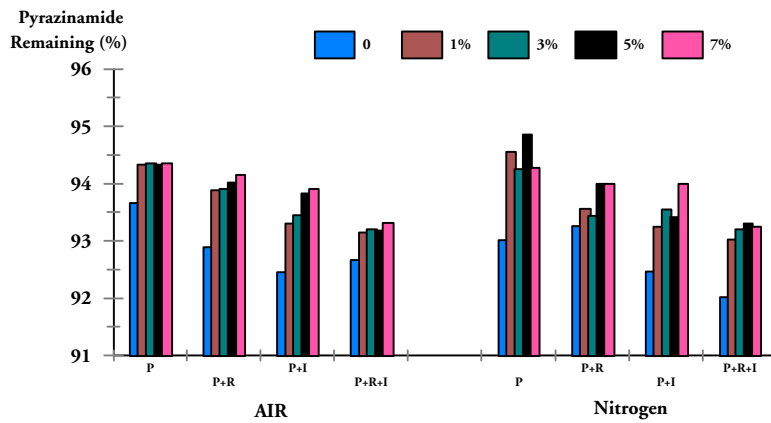


Figure 7.42 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Hydroxypropyl-β-Cyclodextrin at 40EC

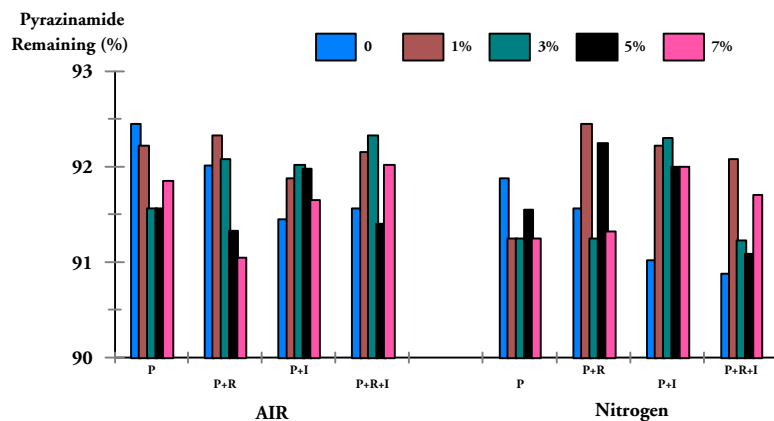


Figure 7.43 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of Hydroxypropyl-β-Cyclodextrin at 60EC

7.2.6 SOLUBILITY STUDIES OF RIF, INH, PZA AND VARIOUS COMBINATIONS IN THE PRESENCE OF β -CYCLODEXTRIN

1.0, 2.0 3.0, 4.0, 5.0, 6.0 and 8.0% m/v β -cyclodextrin solutions were produced by accurately weighing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.8g of β -cyclodextrin and dissolving in 10ml of water. These solutions were saturated with the rifampicin, isoniazid, pyrazinamide, rifampicin with isoniazid, rifampicin with pyrazinamide, isoniazid with pyrazinamide and rifampicin with both isoniazid and pyrazinamide. The supernatants were analyzed by HPLC after 12 hours of constant shaking at a $25 \pm 2^\circ\text{C}$ in a waterbath. Figures 7.44 to 7.46 represented the solubility studies results and the corresponding data can be examined in tables 7.8.

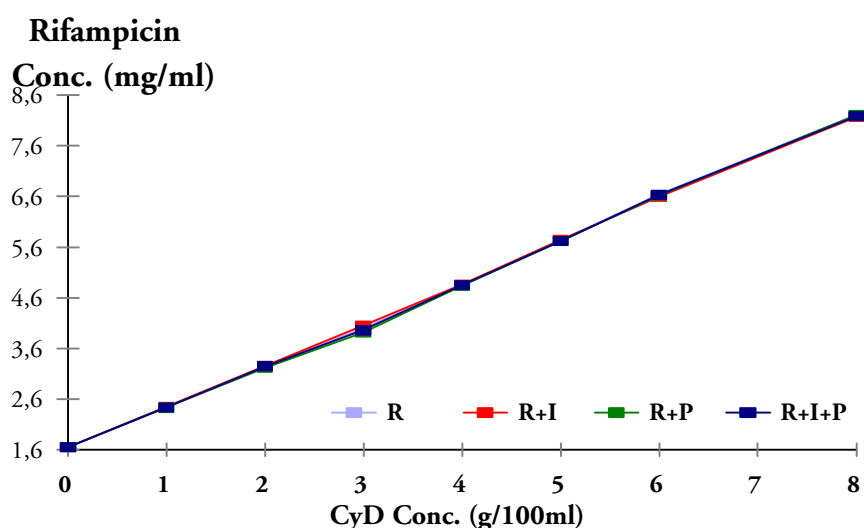


Figure 7.44 Solubility Curves of Rifampicin (RIF Alone, RIF With INH, RIF With PZA or RIF With Both INH or PZA) in the Presence of β -Cyclodextrin

The above figure clearly demonstrates that the target concentration (75mg/10ml)rifampicin could be reached by incorporating about 0.7112g of β -cyclodextrin when combining with isoniazid and pyrazinamide in 10ml.

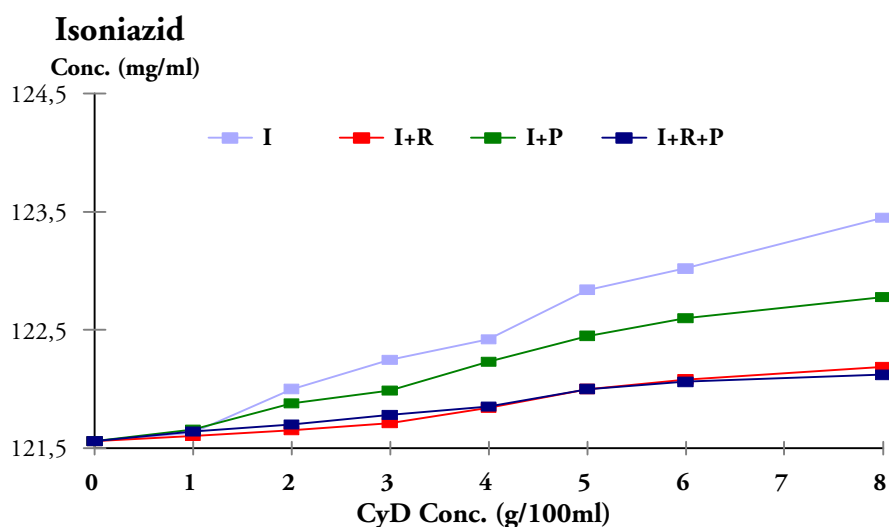


Figure 7.45 Solubility Curves of Isoniazid (INH Alone, INH With RIF, INH, With PZA or INH With Both RIF And PZA) in the Presence of (-Cyclodextrin

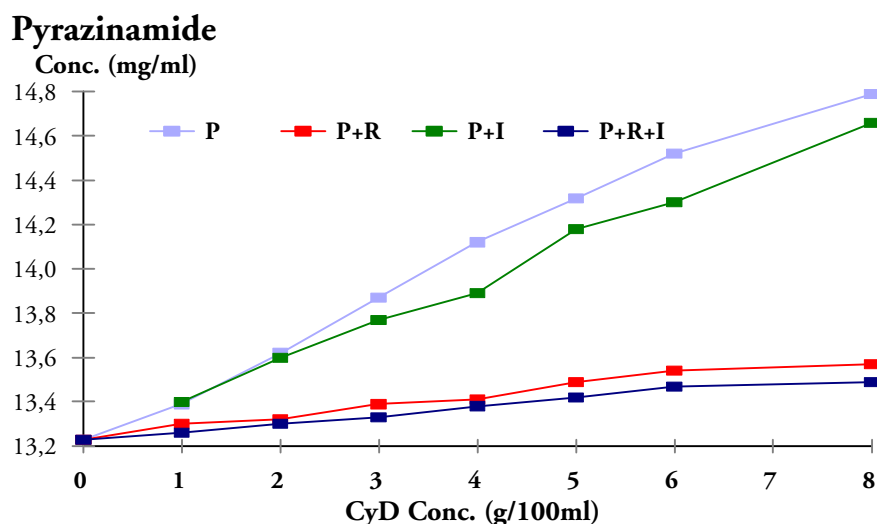


Figure 7.46 Solubility Curves of Pyrazinamide (PZA Alone, PZA With RIF, PZA With INH, or PZA With Both RIF And INH) in the Presence of (-Cyclodextrin

The target concentration of pyrazinamide (100mg/10ml) was unable to be reached even when 0.8g of (-cyclodextrin was incorporated into the 10ml water. Once again, the competition for interaction with cyclodextrins amongst the drugs is shown in above figures. In the presence of rifampicin, both isoniazid and pyrazinamide showed minimal solubility enhancement and isoniazid and pyrazinamide further caused the reduction of each other's solubility.

Table 7.8 Rifampicin, Isoniazid and Pyrazinamide Solubility Curves in the Presence of β -Cyclodextrin

Drug- β -CyD Solubility Curve Data				
Analyte	Cyclodextrins [plus other drug(s)]	Equation	Linear Correlation Factor	Interaction Ratio Drug : β -CyD (mole : mole)
RIF	β -CyD	$y = 0.828x + 1.595$	0.9994	1.292 : 1
INH	β -CyD	$y = 0.094x + 121.537$	0.9844	2.413 : 1
PZA	β -CyD	$y = 0.102x + 13.238$	0.9904	2.201 : 1
RIF	INH +HP- β -CYD	$y = 0.821x + 1.625$	0.9998	1.286 : 1
INH	RIF +HP- β -CYD	$y = 0.021x + 121.559$	0.9816	0.385 : 1
RIF	PZA + HP- β -CYD	$y = 0.828x + 1.597$	0.9990	1.293 : 1
PZA	RIF + HP- β -CYD	$y = 0.0313x + 13.253$	0.9866	0.804 : 1
INH	PZA + HP- β -CYD	$y = 0.0591x + 121.554$	0.9957	1.601 : 1
PZA	INH + HP- β -CYD	$y = 0.0733x + 13.258$	0.9976	1.828 : 1
RIF	INH + PZA + HP- β -CYD	$y = 0.825x + 1.600$	0.9992	1.287 : 1
INH	RIF + PZA +HP- β -CYD	$y = 0.076x + 121.561$	0.9817	0.495 : 1
PZA	RIF + INH +HP- β -CYD	$y = 0.037x + 13.25$	0.9797	0.493 : 1

Although phase solubility can not yield precise information regarding the exact ratio of higher order complexation. A conclusive determination can be achieved only from two dimensional NMR and single X-ray diffraction studies. However, the drug to β -cyclodextrin solubilizing ratio listed in table 7.8 could suggest the following:

- < The interaction ratio between rifampicin and β -cyclodextrin is approximately 1.3 to 1 (mol. : mol.) which suggests that either every four β -cyclodextrins may be complexed with two molecules of rifampicin or rifampicin may interact with part of the β -cyclodextrin outside of the hydrophobic cavity.

- < The interaction ratio between isoniazid and (-cyclodextrin is approximately 2.4 to 1 (mol. : mol.) which suggests that either five isoniazids are complexed with two (-cyclodextrins either through inclusion complexation or external interaction. Pyrazinamide shows a similar interaction with (-cyclodextrin with the ratio of 2.2 to 1 (mol. : mol.).
- < When rifampicin combines with isoniazid in the presence of (-cyclodextrin, the ratio is approximately 8 : 2 : 10 (RIF : PZA : (-CyD; mol. : mol. : mol.), but when rifampicin combines with pyrazinamide the ratio difference is decreased to approximately 6 : 4 : 10 (RIF : PZA : (-CyD; mol. : mol. : mol.). Therefore this could suggest that the affinity of drug-(-cyclodextrin interaction is in the order of RIF > PZA > INH.
- < When isoniazid combines with pyrazinamide in the presence of (-cyclodextrin, the ratio is approximately 8 : 9 : 17 (INH : PZA : (-CyD; mol. : mol. : mol.) which suggests the isoniazid has a slightly weaker interaction than pyrazinamide.
- < Furthermore, when rifampicin, isoniazid and pyrazinamide were all combined together in the presence of (-cyclodextrin, the ratio is approximately 12 : 5 : 5 : 22 (RIF : INH : PZA : (-CyD; mol. : mol. : mol.).

The classification of the phase solubility curves are presented in table 7.9.

Table 7.9 Classification of (-Cyclodextrin Phase-Solubility Curves

(-Cyclodextrin Phase-Solubility Curves			
<i>Additional Factors</i>	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
(-CyD only	A _p	A _p	A _p
(-CyD + RIF	A _p	Minor A _p	Minor A _p
(-CyD + INH	A _p	A _p	A _p
(-CyD + PZA	A _p	A _p	A _p
(-CyD + three Drugs	A _p	Minor A _p	Minor A _p

7.2.7 STABILITY STUDIES OF RIF, INH, PZA IN THE PRESENCE OF β -CYCLODEXTRIN

The same method as in section 7.2.5 was repeated by replacing the hydroxypropyl- β -cyclodextrin with β -cyclodextrin. 1.0, 3.0, 5.0 and 7.0 % of β -cyclodextrin solutions were made up by dissolving 0.1, 0.3, 0.5 or 0.7g of β -cyclodextrin in 10 ml water. The stability assessments of rifampicin, isoniazid and pyrazinamide in the presence of β -cyclodextrin were conducted as per the method used in section 7.2.3 and the observations are as follows:

- < Increasing the cavity size did not enhance the stability of rifampicin and the stability reduction is dependent on the β -cyclodextrin concentration.
- < Isoniazid showed no significant increase in its aqueous stability, whereas a slight stability enhancement of 2.9 % was observed for pyrazinamide.
- < The general stabilities of the three drugs in combinations were also improved. β -cyclodextrin reduced the drug-drug interaction especially when rifampicin, isoniazid and pyrazinamide were stored together at the 25 or 40°C, the enhancements in stability up to 5.6, 3.8 and 2.4% were clearly shown.

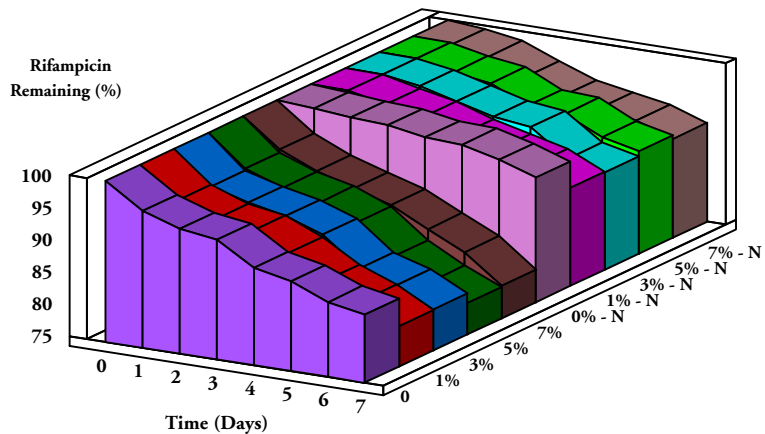


Figure 7.47 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 25°C

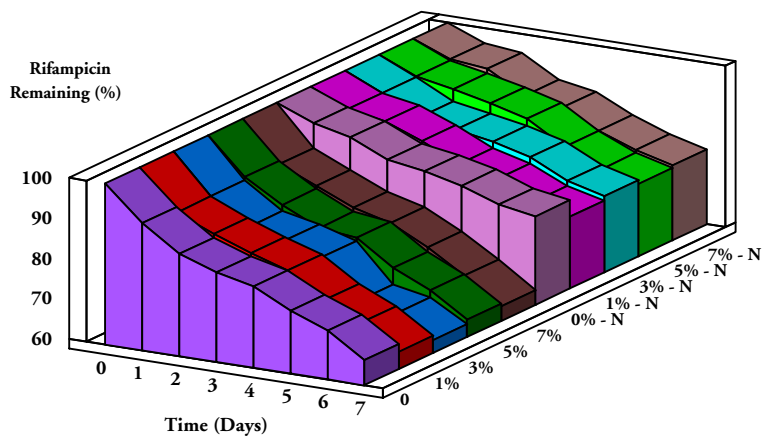


Figure 7.48 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 40°C

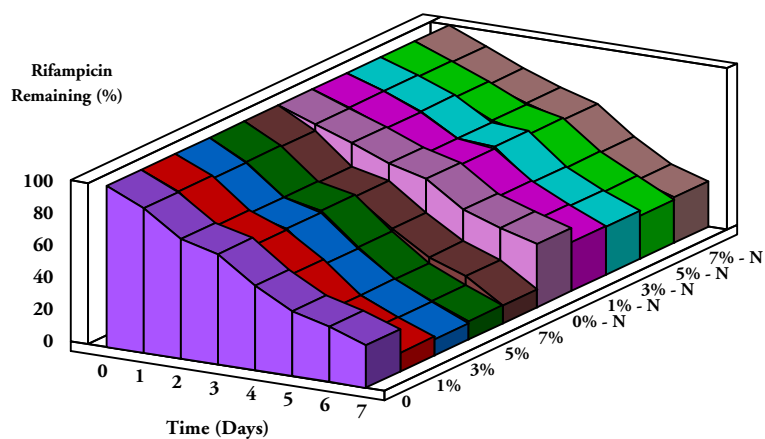


Figure 7.49 Stability Profiles of Rifampicin in the Presence of β -Cyclodextrin at 60°C

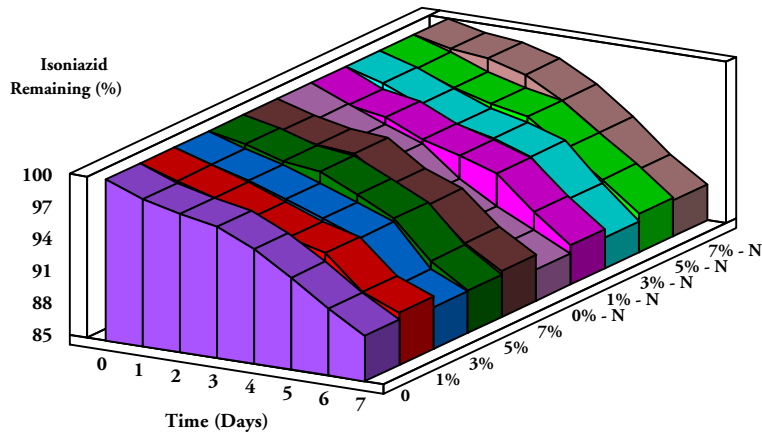


Figure 7.50 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 25EC

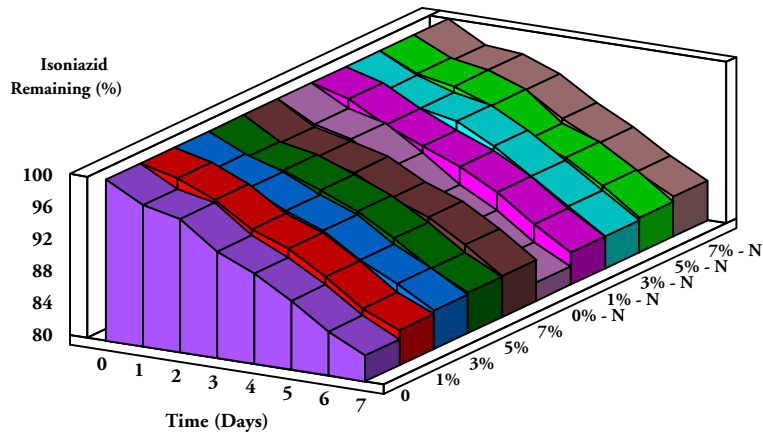


Figure 7.51 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 40EC

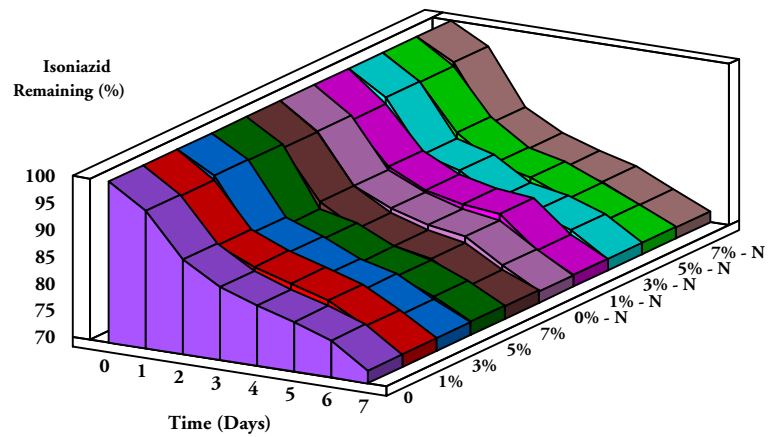


Figure 7.52 Stability Profiles of Isoniazid in the Presence of β -Cyclodextrin at 60EC

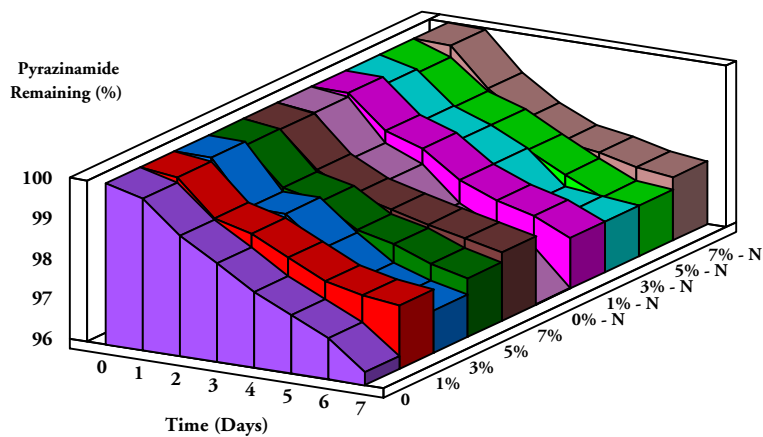


Figure 7.53 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 25°C

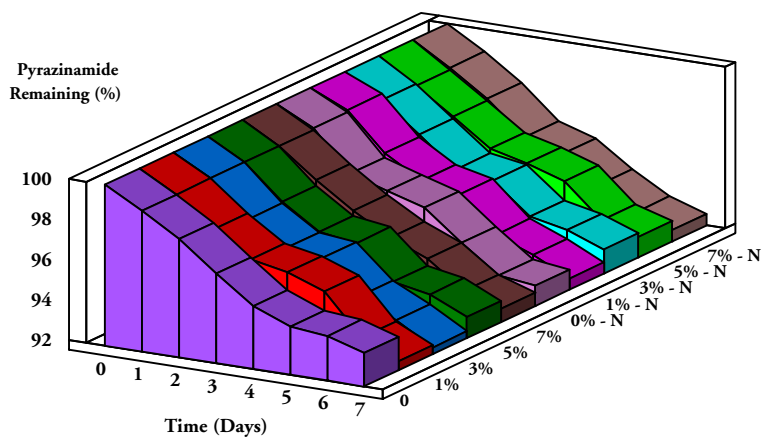


Figure 7.54 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 40°C

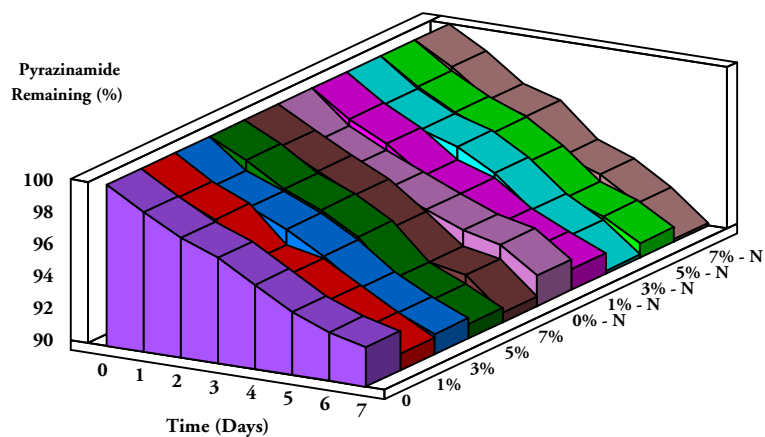


Figure 7.55 Stability Profiles of Pyrazinamide in the Presence of β -Cyclodextrin at 60°C

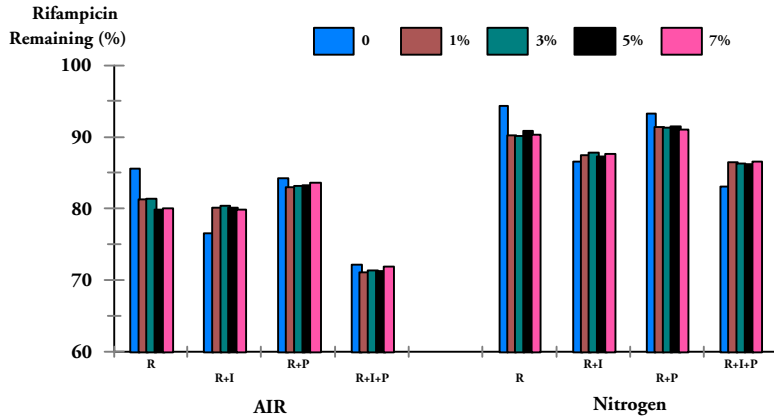


Figure 7.56 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 25EC

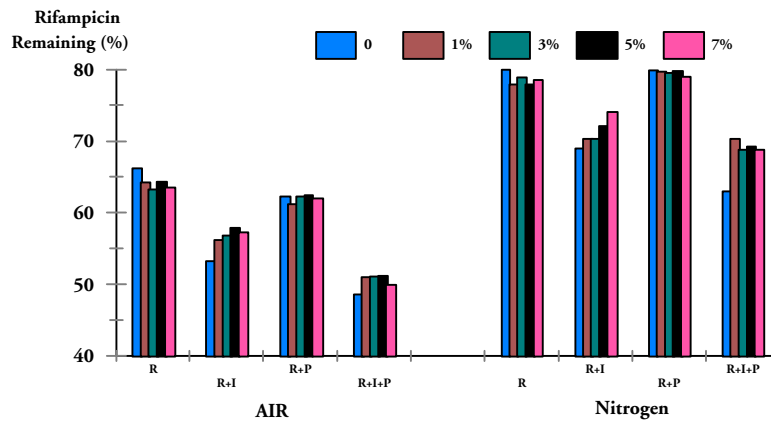


Figure 7.57 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 40EC

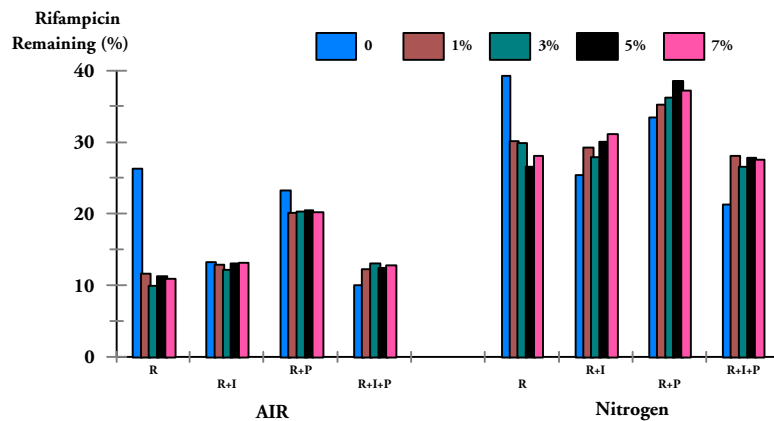


Figure 7.58 Stability Profiles of Rifampicin (RIF, RIF with INH, RIF with PZA and RIF with Both INH and PZA) in the Presence of β -Cyclodextrin at 60EC

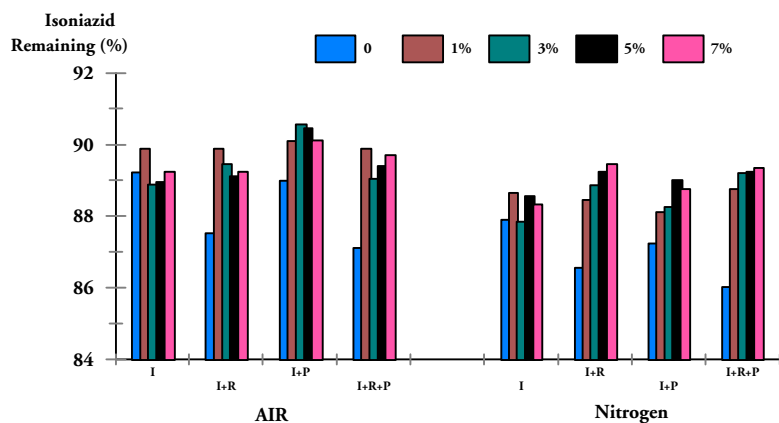


Figure 7.59 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 25°C

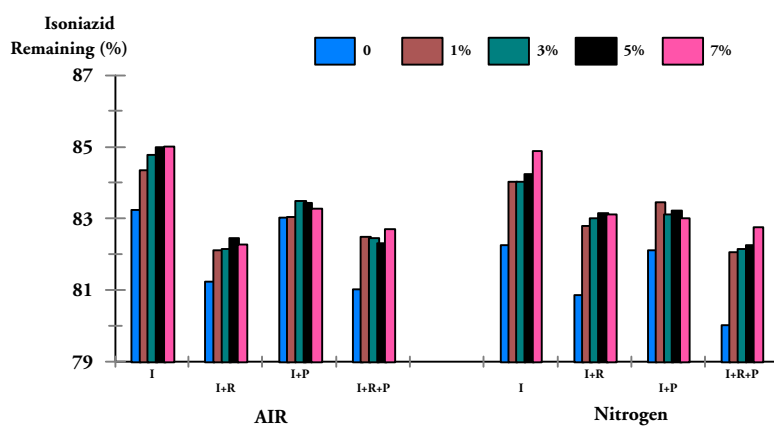


Figure 7.60 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 40°C

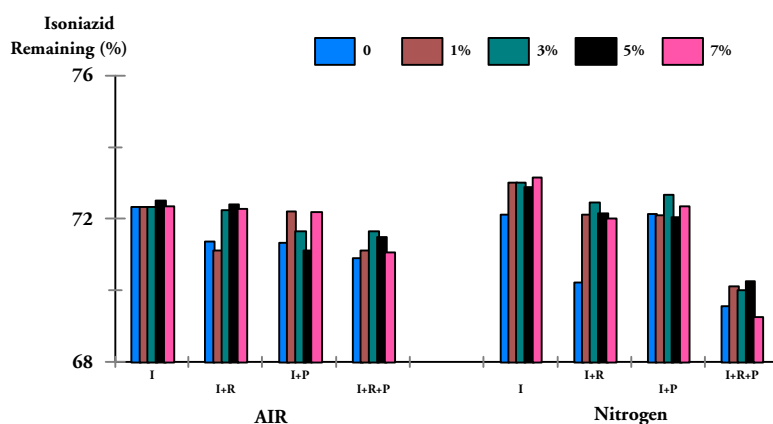


Figure 7.61 Stability Profiles of Isoniazid (INH, INH with RIF, INH with PZA or INH with Both RIF and PZA) in the Presence of β -Cyclodextrin at 60°C

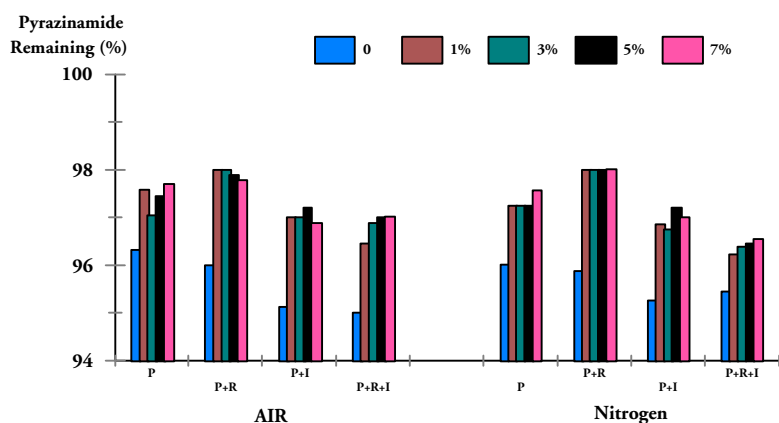


Figure 7.62 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 25°C

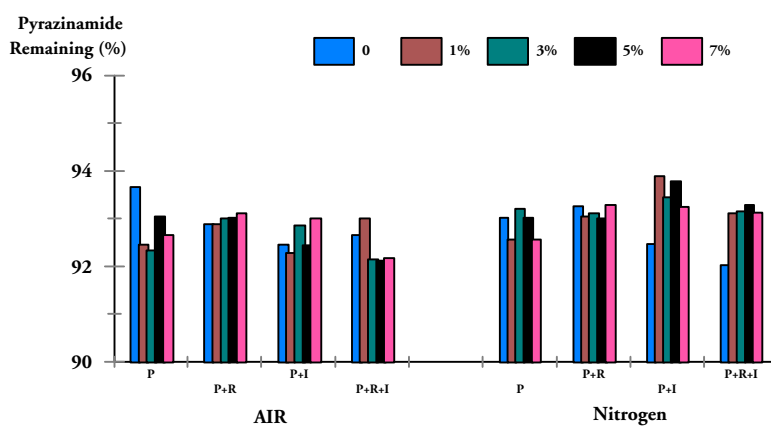


Figure 7.63 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 40°C

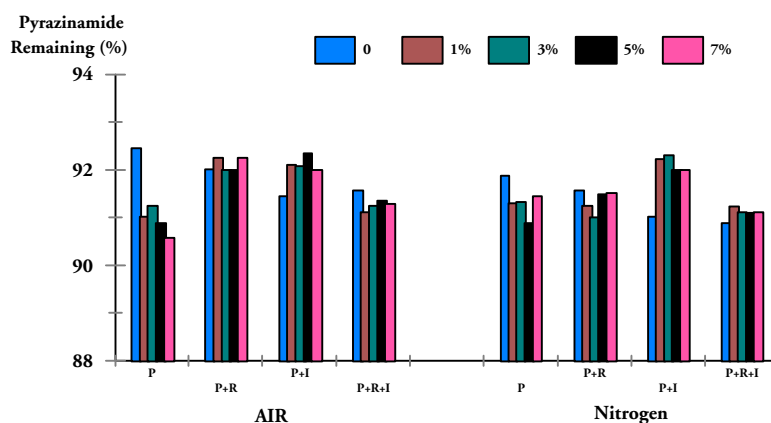


Figure 7.64 Stability Profiles of Pyrazinamide (PZA, PZA with RIF, PZA with INH or PZA with Both RIF and INH) in the Presence of β -Cyclodextrin at 60°C

7.2.8 COMPUTER MODELLING OF β -CYCLODEXTRIN AND α -CYCLODEXTRIN INCLUSION COMPLEXATION WITH RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE

Computer modelling has been frequently used in the field of chemistry. It serves as an excellent tool which enables the researchers to understand sophisticated problems by examining the statistical and mathematical data generated. In the application of host-guest inclusion, the technique of computer force-field simulation analyses is termed as the “docking” experiment. Backensfeld and his co-researchers [Backensfeld et al., 1991] presented the complexation computer models of indomethacin and diclofenac with β -cyclodextrin which has served as a classic example of the usefulness of computer modelling.

The size of the cyclodextrin ring is the essential complexation determining factor to be considered, whereas the alkylation functional groups such as in the hydroxypropyl cyclodextrin derivative do not play an important role in inclusion complexation but are the beneficial factor in the aqueous solubility [Duchêne et al., 1990].

In this section, the docking experiments were attempted in order to provide a crude understanding of the effect of the different cyclodextrin ring cavity size on the rifampicin, isoniazid and pyrazinamide inclusion. Due to the lack of certain information, the following drawbacks should be taken into consideration:

- < Due to the fact that the software used was unable to create a “solvent box”, the actual shape of the cyclodextrin in the presence of water molecules at the particular temperature and given pressure was unable to be determined. The shape and the conformational changes of cyclodextrin determine the outcome of the complexation [Becker et al., 1991].
- < The potential energy difference serves as an guide of the tendency of the drug-cyclodextrin complexation. The accurate potential energy difference (P_o) during the complexation process can be summarised as equation 7.1 which incorporates the essential point of complexation stated in section 7.31.:

$$P_o = E_{\text{Water-CyD}} + E_{\text{Drug}} - E_{\text{Drug-CyD}} \dots\dots\dots \text{Eq. 7.1.}$$

Where

$E_{\text{Water-CyD}}$ = The total potential energy of conformation of water complexing with cyclodextrin at the particular temperature and given pressure

$E_{\text{Water-Drug}}$ = The potential energy of the drug conformation at the particular temperature and the given pressure

$E_{\text{Drug-CyD}}$ = The potential energy of the conformation of drug complexing with cyclodextrin at the particular temperature and the given pressure

Therefore, the actual potential energy of the above situation is difficult to determine without the adequate determination of the proper conformation of the molecule/molecule complexes within the solvent.

< The solvation arrangement of the molecule around cyclodextrin in that not only the water molecules may surround the cyclodextrin molecules but also the drug molecule could also have affinity towards the exterior of the cyclodextrin when in the presence of water molecules. This could provide an understanding of the solubility outcome. However, without the "solvent box", rifampicin, isoniazid and pyrazinamide preferred to situated themselves away from the exterior of cyclodextrin

As these docking experiments are only intended to served as a crude understanding of complexation, the docking experiments were conducted without the "solvent box" as follows:

< Using the three dimensional conformation of both β -cyclodextrin and α -cyclodextrin obtained from the Cambridge x-ray crystallography data, they were further trajectory simulated for 100000 steps at 25EC and 1 atmosphere pressure. In each and every trajectory step, the computer made slight adjustments to the model conformation and output with the corresponding energy data. The conformation with the lowest local

potential energies minimum (CyD_{min}) was selected and the potential energy of this conformation (E_{CyD}) was computed.

- < Rifampicin, isoniazid and pyrazinamide were manually constructed and further trajectory simulated for 100000 steps at 25EC and 1 atmosphere pressure. The potential energy of the drugs (E_{Drug}) of the drug conformation with the lowest local minimum energy (D_{min}) was computed.
- < Docking D_{min} with CyD_{min} and trajectory simulated for 100000 steps at 25EC and 1 atmosphere pressure, the inclusion complexes with the lowest potential energy (COM_{min}) were examined and the $E_{Drug-CyD}$ of this COM_{min} complex was computed.
- < The sum of E_{Drug} of the D_{min} and the E_{CyD} of the CyD_{min} was calculated and further subtracted from $E_{Drug-CyD}$ of the COM_{min} . The potential energy difference (P_d) indicates the tendency of the inclusion complexation formation.

The scalar value of the estimated potential difference [$P.D.$ value], i.e. without taking water into consideration] obtained served only as a rough indication of the tendency of inclusion complexation between drugs and cyclodextrin since, the E_{CyD} of β -cyclodextrin CyD_{min} was found to be 283.22Kcal/mol but when one molecule of water is present $E_{Water-CyD}$ of β -cyclodextrin reached approximately 360Kcal/mol depending on the orientation of the water molecule. The E_{CyD} of α -cyclodextrin CyD_{min} was found to be 342.631Kcal/mol but when one molecule of water is present, the $E_{Water-CyD}$ of α -cyclodextrin reached approximately 410Kcal/mol depending on the orientation of the water molecule. Furthermore, both cyclodextrins are able to complex with more than one water molecule in their cavity. Therefore, a higher negative value of $P.D.$ value is expected *in vivo*.

The docking experiments, represented by figures 7.65 to 7.89, indicate various inclusion complexes as some of the drug molecules could have more than one site which is suitable for complexation [Fronza, 1992]. The potential energy values of the drugs and cyclodextrins at 25EC and 1 atmosphere pressure are as follows:

E_{Drug}	(D_{min} of RIF)	=	252.208Kcal/mol
E_{Drug}	(D_{min} of INH)	=	87.867Kcal/mol
E_{Drug}	(D_{min} of PZA)	=	49.077Kcal/mol
$E_{Drug-CyD}$	(CyD_{min} of β -CyD)	=	283.266Kcal/mol
$E_{Drug-CyD}$	(CyD_{min} of α -CyD)	=	342.631Kcal/mol

Note:

P.D. = The estimated potential difference of the complexation without taking water molecules into consideration. The sum E_{CyD} of CyD_{min} and E_{Drug} of D_{min} subtracting COM_{min} and the negative value indicates a possibility of inclusion complexation

“Side” View = Vision plane is parallel to glycopyranosyl residues

“Top” View = Vision plane is perpendicular to glycopyranosyl residues with the two secondary hydroxyl groups at the C2 and C3 position on the top surface of the model

“Bottom” View = Vision plane is perpendicular to glycopyranosyl residues with the two primary hydroxyl groups at the C6 position on the top surface of the model

Ball Shape models = The space filling model resembles the actual shape and the size of the drug corresponding to the particular cyclodextrin

Purple Models = The dotted space filling model demonstrates actual dimensions of the cyclodextrin and the stick model represents the glycopyranosyl residues

Green Arrows = Indicates the major degradation sites of that particular drug

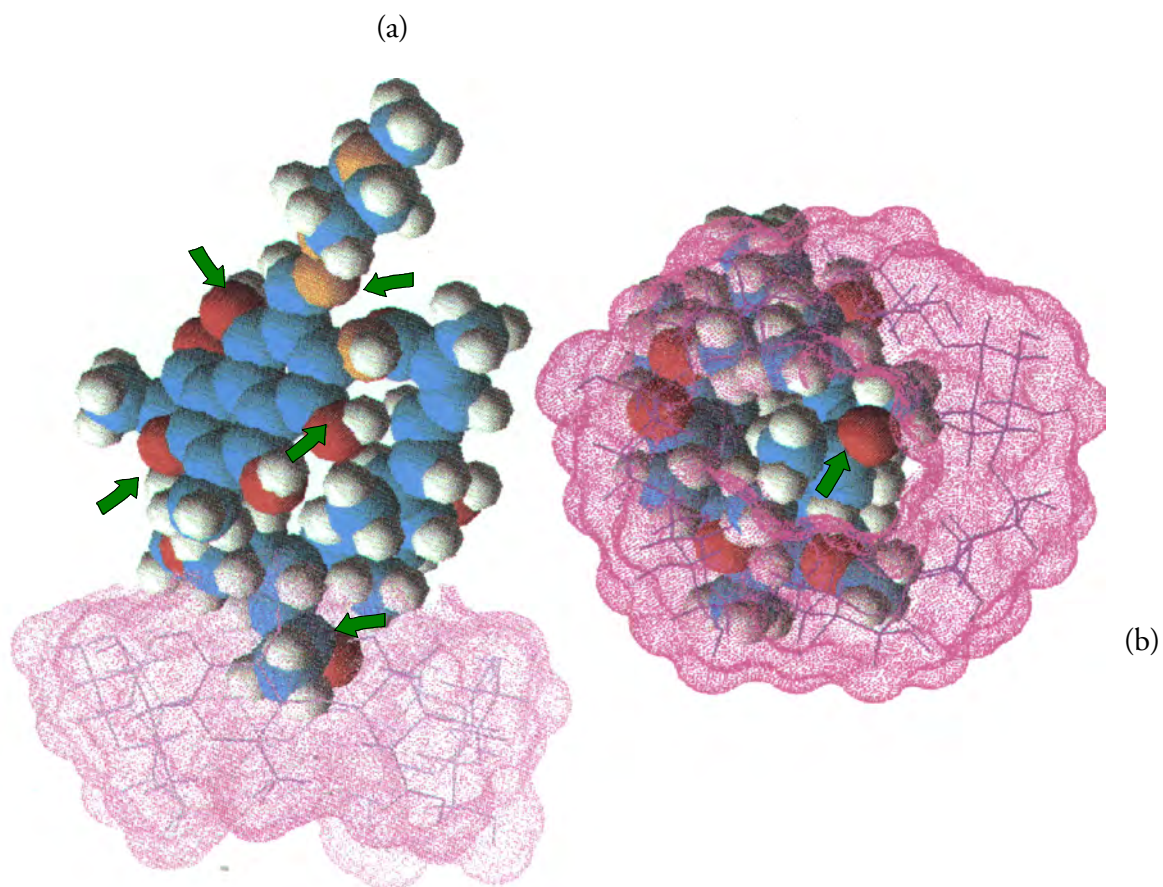


Figure 7.65. Rifampicin- β -Cyclodextrin Complexation I (a) "Side" View and (b) "Bottom" View

The local potential minimum of this complex is 441.798Kcal/mol with a resulting *P.D.* value of -93.636Kcal/mol. This model shows rifampicin favourable to complex with β -cyclodextrin. However, the "side" view clearly demonstrates that the degradation sites are excluded from the β -cyclodextrin cavity. Therefore, the stability of rifampicin should not be enhanced .

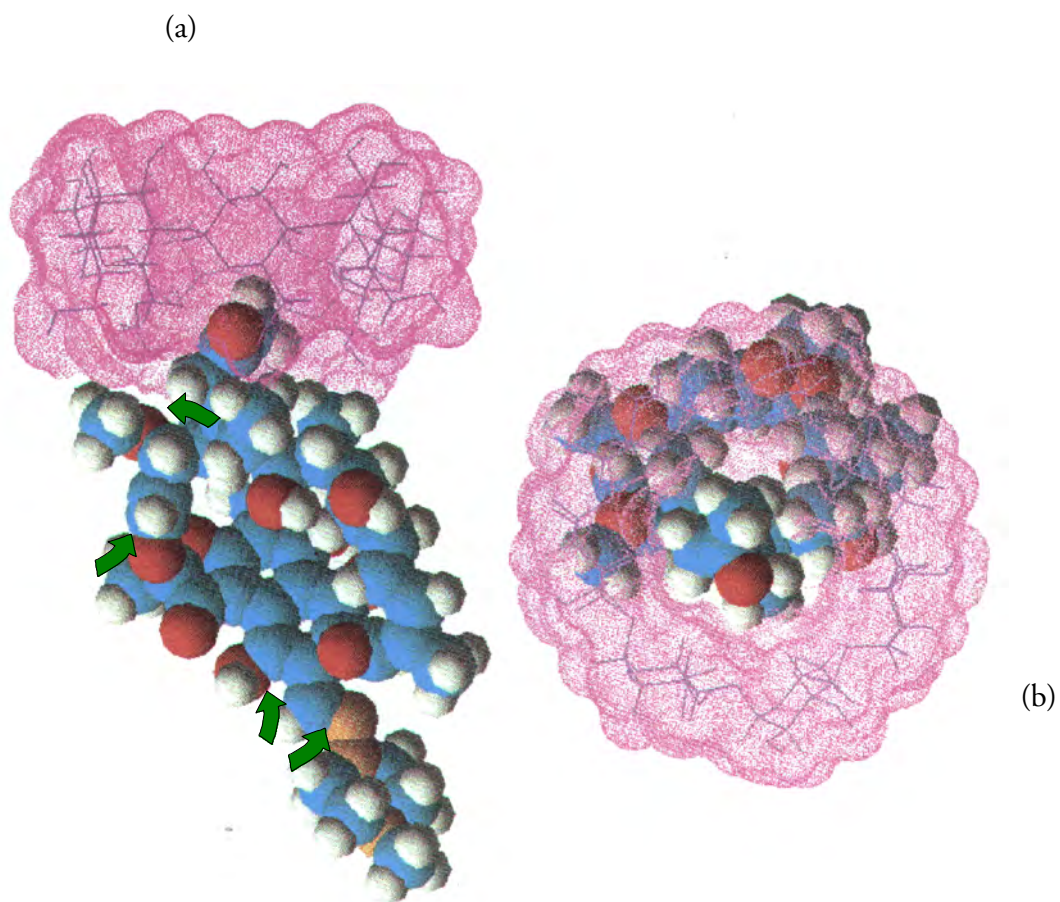


Figure 7.66. Rifampicin-Cyclodextrin Complexation II (a) “Side” View and (b) “Top” View

The local potential minimum of this complex is 480.796Kcal/mol with a resulting *P.D.* value of -54.938Kcal/mol. This complexation is the least favourable amongst the four complexations found. The model ((a) “side” view) clearly demonstrates that the degradation sites are still outside of the cavity, hence, the complexation does not offer protection for rifampicin, but this complexation has a lower occurrence possibly due to the slightly higher *P.D.* value compared to all other four complexes.

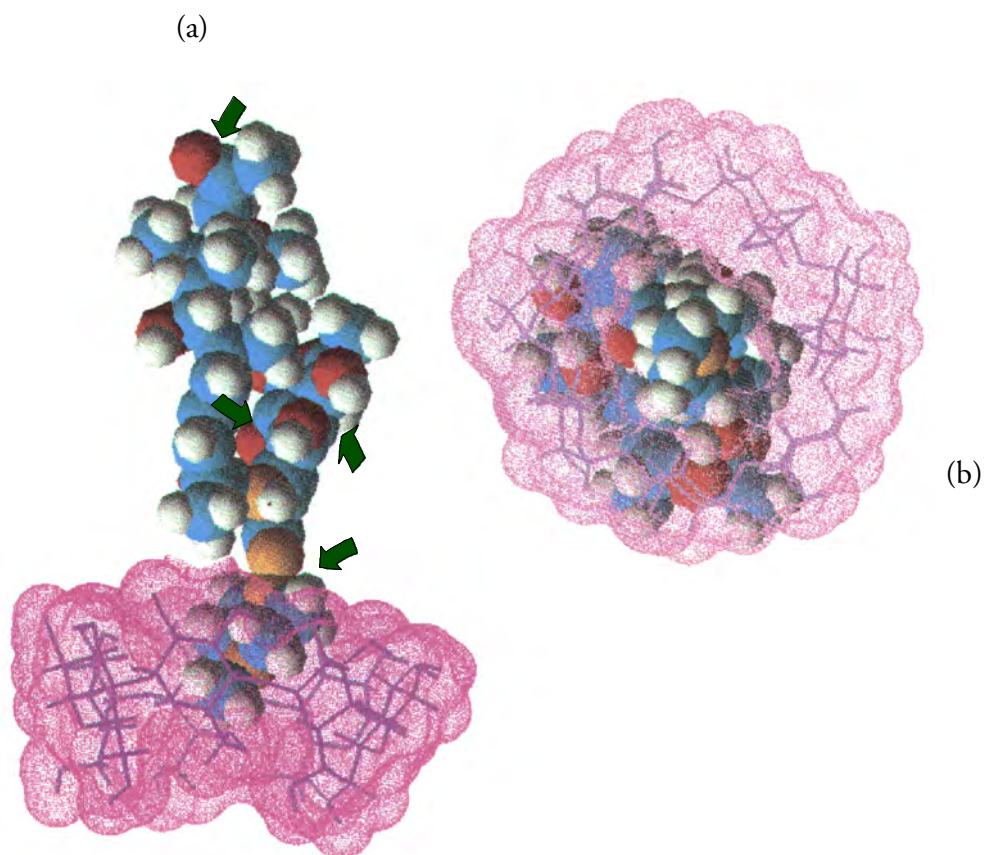


Figure 7.67 Rifampicin- β -Cyclodextrin Complexation III (a) "Side" View and (b) "Bottom" View

The local potential minimum of this complex is 418.984Kcal/mol and the resulting *P.D.* value is -116.450Kcal/mol. This is the most favourable inclusion complexation between rifampicin and β -cyclodextrin. However, the "side" view clearly demonstrates that the most of the degradation sites (except the azomethine bond on the side chain) are out of the cavity. Therefore, the stability of rifampicin should not be enhanced in the presence of β -cyclodextrin which coincides with the experimental stability studies results. Rifampicin is a large molecule with potential degradation sites situated all around the molecule and the cavity of β -cyclodextrin is simply too small to protect all of the potential degradation sites.

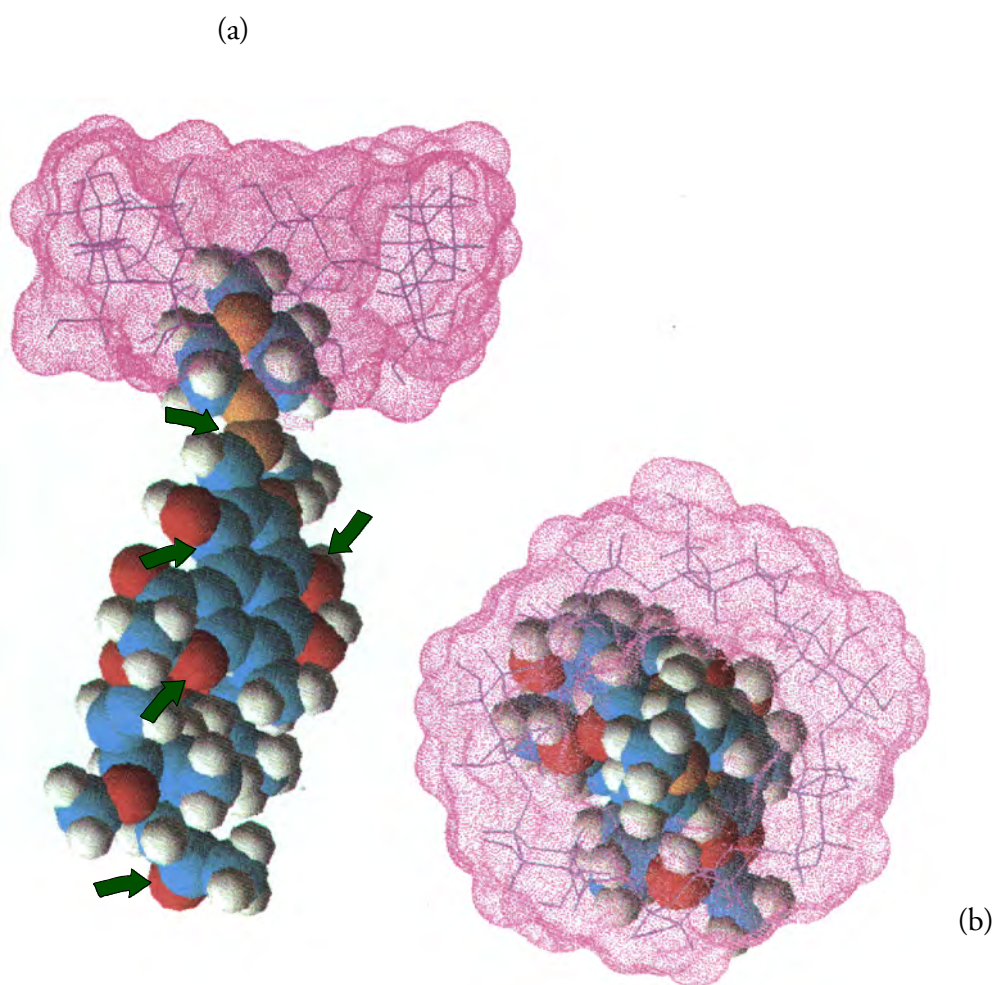


Figure 7.68 Rifampicin- β -Cyclodextrin Complexation IV (a) “Side” View and (b) “Top” View

The local potential minimum of this complex is 453.967Kcal/mol and the resulting *P.D.* value is -86.457Kcal/mol. This complexation is similar to complex III except the piperazine side chain enters from the “top” part of the β -cyclodextrin. However, this complexation is still not able to provide the total protection of the rifampicin degradation sites.

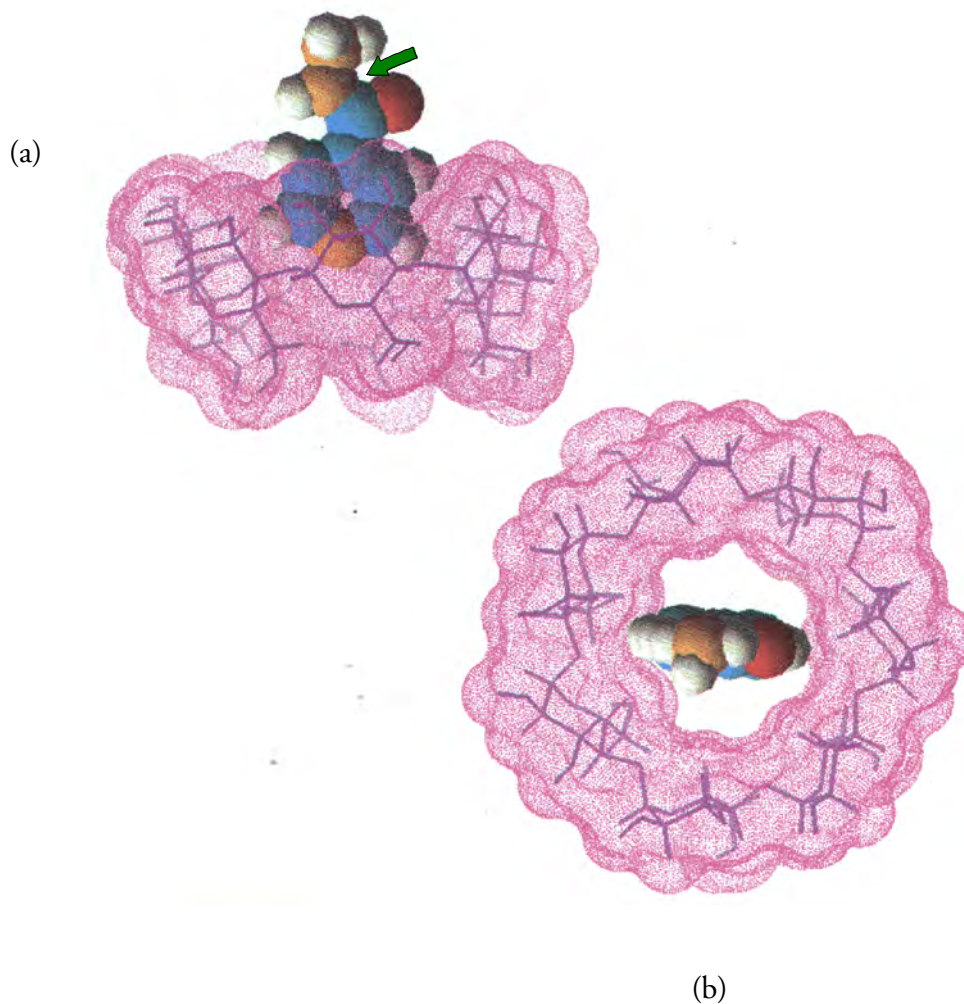


Figure 7.69 Isoniazid- β -Cyclodextrin Complexation I (a) “Side” View and (b) “Top” View

The *P.D.* value is -17.331Kcal/mol and the potential energy minimum of this complex was found to be 353.762l. This complex is not the most favourable arrangement between isoniazid and β -cyclodextrin and the tendency of occurrence of this arrangement is not expected to be high. This arrangement does not offer protection against the hydrolysis taking place on the carboxyhydrazide group of the isoniazid.

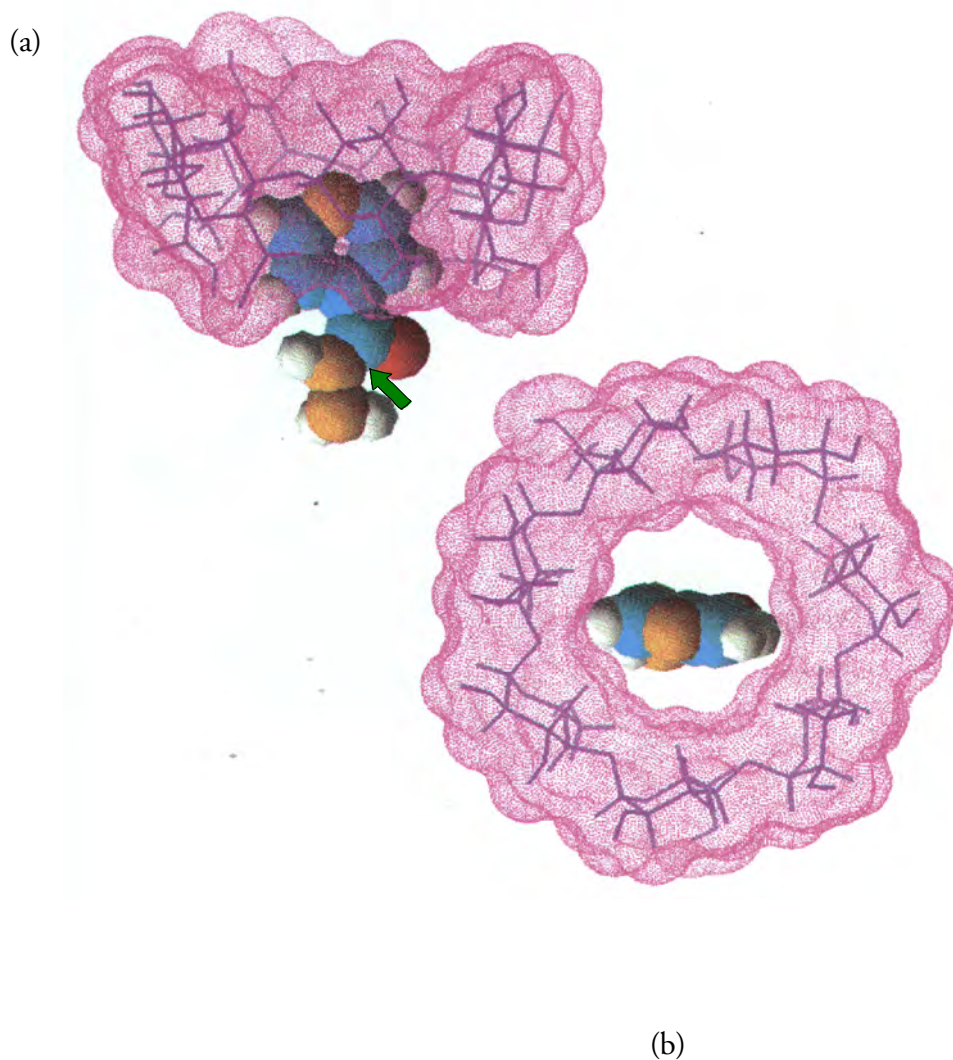


Figure 7.70 Isoniazid-Cyclodextrin Complexation II (a) “Side” View and (b) “Top” View

The *P.D.* value is -21.579Kcal/mol as the local potential energy minimum of this complex was found to be 349.514Kcal/mol . The *P.D.* value of this complex is very close to the complex III but different in the arrangement of the complexation site. The “side” view model shows clearly that the carboxyhydrazide group is situated outside the cavity and the hydrolysis is still free to occur.

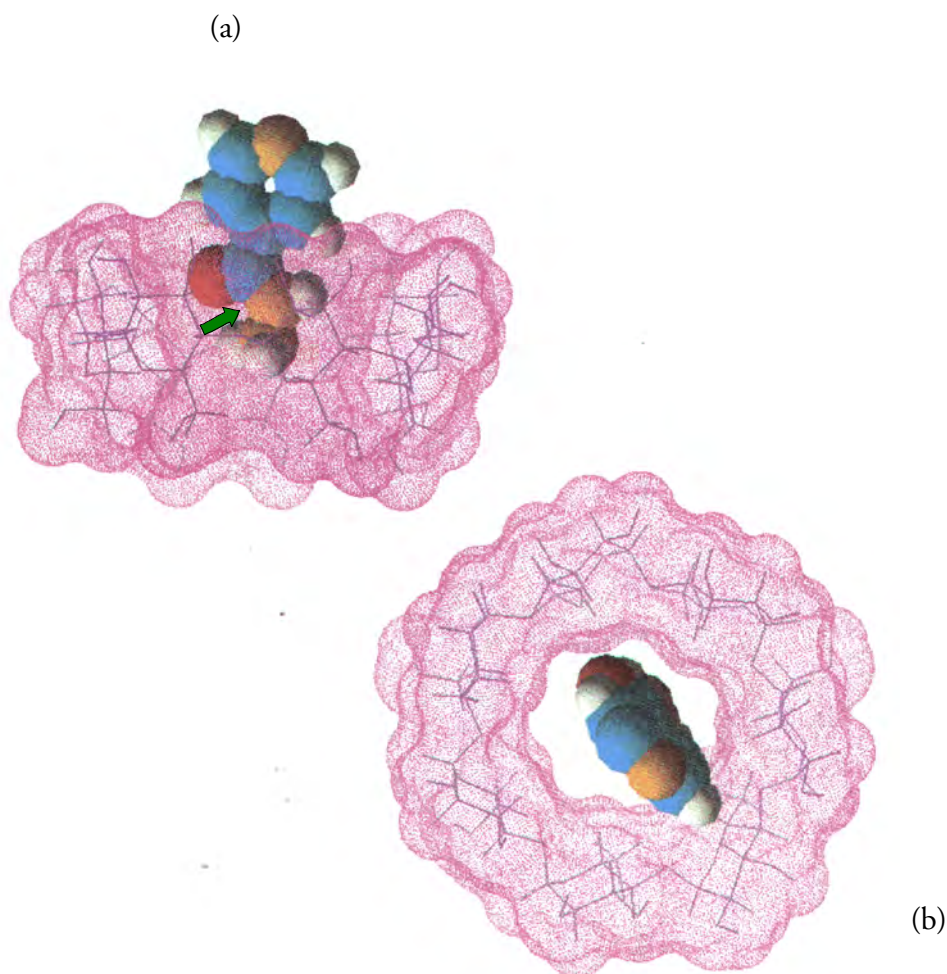


Figure 7.71 Isoniazid- β -Cyclodextrin Complexation III (a) “Side” View and (b) “Top” View

As the local potential energy minimum of this complex was found to be 348.329Kcal/mol and the *P.D.* value is -21,579Kcal/mol. This complex is the more favourable arrangement between isoniazid and β -cyclodextrin. The *P.D.* values of both complexation II and III are very close and thus the occurrence possibility of these two complexes should be almost equal, but the difference in the arrangement at the complexation site would result in a completely different isoniazid stability outcome. As the stability profile suggests a slight enhancement of the isoniazid stability, this could be the indication of complex III as the predominant complexation arrangement.

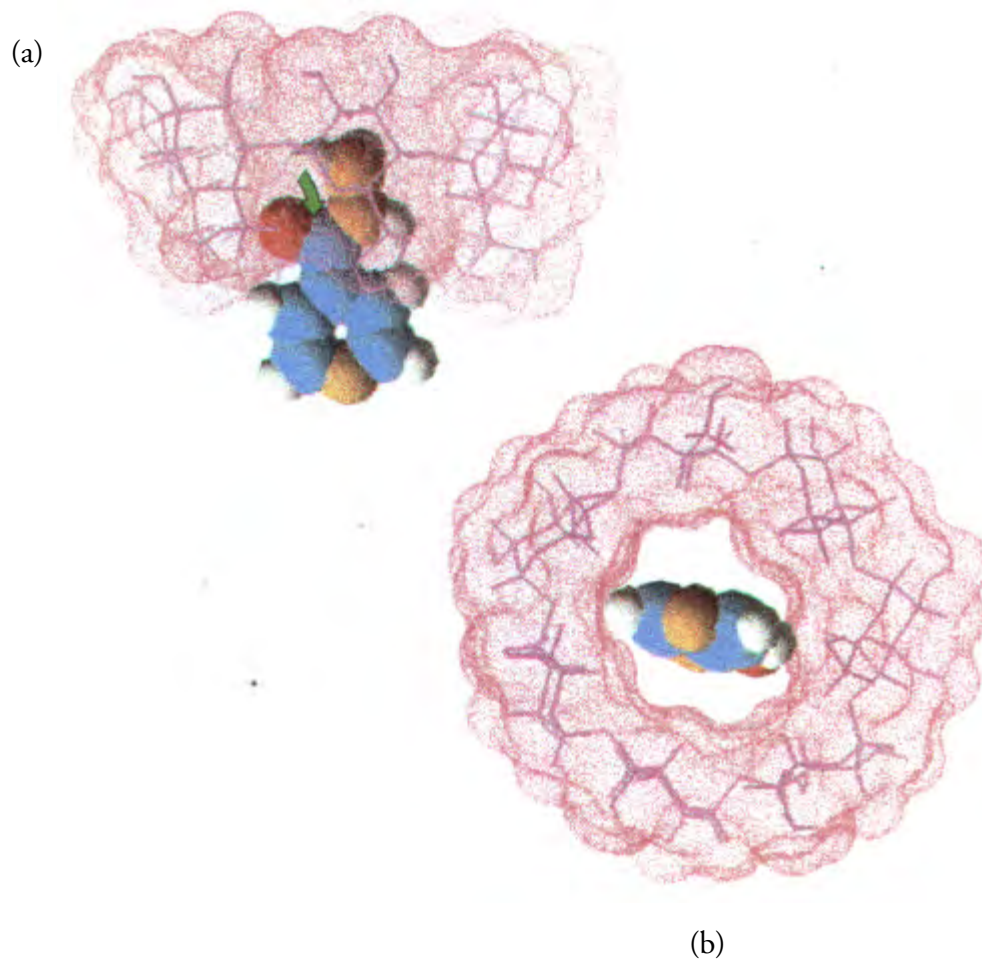


Figure 7.72 Isoniazid- β -Cyclodextrin Complexation IV (a) “Side” View and (b) “Bottom” View

This complex has the local potential energy minimum of 359.372Kcal/mol and the *P.D.* value of -11.721Kcal/mol which makes this complex the least favourable complexation amongst the four other complexes. However, if taking the actual $E_{\text{Water-CyD}}$ into consideration, the difference between the *P.D.* values of complexation IV and the other complexations could appear to be marginal

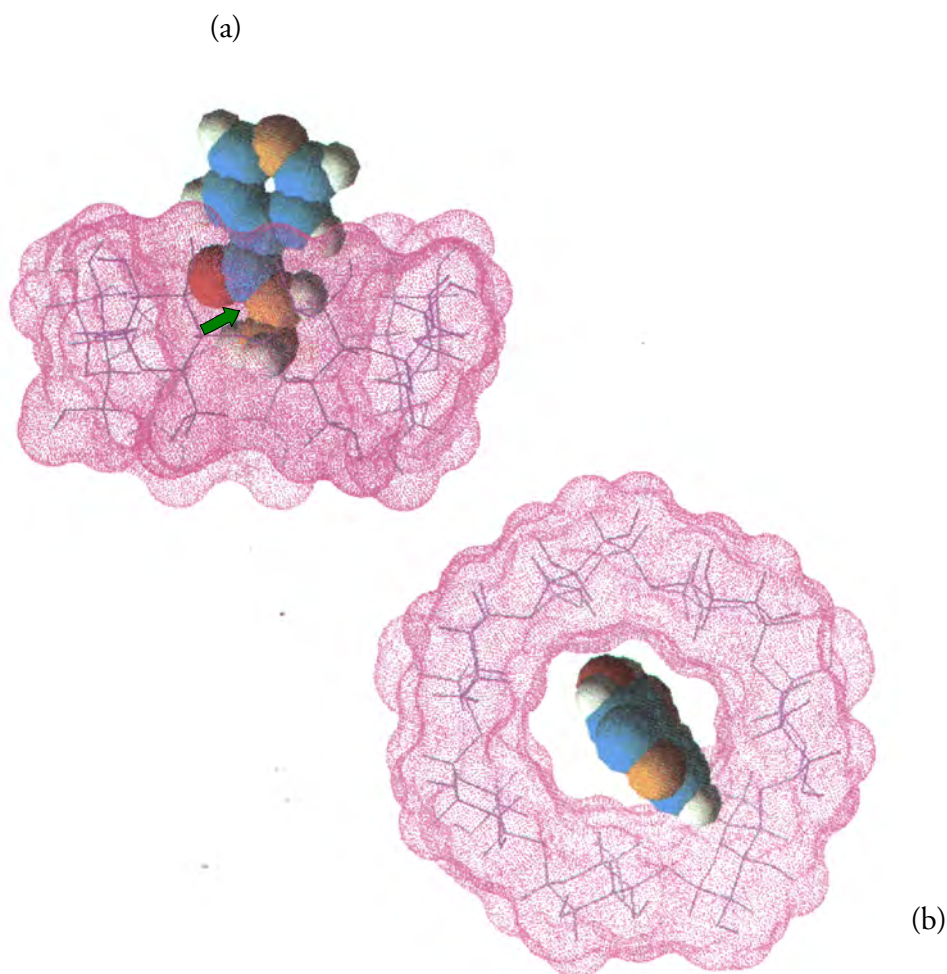


Figure 7.71 Isoniazid- β -Cyclodextrin Complexation III (a) “Side” View and (b) “Top” View

As the local potential energy minimum of this complex was found to be 348.329Kcal/mol and the *P.D.* value is -21,579Kcal/mol. This complex is the more favourable arrangement between isoniazid and β -cyclodextrin. The *P.D.* values of both complexation II and III are very close and thus the occurrence possibility of these two complexes should be almost equal, but the difference in the arrangement at the complexation site would result in a completely different isoniazid stability outcome. As the stability profile suggests a slight enhancement of the isoniazid stability, this could be the indication of complex III as the predominant complexation arrangement.

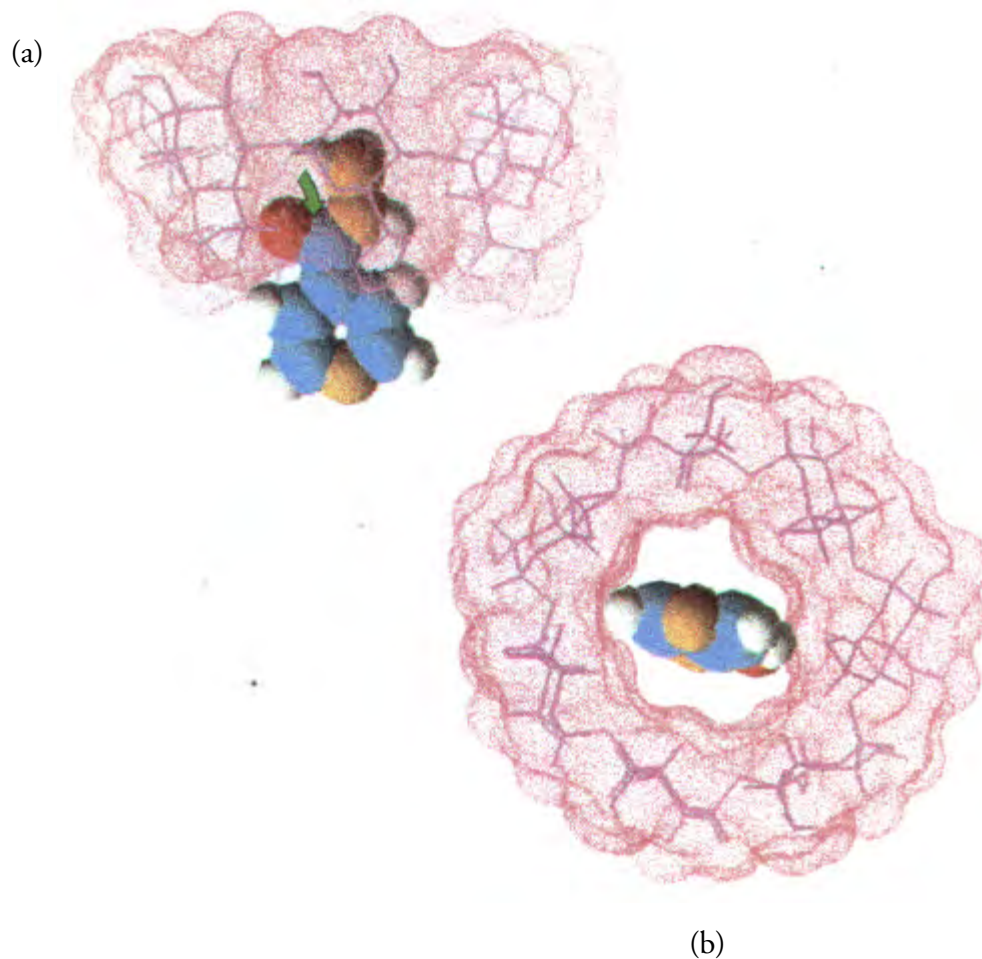


Figure 7.72 Isoniazid-Cyclodextrin Complexation IV (a) “Side” View and (b) “Bottom” View

This complex has the local potential energy minimum of 359.372Kcal/mol and the *P.D.* value of -11.721Kcal/mol which makes this complex the least favourable complexation amongst the four other complexes. However, if taking the actual $E_{\text{Water-CyD}}$ into consideration, the difference between the *P.D.* values of complexation IV and the other complexations could appear to be marginal

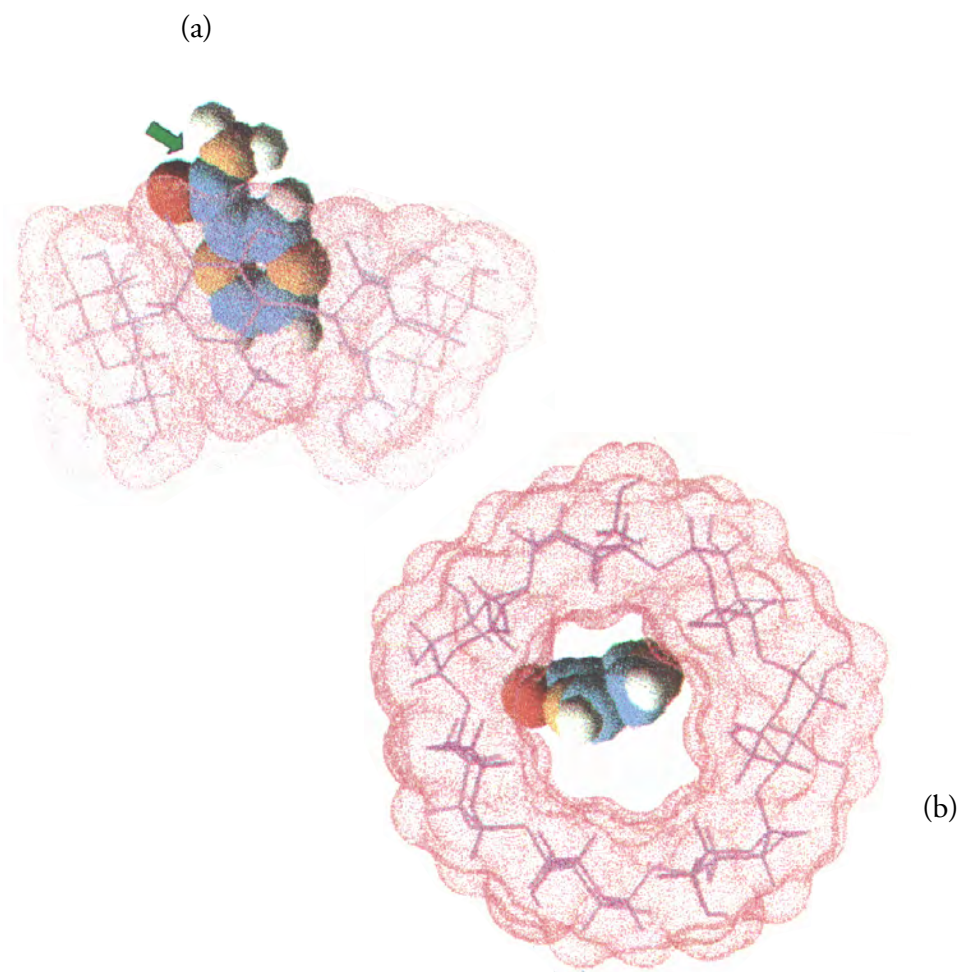


Figure 7.73 Pyrazinamide- β -Cyclodextrin Complexation I (a) “Side” View and (b) “Bottom” View

These models represent the most favourable complexation between pyrazinamide and β -cyclodextrin as the $E_{\text{Drug-CyD}}$ is 310.570Kcal/mol which results in a *P.D.* value of -21.733Kcal/mol. The pyrazine is the complexation site thus the carboxamide is not being protected and free from hydrolysis. However, the stability profile indicates that the presence of β -cyclodextrin enhances the pyrazinamide stability. This could be due to the water solvation shell which may be situated far from the carboxamide and therefore does not interfere with the stability.

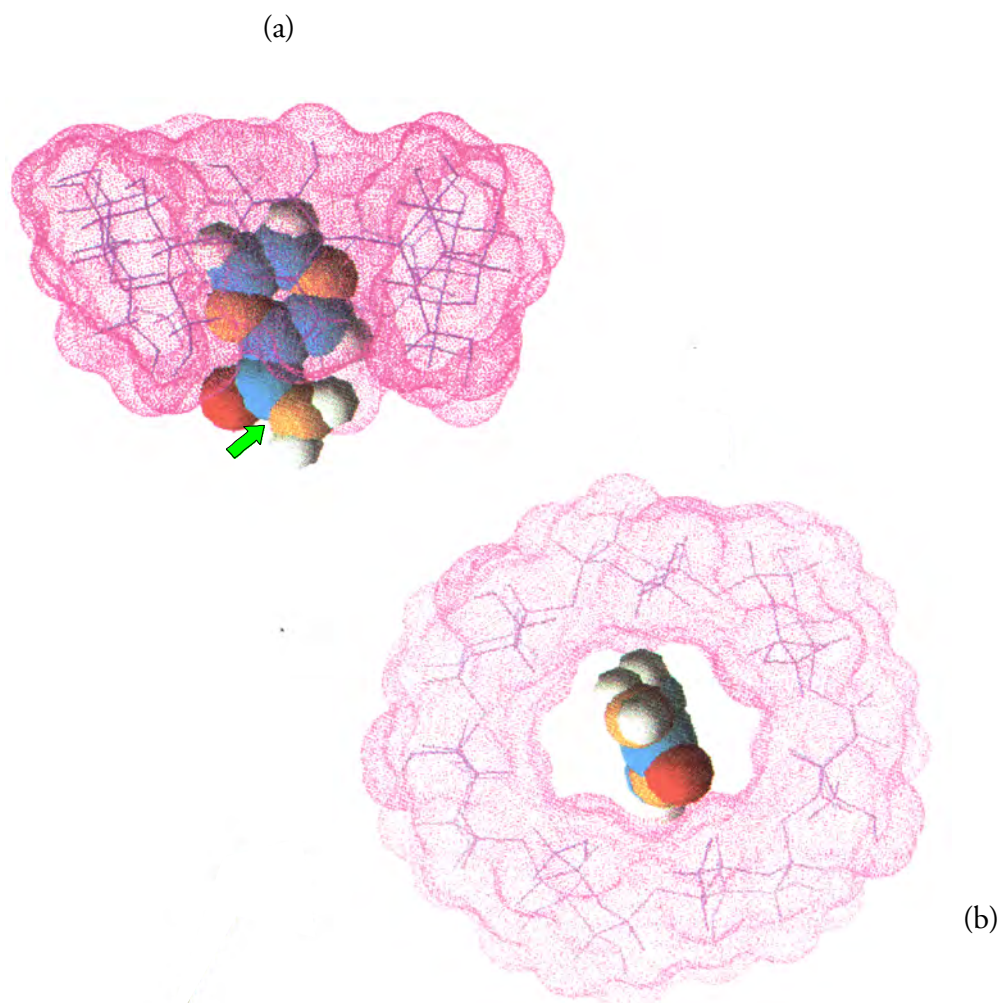


Figure 7.74 Pyrazinamide- β -Cyclodextrin Complexation II (a)“Side” View and (b) “Bottom” View

This complexation has the $E_{\text{Drug-CyD}}$ (COM_{min}) of 312.424Kcal/mol and the $P.D.$ value of -19.879Kcal/mol. The negative $P.D.$ value indicates that complexation is possible and hence the pyrazinamide solubility should be enhanced. The solubility studies are proof of the above statement. However, the carboxamide is still outside of the β -cyclodextrin cavity and the stability enhancement should not be expected, a contradiction with the stability profiles. Therefore, unless the arrangement of water around the pyrazinamide- β -cyclodextrin complex can be created, this cannot be explained.

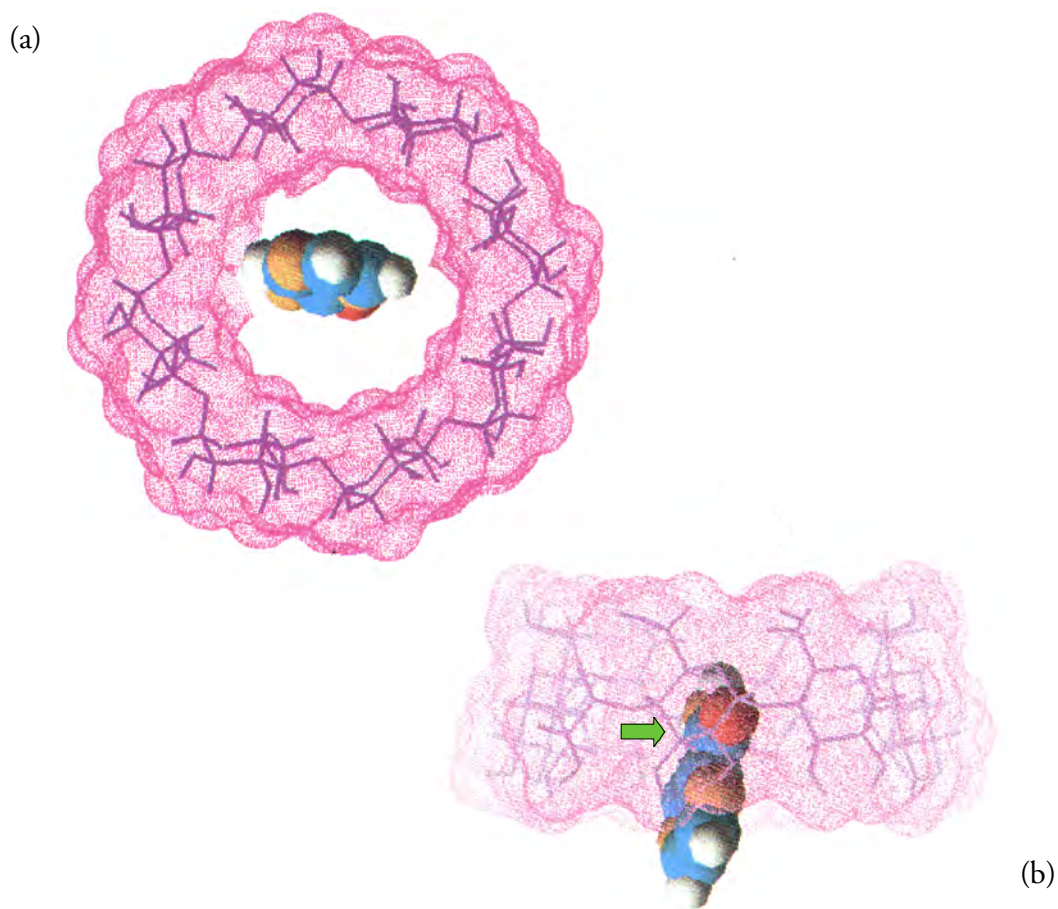


Figure 7.75 Pyrazinamide- β -Cyclodextrin Complexation III (a) "Top" View and (b) "Side" View

This complexation has the local potential energy minima of 313.363Kcal/mol and the negative $P.D.$ value of -18.940Kcal/mol. The negative $P.D.$ value indicates that complexation is possible and hence the pyrazinamide solubility should be enhanced. The solubility studies coincide with above statement. The carboxamide is protected by the β -cyclodextrin cavity and the stability enhancement should be expected. Since there is only about 4Kcal/mol energy difference between complexes I and III the difference is marginal, taking the actual $E_{\text{Water-CyD}}$ into account.

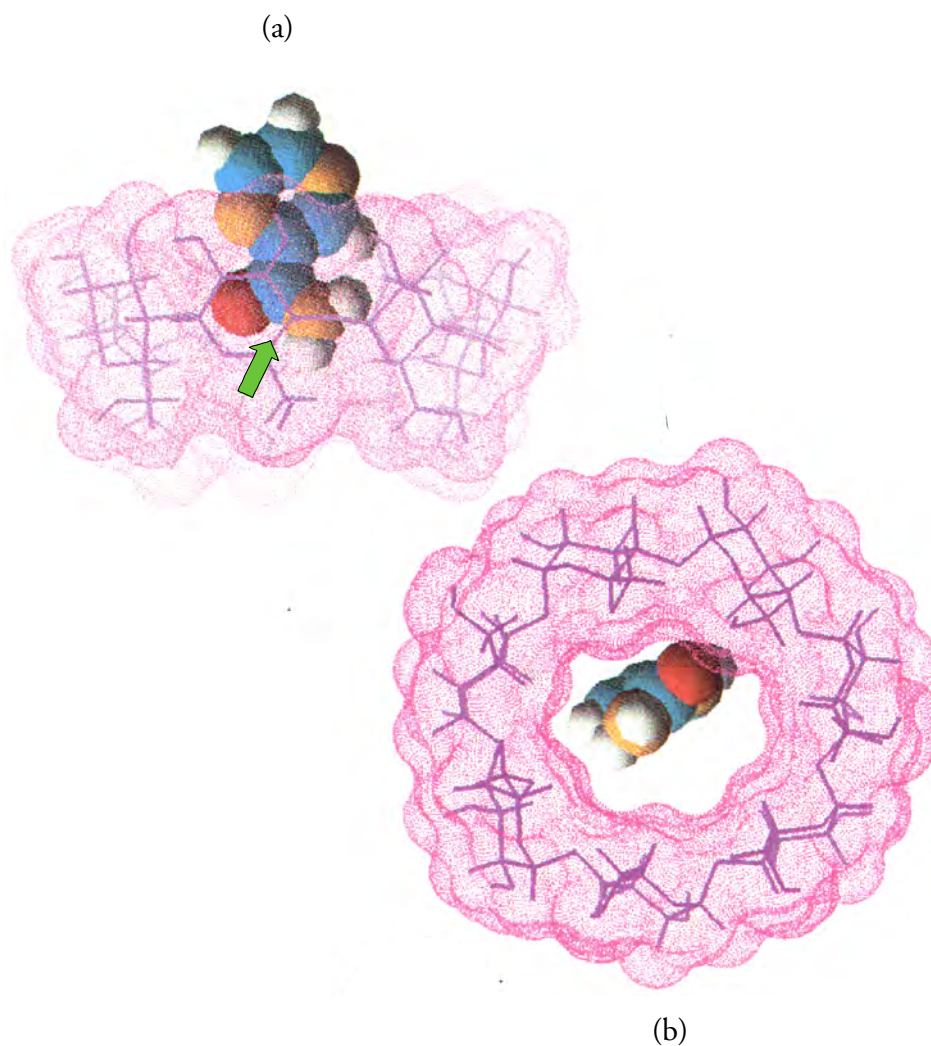


Figure 7.76 Pyrazinamide- β -Cyclodextrin Complexation IV (a) “Side” View and (b) “Bottom” View

This complexation has a local potential energy minima of 315.376Kcal/mol and the *P.D.* value of -16.927Kcal/mol. The negative *P.D.* value indicates that formation of this complex is possible and hence the pyrazinamide solubility should be enhanced. The solubility studies again confirm the above statement. The carboxamide is within the β -cyclodextrin cavity and the stability enhancement should be expected.

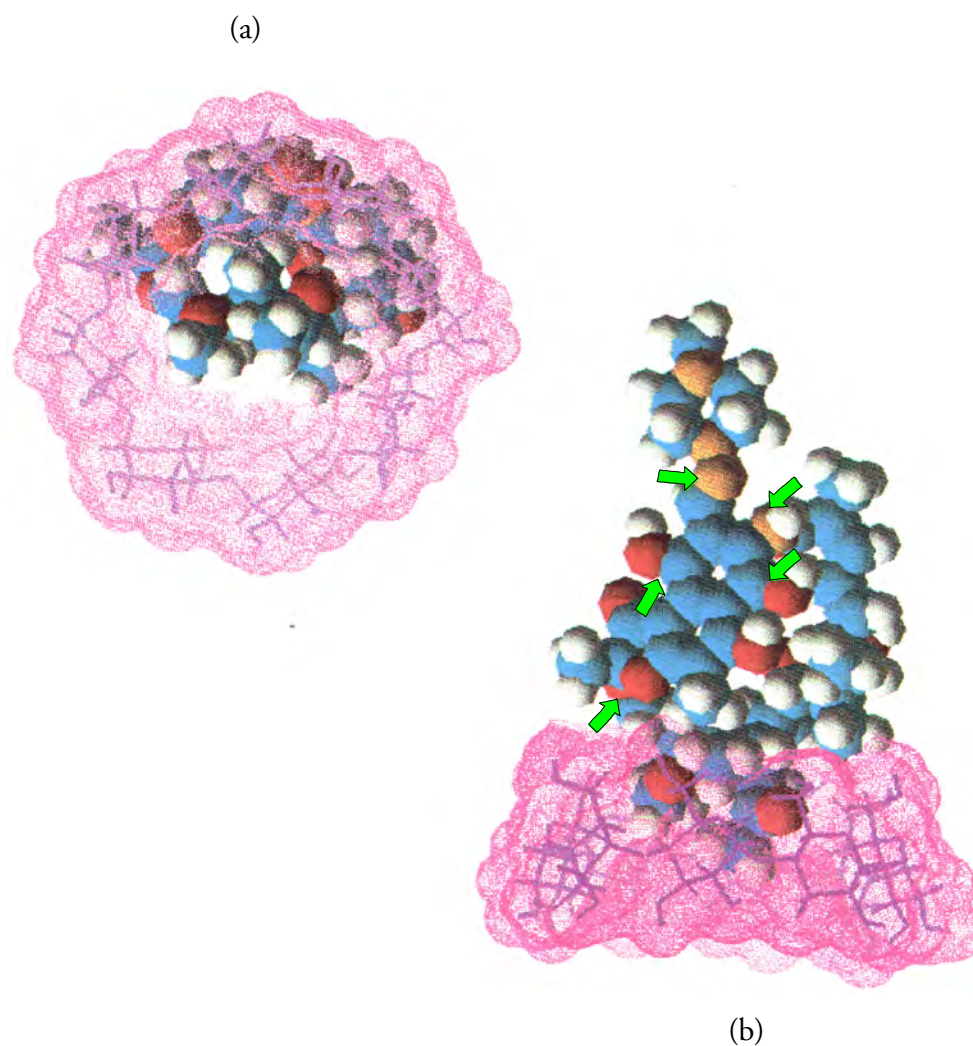


Figure 7.77 Rifampicin-(Cyclodextrin Complexation I (a) "Top" View and (b) "Side" View

The local potential energy minimum of 259.081Kcal/mol and the *P.D.* value of -65.758Kcal/mol are the energy data for to this complexation. The negative *P.D.* value corresponds to the rifampicin solubility enhancement in the presence of (Cyclodextrin which is demonstrated by the solubility studies (section 7.2.6). However, the degradation sites, the azomethine bond, the C1 and C4 hydroxy groups, the oxygen atom attached to C12 and nitrogen atoms attached to C2, are all found outside the (Cyclodextrin cavity.

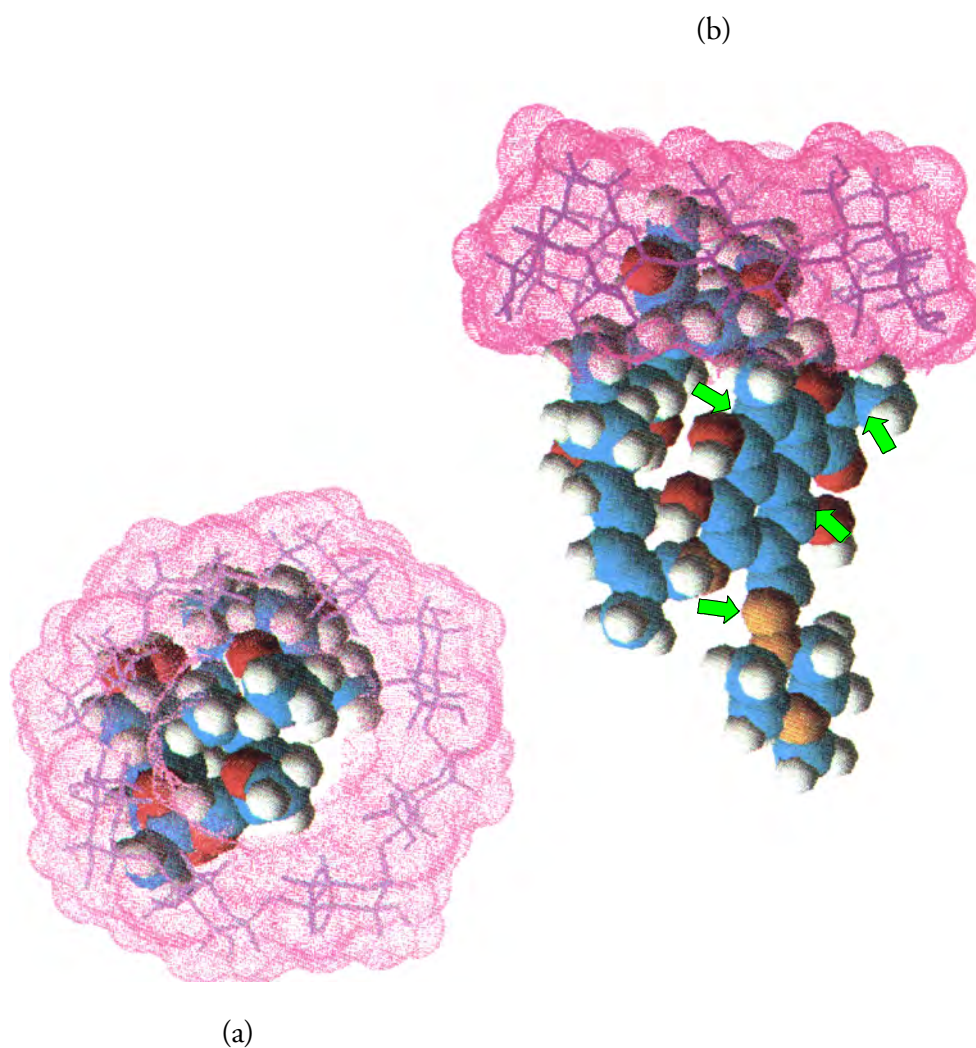


Figure 7.78 Rifampicin-β-Cyclodextrin Complexation II (a) “Bottom” View and (b) “Side” View

The local potential energy minimum of 543.265Kcal/mol and the *P.D.* value of -51.574Kcal/mol was the found for the proposed complexation II. The negative *P.D.* value suggests the tendency of rifampicin solubility enhancement. However, the degradation sites are still found to be situated outside the β-cyclodextrin cavity.

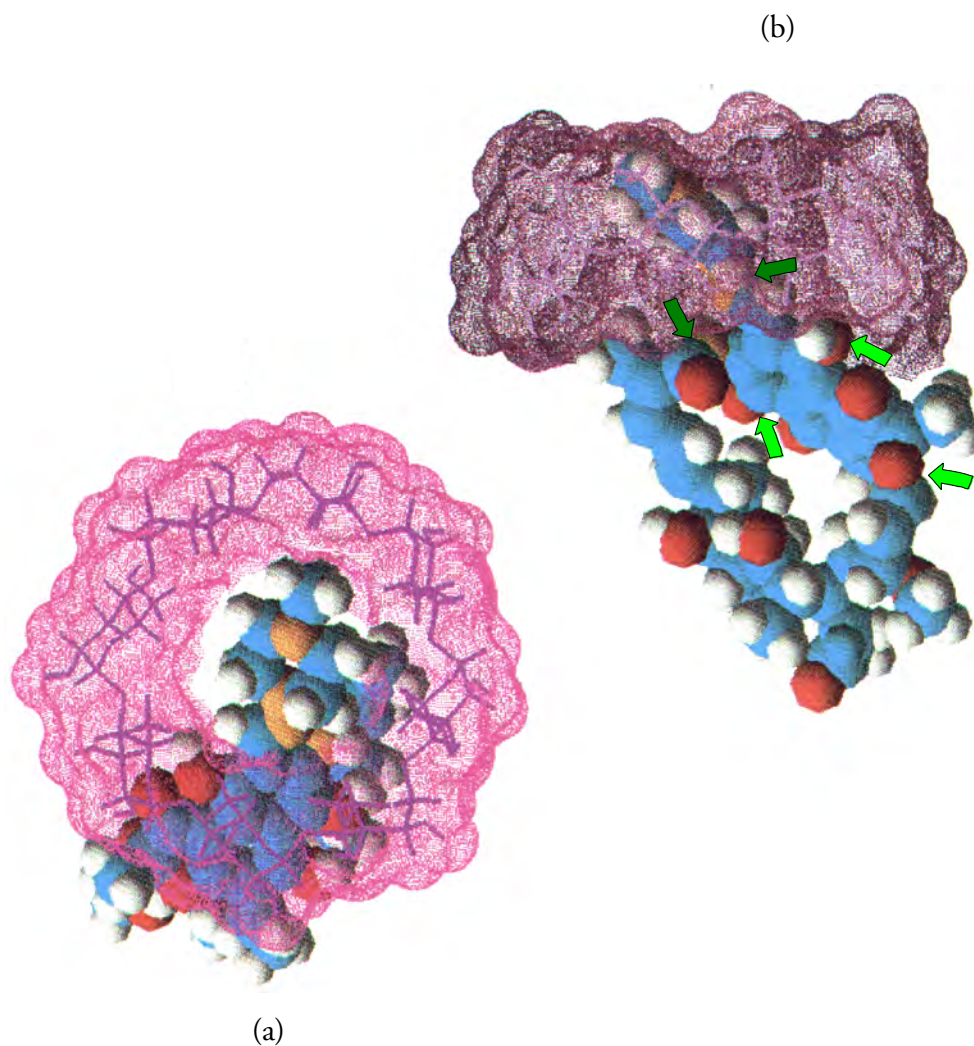


Figure 7.79 Rifampicin- β -Cyclodextrin Complexation III (a) "Top" View and (b) "Side" View

As the piperazine side chain of rifampicin complexes with the β -cyclodextrin cavity through the "top", the local potential energy minimum and the *P.D.* value were found to be 486.769Kcal/mol and -108.070Kcal/mol. As for rifampicin complexed with β -cyclodextrin in similar manner, this complexation was found to be most favourable amongst all four RIF- β -CyD complexations. However, $E_{\text{Drug-CyD}}$ of RIF- β -CyD is lower than the $E_{\text{Drug-CyD}}$ of RIF- β -CyD, thus implying superior complexation with β -cyclodextrin. Moreover, the azomethine bond is protected and the degradation should thus occur through the other sites.

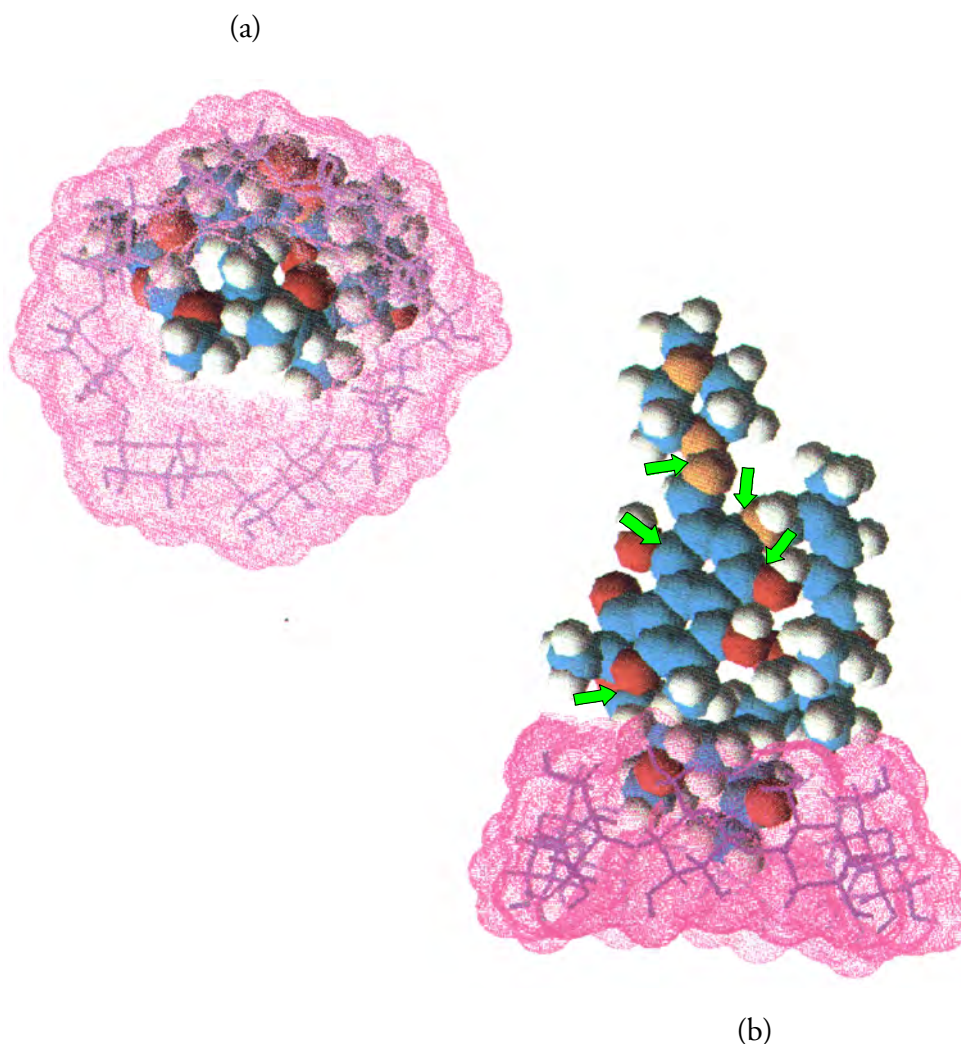


Figure 7.80 Rifampicin-β-Cyclodextrin Complexation IV (a) “Bottom” View and (b) “Side” View

When the piperazine side chain of rifampicin complexes with β-cyclodextrin cavity through the “bottom”, the local potential energy minimum and the *P.D.* value were found to be 544.212Kcal/mol and -55.627Kcal/mol respectively. This complexation becomes the least favourable amongst the four rifampicin-β-cyclodextrin complexes found. The low tendency of the formation of this complex is expected.

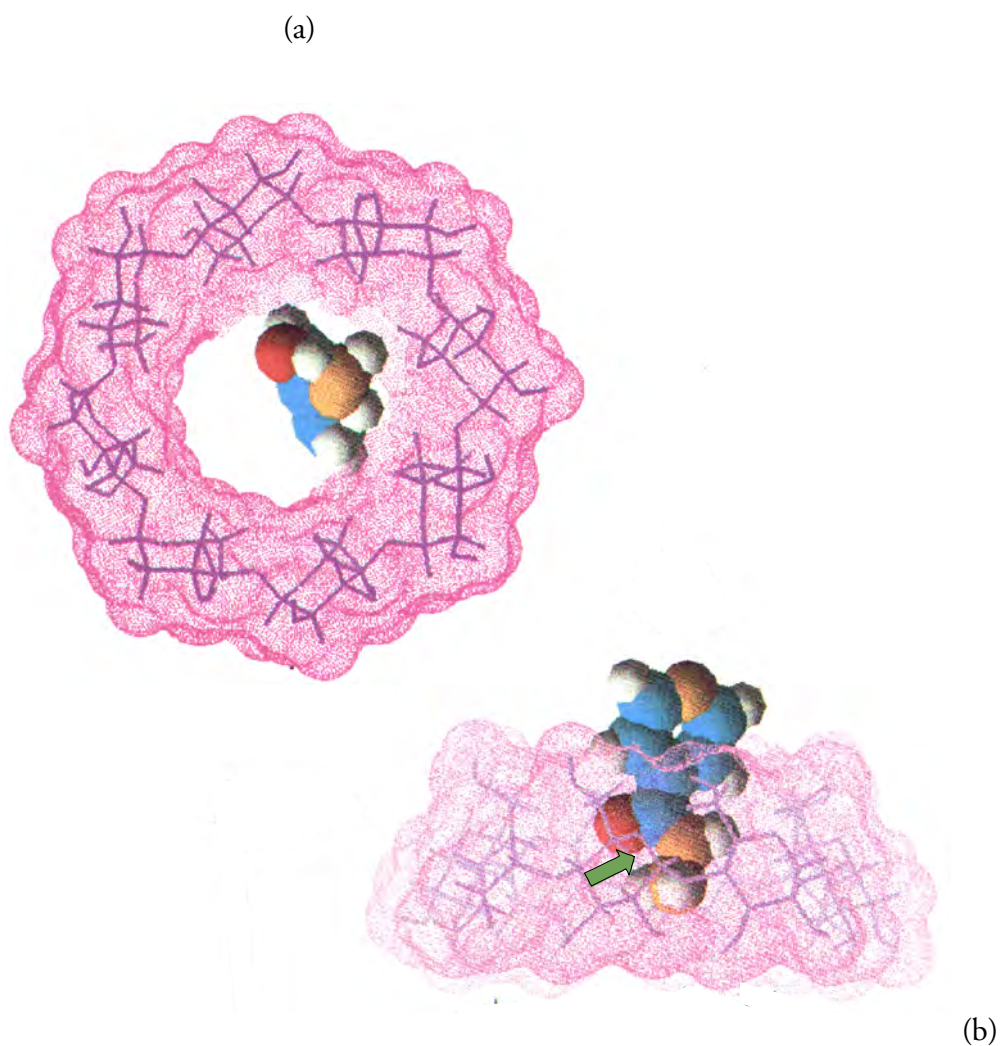


Figure 7.81 Isoniazid-(Cyclodextrin Complexation I (a) "Top" View and (b) "Side" View

As the carboxyhydrazide is unprotected in this complexation, the failure to improve the stability of isoniazid is expected. However, the local potential energy minimum and the *P.D.* value of this complexation were found to be 407.364Kcal/mol and -23.134Kcal/mol. By comparing with the energy data of other four INH-(CyD complexes found, this complex is one of the two least favourable. Therefore, a low occurrence tendency is expected.

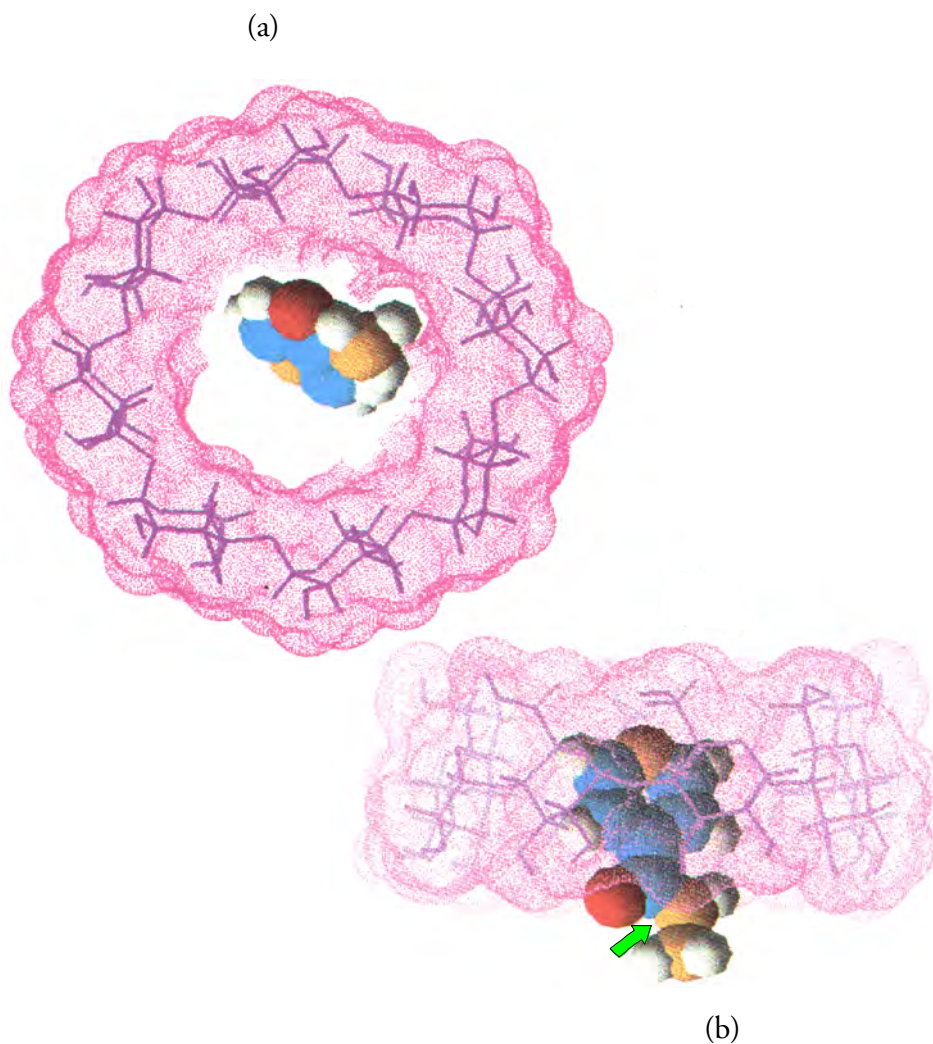


Figure 7.82 Isoniazid-(α -Cyclodextrin Complexation II (a) “Bottom” View and (b) “Side” View

The carboxyhydrazide is also unprotected in this complexation, the failure to enhance the stability of isoniazid should also be expected. However, the local potential energy minimum (407.875Kcal/mol) and the *P.D.* value (-22.623Kcal/mol) indicates that this complexation is also the one of the two less favourable to occur. Therefore, a low occurrence tendency is expected. It is noticeable that there seems to be extra space within the cavity even after the isoniazid complexation (Figure 7.81 (a) “Top” view). This could imply a possibility of additional inclusion with another molecule.

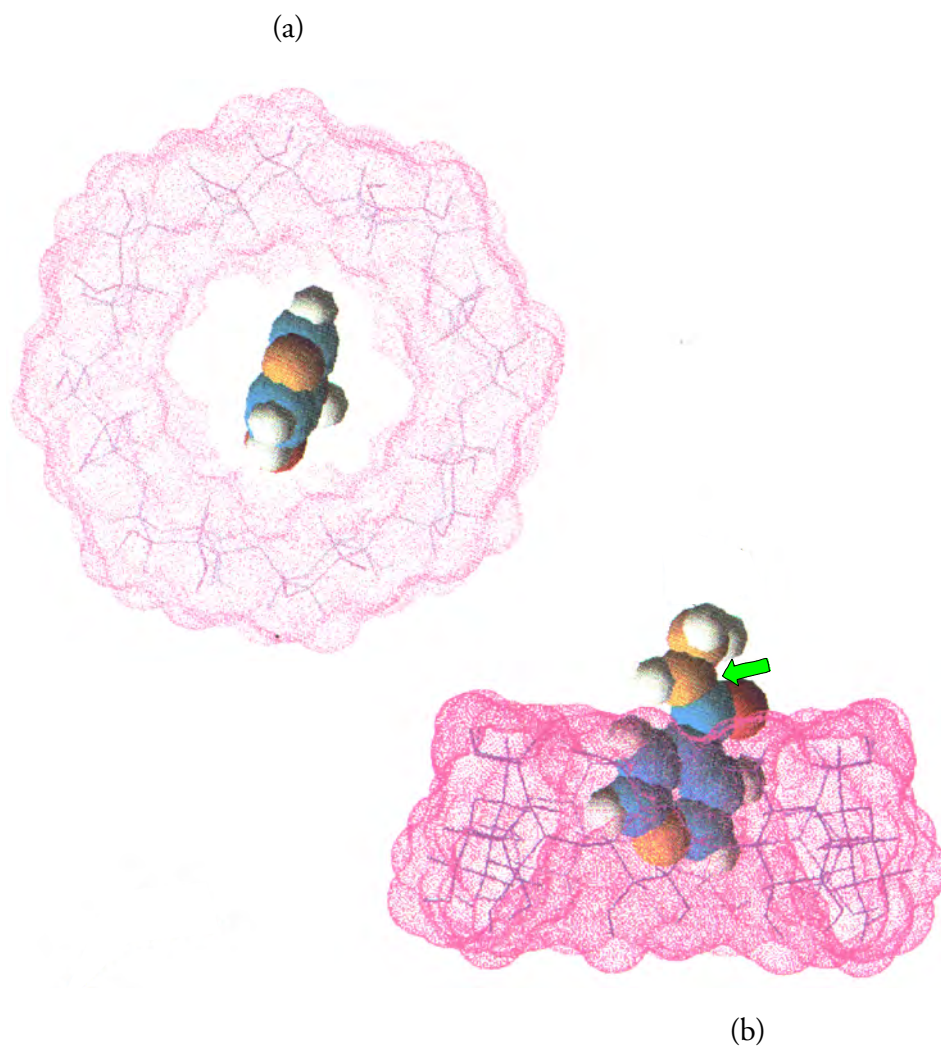


Figure 7.83 Isoniazid-(-Cyclodextrin Complexation III (a) “Top” View and (b) “Side” View

As the carboxyhydrazide is complexed with the (-cyclodextrin cavity, a stability enhancement of isoniazid should be expected. The local potential energy minimum (404.094Kcal/mol) and the *P.D.* value -26.404Kcal/mol of this complexation indicates that this complex is one of the more favourable and correlates with the stability profiles of isoniazid in the presence of (-cyclodextrin. The extra space within the cavity after isoniazid complexation is far more noticeable in complexation III and this could imply that dual complexation may occur.

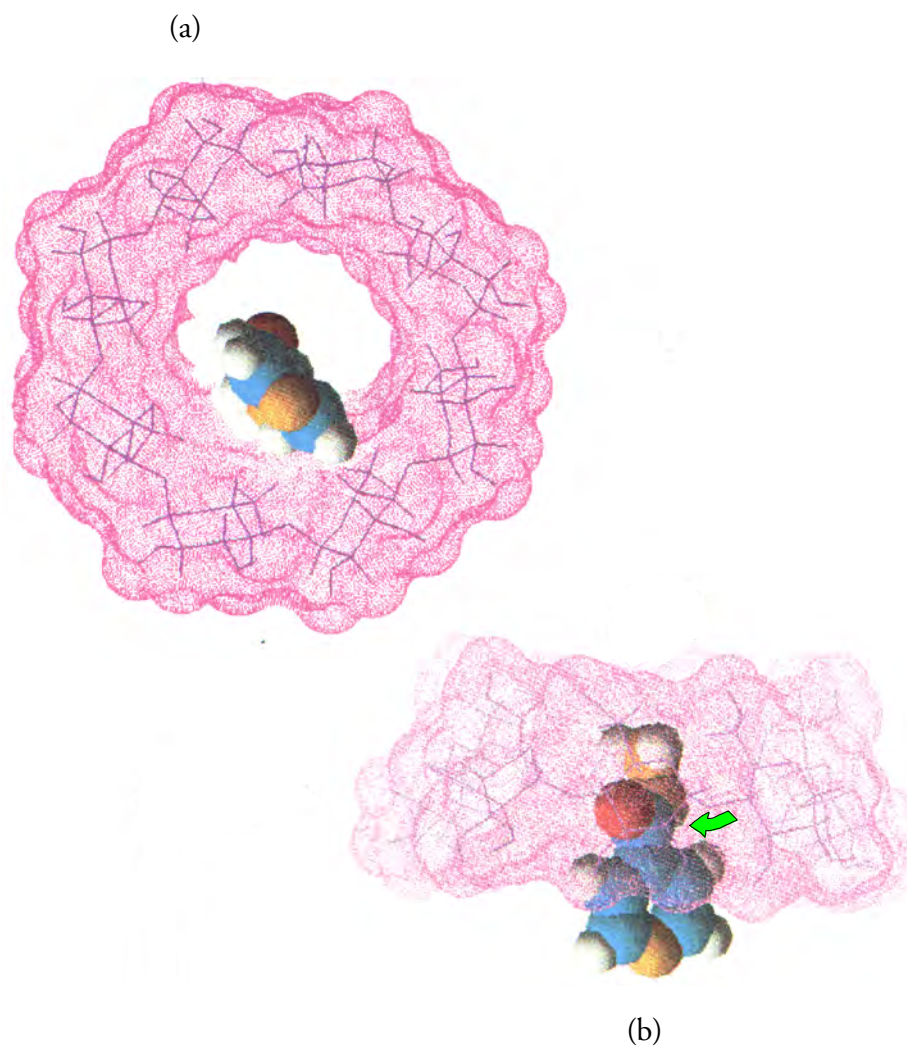


Figure 7.84 Isoniazid-(-Cyclodextrin Complexation IV (a) "Bottom" View and (b) "Side" View

Although the carboxyhydrazide is complexed with the (-cyclodextrin cavity and this may result in the enhancement of isoniazid stability, this complexation has a higher local potential energy minimum (406.666Kcal/mol) and resulting a higher *P.D.* value of -23.134Kcal/mol which indicates that complexation IV has a lesser chance of occurrence. Similar to complexation III, the extra space could imply there is space for (-cyclodextrin to complex with another molecule such as water which could react with rifampicin and inducing further degradation.

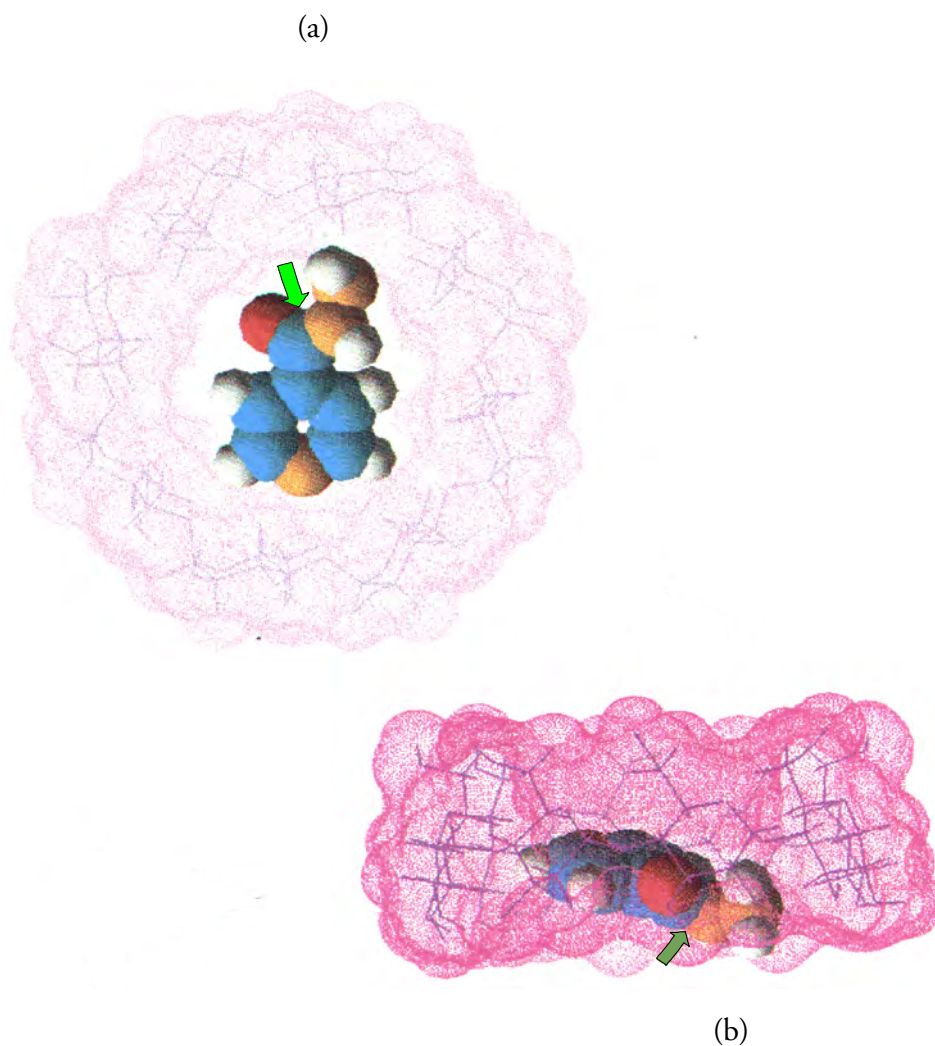


Figure 7.85 Isoniazid-(-Cyclodextrin Complexation V (a) “Bottom” View and (b) “Side” View

The cavity size (8D) of (-cyclodextrin is significantly larger than the cavity size (6D) of α -cyclodextrin. Therefore, isoniazid could also complex with (-cyclodextrin in the above manner. The orientation of isoniazid within the (-cyclodextrin cavity shows a complete inclusion. This complexation, the most favourable amongst all the INH-(-CyD complexations found, has a local potential energy minimum of 402.452Kcal/mol and resulting a *P.D.* value of -28.046Kcal/mol.

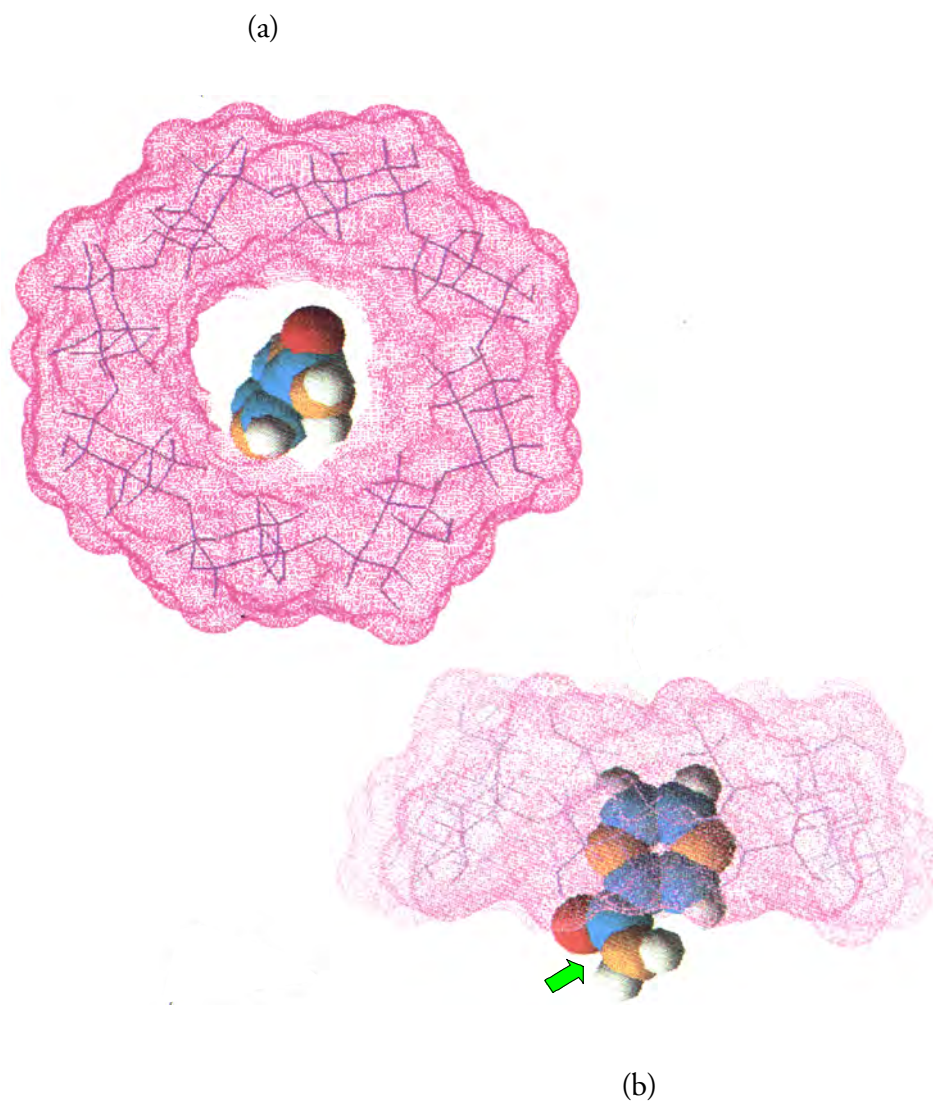


Figure 7.86 Pyrazinamide-(α -Cyclodextrin Complexation I (a) “Top” View and (b) “Side” View

This complexation has a local potential energy minimum of 372.453Kcal/mol and resulting a *P.D.* value of -19.255Kcal/mol. Although the carboxamide group of pyrazinamide is unprotected in this complex, the tendency of this complex formation is low when comparing its *P.D.* value to the *P.D.* values of other PZA-(α -CyD complexes.

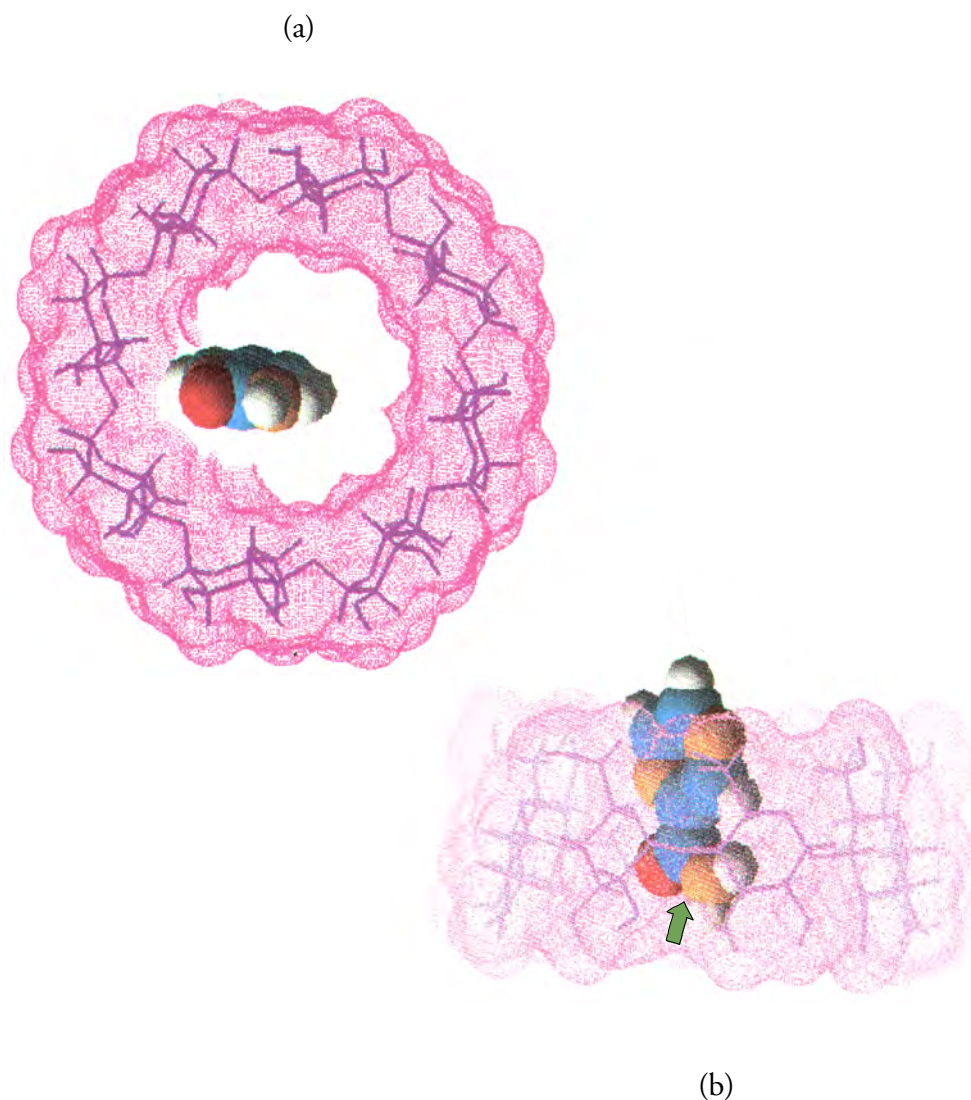


Figure 7.87 Pyrazinamide-β-Cyclodextrin Complexation II (a) “Top” View and (b) “Side” View

This complexation was found to have a local potential energy minimum of 369.723Kcal/mol and a *P.D.* value of -21.985Kcal/mol. The carboxamide group of pyrazinamide was protected by the β-cyclodextrin cavity and therefore, enhancements on both the solubility and stability of isoniazid is expected. This statement corresponds to the solubility and stability findings. However, the excess space within the cavity suggests the further inclusion with other molecules of adequate size and polarity could be possible.

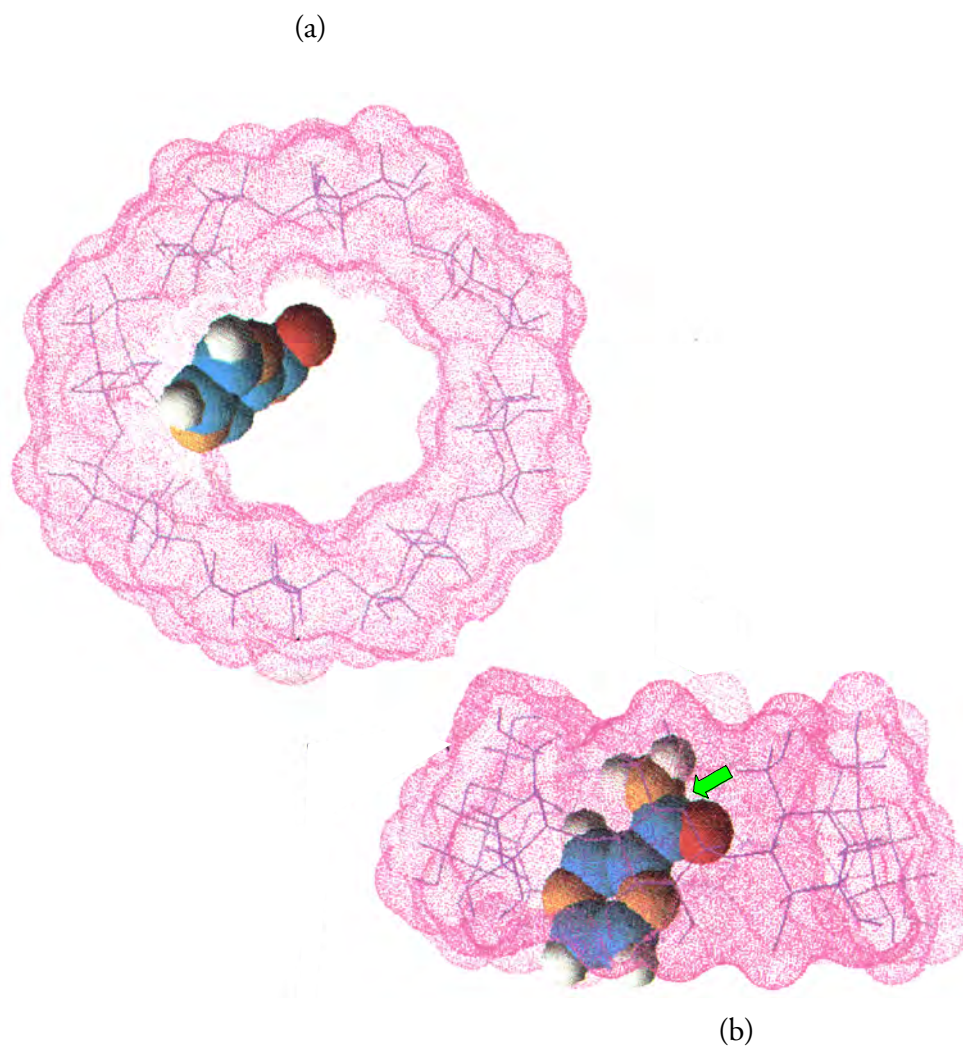


Figure 7.88 Pyrazinamide- β -Cyclodextrin Complexation III (a) “Top” View and (b) “Side” View

This complexation was found to have a local potential energy minimum of 366.221Kcal/mol and a *P.D.* value of -25.487Kcal/mol. The carboxamide group of pyrazinamide is protected by the β -cyclodextrin cavity and therefore, enhancements of both the solubility and stability of isoniazid are expected. There seems to be an excess space within the β -cyclodextrin cavity and this suggests that further inclusion could be possible.

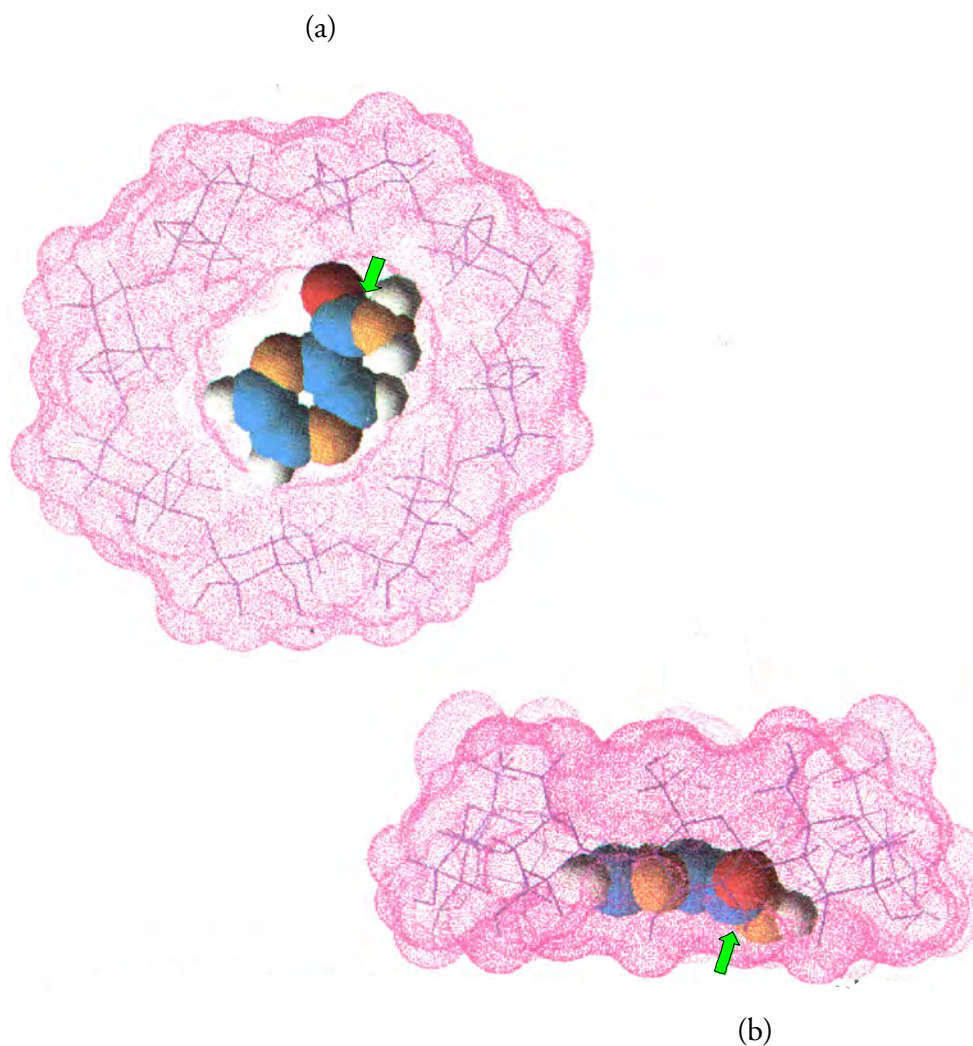


Figure 7.89 Pyrazinamide-(-Cyclodextrin Complexation IV (a) "Top" View and (b) "Side" View

This is the most favourable complexation of pyrazinamide and (-cyclodextrin. It was found having a local potential energy minimum of 361.022Kcal/mol and a *P.D.* value of -30.686Kcal/mol. The orientation of the pyrazinamide within the cavity of (-cyclodextrin allows a maximum protection, as pyrazinamide is completely included with the cavity. Therefore explains the pyrazinamide solubility and stability enhancement in the presence of (-cyclodextrin. However, there is still a possibility of water molecule entering the cavity from either the top or the bottom of the cyclodextrin and reacting with pyrazinamide. Thus the degradation of pyrazinamide still can occur.

7.3 CONCLUSION

7.3.1 SOLUBILITY STUDIES

The comparative solubility curves of rifampicin, isoniazid and pyrazinamide (presented as mol./ml) in the presence of β -cyclodextrin (CyD-1), hydroxypropyl- β -cyclodextrin (CyD-2) or γ -cyclodextrin (CyD-3) are shown in figures 7.90 to 7.92.

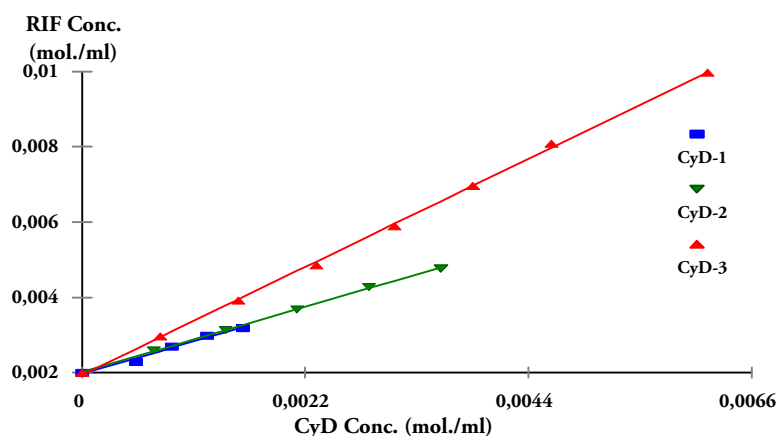


Figure 7.90 Solubility Curves of Rifampicin in the Presence of β -CyD (\bullet), HP- β -CyD (\blacktriangledown) and γ -CyD (\blacktriangle)

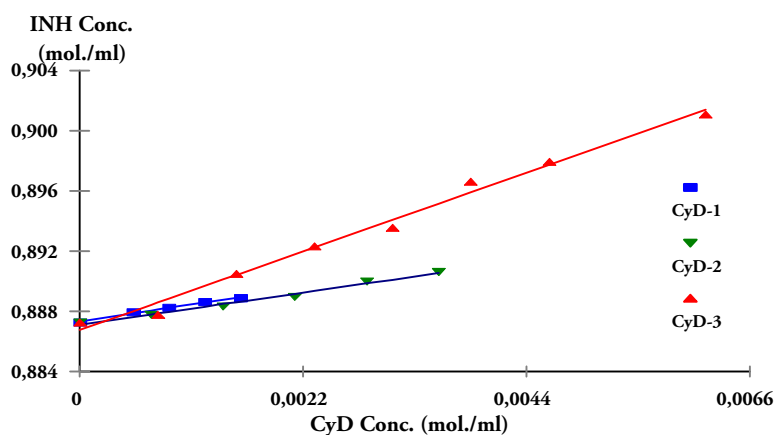


Figure 7.91 Solubility Curves of Isoniazid in the Presence of β -CyD (\bullet), HP- β -CyD (\blacktriangledown) and γ -CyD (\blacktriangle)

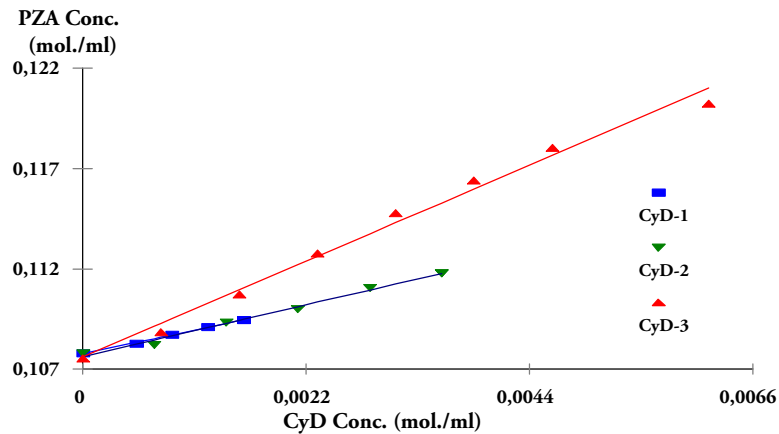


Figure 7.92 Solubility Curves of Pyrazinamide in the Presence of β -CyD (\bullet), HP- β -CyD (---) and α -CyD (\blacktriangle)

Regarding the solubility studies the following conclusions can be made:

- < Rifampicin solubility enhancing effect in the presence of various cyclodextrins is in the order of (α -CyD > HP- β -CyD > β -CyD).
- < Isoniazid solubility enhancement effect in the presence of various cyclodextrins is in the order of (α -CyD > HP- β -CyD > β -CyD).
- < The inclusion tendency between the three drugs and β -cyclodextrin (concluded by referring to the observation of the solubility ratio between the drugs in the presence of β -cyclodextrin listed in tables 7.3 and 7.4) is as follows: RIF > PZA . INH.

The potential energy difference of the most favourable drug- β -cyclodextrin complexations (refer to table 7.11) coincide with the above statement since:

$$\text{P.D. Value}_{(\text{RIF-}\beta\text{-CyD})} (-116.450\text{Kcal/mol}) > \text{P.D. Value}_{(\text{PZA-}\beta\text{-CyD})} (-24.733\text{Kcal/mol}) . \text{P.D. Value}_{(\text{INH-}\beta\text{-CyD})} (-22.7639\text{Kcal/mol}).$$

- < The inclusion tendency between the three drugs and hydroxypropyl- β -cyclodextrin is as follows: RIF > INH > PZA. This statement was concluded by referring to the observation of the solubility ratio between the drugs in the presence of hydroxypropyl- β -cyclodextrin listed in table 7.6.

- < The inclusion tendency between the three drugs and β -cyclodextrin (concluded by referring to the observation of the solubility ratio between the drugs in the presence of β -cyclodextrin listed in table 7.3 and 7.4) is as follows: RIF > PZA > INH.

The potential energy difference of the most favourable drug- β -cyclodextrin complexations (refer to table 7.11) coincides with the above statement since :

$$\text{P.D. Value}_{(\text{RIF}-\beta\text{-CyD})} (-108.070\text{Kcal/mol}) > \text{P.D. Value}_{(\text{PZA}-\beta\text{-CyD})} (-30.686\text{Kcal/mol}) .$$

$$\text{P.D. Value}_{(\text{INH}-\beta\text{-CyD})} (-28.046\text{Kcal/mol})$$

- < The solubility constants of each drugs in the presence of each cyclodextrin are listed in the table 7.10. All of the constants showed that the solubility of the drugs is enhanced by incorporating the cyclodextrins

Table 7.10 The Solubility Constants of Rifampicin, Isoniazid and Pyrazinamide in the Presence of Various Cyclodextrins

Solubility Constants			
	<i>Rifampicin</i>	<i>Isoniazid</i>	<i>Pyrazinamide</i>
β -CyD	1732.46 (enhancement)	1.61 (enhancement)	15,28 (enhancement)
HP- β -CyD	1811.83 (enhancement)	1.53 (enhancement)	16,45 (enhancement)
α -CyD	2207.17 (enhancement)	1.65 (enhancement)	18,50 (enhancement)

The high Solubility constants generated from the interactions between rifampicin and the cyclodextrins could imply the occurrence of the inclusion complexation, this make sense since rifampicin is a fairly non-polar molecule, whereas the low magnitude of the solubility constants generated between isoniazid and pyrazinamide suggest the inclusion complexation could be unfavourable.

7.3.2 STABILITY STUDIES AND COMPUTER FORCE FIELD INCLUSION COMPLEXATION MODELLING

In general, although the stability of rifampicin is enhanced when stored under nitrogen, the general stability of rifampicin in the presence of β -cyclodextrin, hydroxypropyl- β -cyclodextrin or α -

cyclodextrin is found to be poor. This observation concurs with the computer force field generated models as the possible degradation sites (except the azomethine bond on the side chain) of rifampicin are not included within the cavity of both β -cyclodextrin and α -cyclodextrin. The advantage of the hydroxypropylated derivative is only its aqueous solubility, but it has the same inclusion capability as the parent cyclodextrins. Therefore, the inclusion of rifampicin with hydroxypropyl- β -cyclodextrin should follow the similar manner as the complexation between rifampicin and β -cyclodextrin.

Although rifampicin is a bulky molecule consisting of 117 atoms with the molecular mass of 822.95g/mol., rifampicin preferred to form inclusion complexation with β -cyclodextrin than with α -cyclodextrin as the *P.D.* value of the former complexation (-116.450Kcal/mol) was lower than latter complexation (-108.070Kcal/mol) (refer to table 7.11). This could be due to the fact that the cavity of β -cyclodextrin is more suitable for the inclusion of piperazine side chain. It was observed that the piperazine side chain orientated itself in the center of the β -cyclodextrin cavity for maximum hydrophobic attraction between the hydrogen on the piperazine and the two inner hydrogen rings found in the center β -cyclodextrin cavity. This lowered the *P.D.* value. Whereas the piperazine side chain was included off the α -cyclodextrin cavity center and hence could not induce maximum hydrophobic interaction.

Isoniazid and pyrazinamide both showed a slight enhancement in the stability when in the presence of β -cyclodextrin, hydroxypropyl- β -cyclodextrin or α -cyclodextrin. The correlated computer models of the most favourable complexation clearly demonstrated that the degradation sites of isoniazid and pyrazinamide are being protected within the cavity which indicated the possibility of stability enhancement.

Isoniazid preferred to complex with α -cyclodextrin than β -cyclodextrin as was suggested by the energy data of the computer force field simulation. The most favourable isoniazid- α -cyclodextrin complex has a lower *P.D.* value (-28.046Kcal/mol) than the most favourable isoniazid- β -cyclodextrin complex (-22.764Kcal/mol). Isoniazid can be horizontally orientated within the 8D width cavity of α -cyclodextrin which allowed completely inclusion. Whereas β -cyclodextrin and hydroxypropyl- β -cyclodextrin have a 6D width cavity and only allowed isoniazid to be partially included within the cavity. The same behaviour was observed with pyrazinamide as the *P.D.* value of PZA- α -CyD complexation (-30.686Kcal/mol) is lower than *P.D.* value of PZA- β -CyD complexation (-24.733Kcal/mol.) (refer to table 7.11.).

Table: 7.11 Summary of the Energy Data of The Computer Force Field Generated Models

Potential Energy Data of Various Complexes						
<i>CyD</i>	<i>Drug</i>	<i>Complex No.</i>	<i>Figure Represented</i>	<i>Minimum $E_{Drug-CyD}$ (Kcal/mol)</i>	<i>P.D. Value (Kcal/mol)</i>	<i>Comment</i>
\$	RIF	I	7.65.	441.798	-93.636	
\$	RIF	II	7.66.	480.496	-54.938	
\$	RIF	III	7.67.	418.984	-116.45	Most favourable
\$	RIF	IV	7.68.	453.967	-81.467	
\$	INH	I	7.69.	353.762	-17.331	
\$	INH	II	7.70.	350.014	-21.079	
\$	INH	III	7.71.	348.329	-22.764	Most favourable
\$	INH	IV	7.72.	359.372	-11.721	
\$	PZA	I	7.73.	307.590	-24.733	Most favourable
\$	PZA	II	7.74.	312.424	-19.879	
\$	PZA	III	7.75.	314.363	-17.94	
\$	PZA	IV	7.76.	315.376	-16.927	
(RIF	I	7.77.	529.081	-65.758	
(RIF	II	7.78.	543.265	-51.574	
(RIF	III	7.79.	486.739	-108.070	Most Favourable
(RIF	IV	7.80.	544.212	-50.627	
(INH	I	7.81.	407.364	-23.134	
(INH	II	7.82.	407.875	-22.623	
(INH	III	7.83.	404.094	-26.404	
(INH	IV	7.84.	406.666	-23.832	
(INH	V	7.85.	402.452	-28.045	Most favourable
(PZA	I	7.86.	372.453	-19.255	
(PZA	II	7.87.	369.723	-21.985	
(PZA	III	7.88.	366.221	-25.487	
(PZA	IV	7.89.	361.022	-30.686	Most favorable

In the presence of cyclodextrins, the overall stability of the rifampicin-isoniazid combination seems to have been improved. This may be due to the fact that the azomethine bond of rifampicin is protected and 3-formyl rifamycin SV was not produced. Therefore interactions (refer to figure 8.1 and 8.2) between isoniazid and the formyl group of rifampicin were unable to take place. The same pattern was observed when rifampicin combined with pyrazinamide.

In the presence of cyclodextrins, the overall stability of both isoniazid and pyrazinamide was improved when they combined with each other. This could be due to the fact that the degradation sites of both drugs were protected by the cyclodextrins.

CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

In chapter one, the threat caused by tuberculosis is fully reviewed as this disease is contagious and life-threatening. Adequate antitubercular regimens combining rifampicin, isoniazid and pyrazinamide is the cornerstone of effective treatment and are usually initiated immediately once there is evidence of a positive sputum smear [Herfindal et al., 1992]. Despite the excellent health infrastructure provided by the South Africa Department of Health to the people in rural areas, South Africa is one of the 16 nations lagging in controlling tuberculosis with an annual estimation of 10,000 people destroyed by this disease [Klaudt et al., 1998; Owen et al., 1998; Weyer et al., 1995]. The most influential determinant of effective of tuberculosis treatment is patient compliance. Should a patient be non compliant, the consequences are treatment failure, additional treatment, additional expense, increasing chance of MDR-TB and an increase in the mortality rate. However, according to the South Africa Tuberculosis Association (SANTA), of the 102,000 patients treated in 1996, only 70 percent of the patients completed their DOTS treatment and the matter is especially noticeable amongst the paediatric and geriatric patients [Isacson, 1998; Kumareson et al., 1996]. This could be due to the fact that in current pharmaceutical market, most antitubercular drugs are “user unfriendly” for these two groups. The inconvenient methods of administering the tablet (or capsule) dosage form and the frequent dosing regimens due to the lack of formulations including the combination of all three drugs are mainly the problem. This led to the initiating of a project targeting the difficulties of pre-formulation of a non-alcoholic based antitubercular liquid formulation which was requested by Pharmacare-Lennon[®] (Korsten, RSA).

A successful formulation required the satisfaction of the drugs’ solubilities to achieve the desired concentration and their stability conditions to meet the regulatory levels set by FDA or ICH [Carstensen, 1995]. In order to investigate the above matters, the solubility and stability trials

protocol/parameters were required. An adequate HPLC method of analysis based on employing a 4.6×250mm μ -bondapakTM C18 plus a C18 guard column, 10mm/125D (Waters[®], Massachusetts) with the mobile phase consisting of 42.5% : 57.5%, MeCN: tBAH (0.0002M), v/v, with a final pH of 3.10 was used. This method was fully developed and validated in chapter 3 whereas the rifampicin, isoniazid and pyrazinamide analytes were already fully identified and characterized in chapter 2 to ensure the purity of the drugs.

The solubilities and the stabilities of rifampicin, isoniazid and pyrazinamide were studied at pH values ranging from 2-10 with or without phosphate buffer and in the presence of three surfactants (poloxamer 188, poloxamer 407, sorbitols), two suspending agents (carbopol 934 and 974F) or three cyclodextrins (β -cyclodextrin, hydroxypropyl- β -cyclodextrin and α -cyclodextrin). After investigating the solubility and stability profiles of rifampicin, isoniazid and pyrazinamide in the presence of the above mentioned agents with additional information provided by computer force field generated cyclodextrin inclusion models (chapter 4, 5, 6 and 7), it was concluded that the above mentioned agents are not recommended which predominately due to the failure to achieve the desired stability. Literature reports the *in vivo* degradation of rifampicin, isoniazid and pyrazinamide [Gallo et al., 1974; Brewer et al., 1977; Wilson et al., 1977; Felder et al., 1983 ; Hansch et al., 1990; Foye et al., 1995], but the *in vitro* degradation of these drugs in the presence of aqueous medium has not been published. The prediction of the degradation (refer to section 4.1.2.1 to 4.1.2.3) served only as a rough guide of what the major degradation sites would likely to be. These can only be confirmed if the degradants are physically isolated and identified. Besides the individual instability of the drugs, the drug-drug interaction also played an influential role.

During the reversible hydrolytic cleavage of the azomethine bond, the 3-formylrifamycin SV and 1-amino-4methylpiperazine are formed. The 3-formylrifamycin SV and isoniazid could possibly undergo Schiff's reaction to form a soluble complex [Jindal et al., 1994]. The carbonyl groups and the amine group may rearrange to yield an iminium ion. The C-4 hydroxy group enhanced the complex formation tendency by forming a hydrogen bond with the hydrogen atom attached to the nitrogen. This is the basic requirement of the Schiff's reaction [Mathews, 1990]. Pyrazinamide could also react the same way with rifampicin which results in the instabilities of rifampicin which are observed throughout the research.

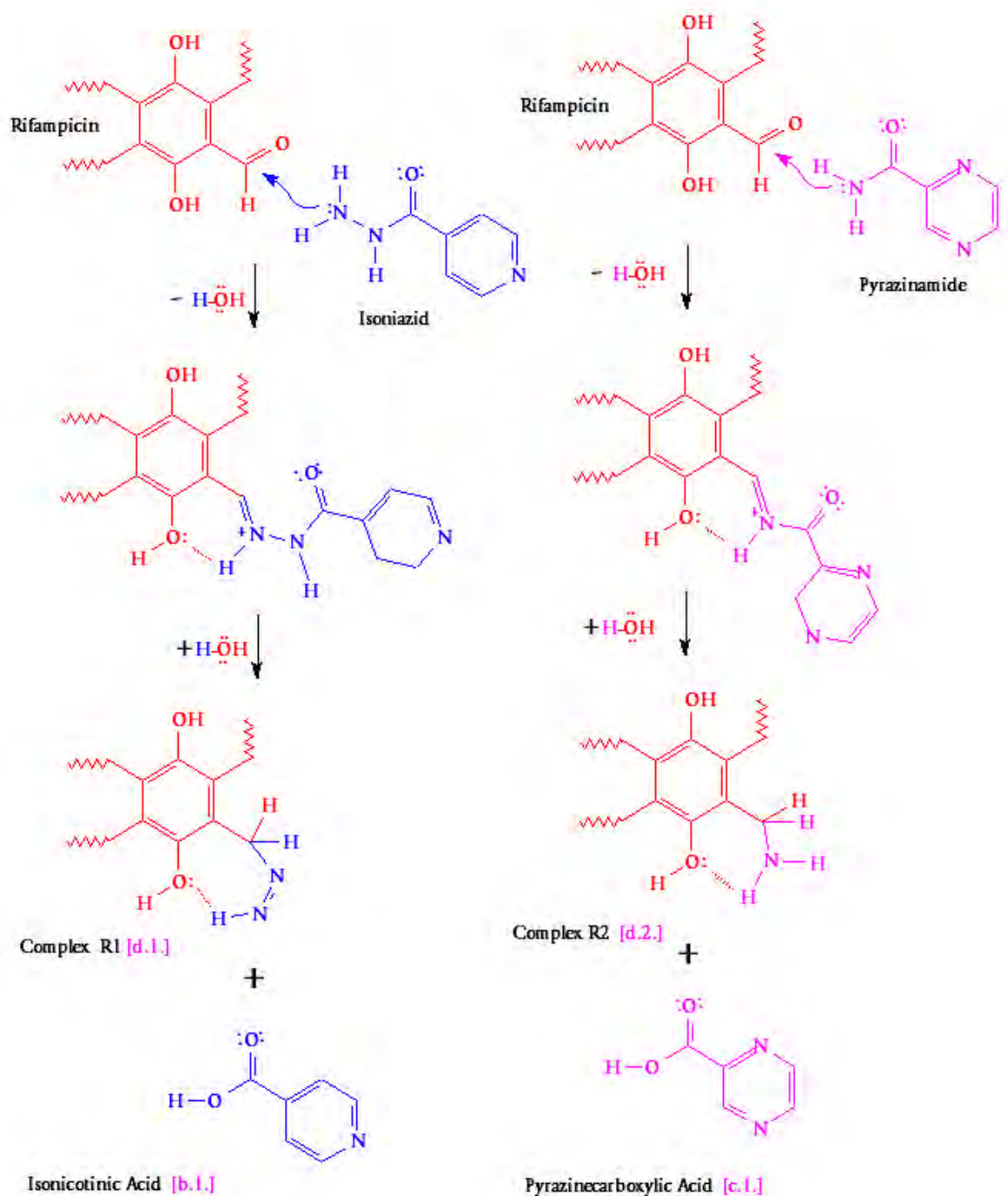


Figure 8.1 Schiff's Reaction of Rifampicin and Isoniazid or Pyrazinamide

Carboxylic acids and alcohols may also undergo carbonyl condensation reactions [McMurry, 1992]. Both isoniazid and pyrazinamide can be able to react with rifampicin as is demonstrated in figure 8.1 to form complex R1 [d.1] and complex R2 [d.2]. This could also account for the instability of rifampicin when present together with isoniazid or pyrazinamide.

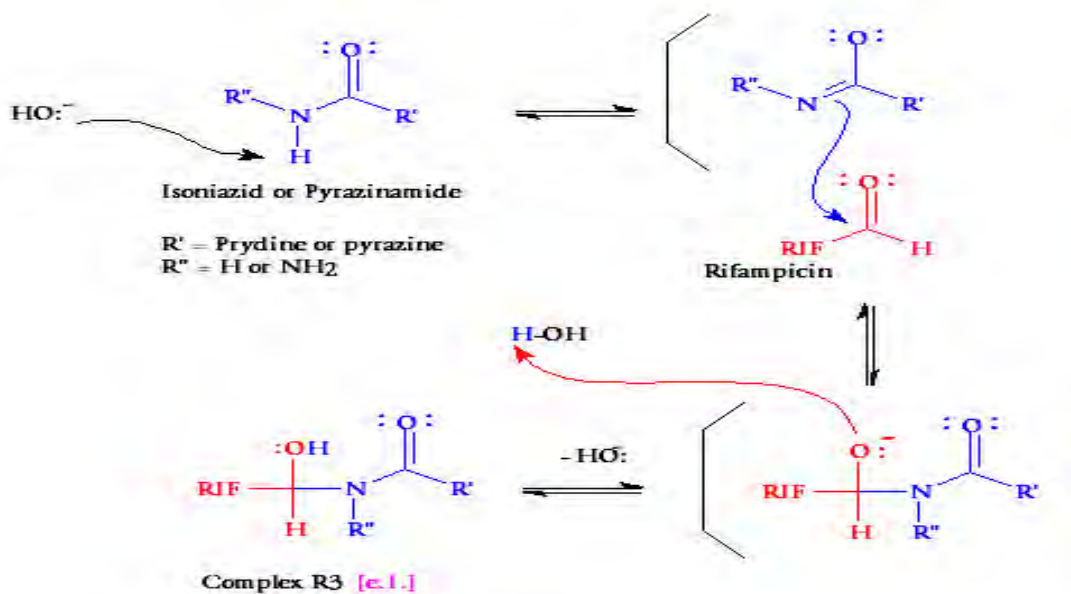


Figure 8.2 Carbonyl Condensation Reaction of Rifampicin and Isoniazid or Pyrazinamide

However, it was frequently observed that isoniazid caused further rifampicin stability reduction compared to pyrazinamide. This could be due to the reason that carboxylic acids and alcohols also undergo Fischer's esterification [McMurry, 1992] to form complex R3 [e.1.]. The hydroxyl groups of rifampicin are readily able to react with the aqueous carboxylic acids degradants yielded by isoniazid and pyrazinamide and form an ester. However, pyrazinamide lacks an electron drawing group such as the secondary nitrogen found on the hydrazide group of isoniazid, therefore less tendency for reaction should be expected between pyrazinamide and rifampicin. Figure 8.3 demonstrates the reaction between isoniazid and rifampicin to form complex R4 [f.1.].

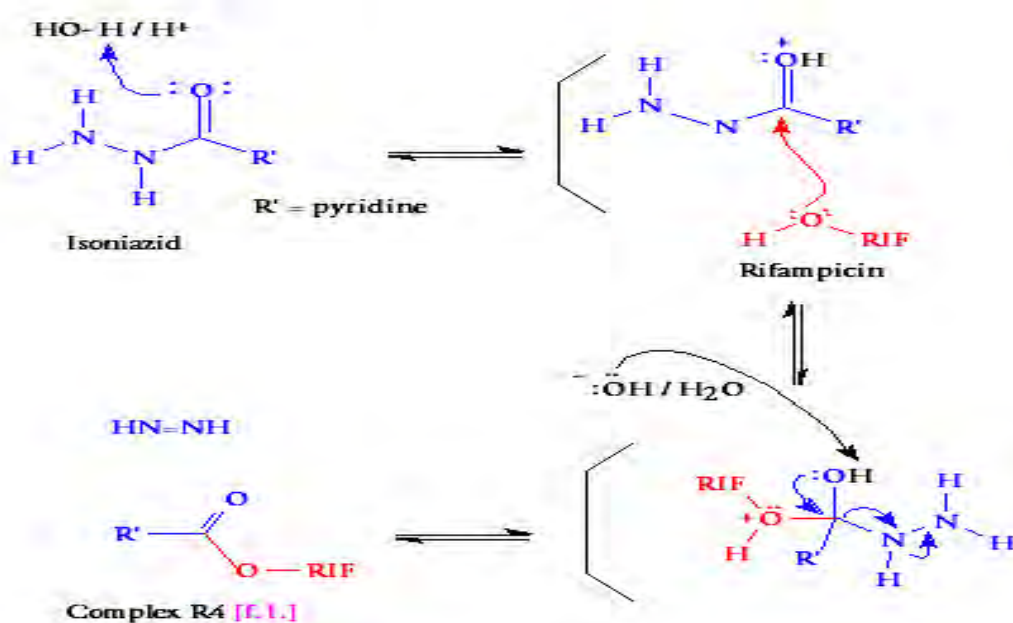


Figure 8.3 Fischer's Esterification Reaction between Rifampicin and Isoniazid

The instability reduction of isoniazid and pyrazinamide was observed during the experiments of combining isoniazid and pyrazinamide. This could be due to isoniazid and pyrazinamide undergoing the carbonyl condensation reaction with each other to cause the formation of complex IP [g.1.] and hydrazine [g.2.] (figure 8.4).

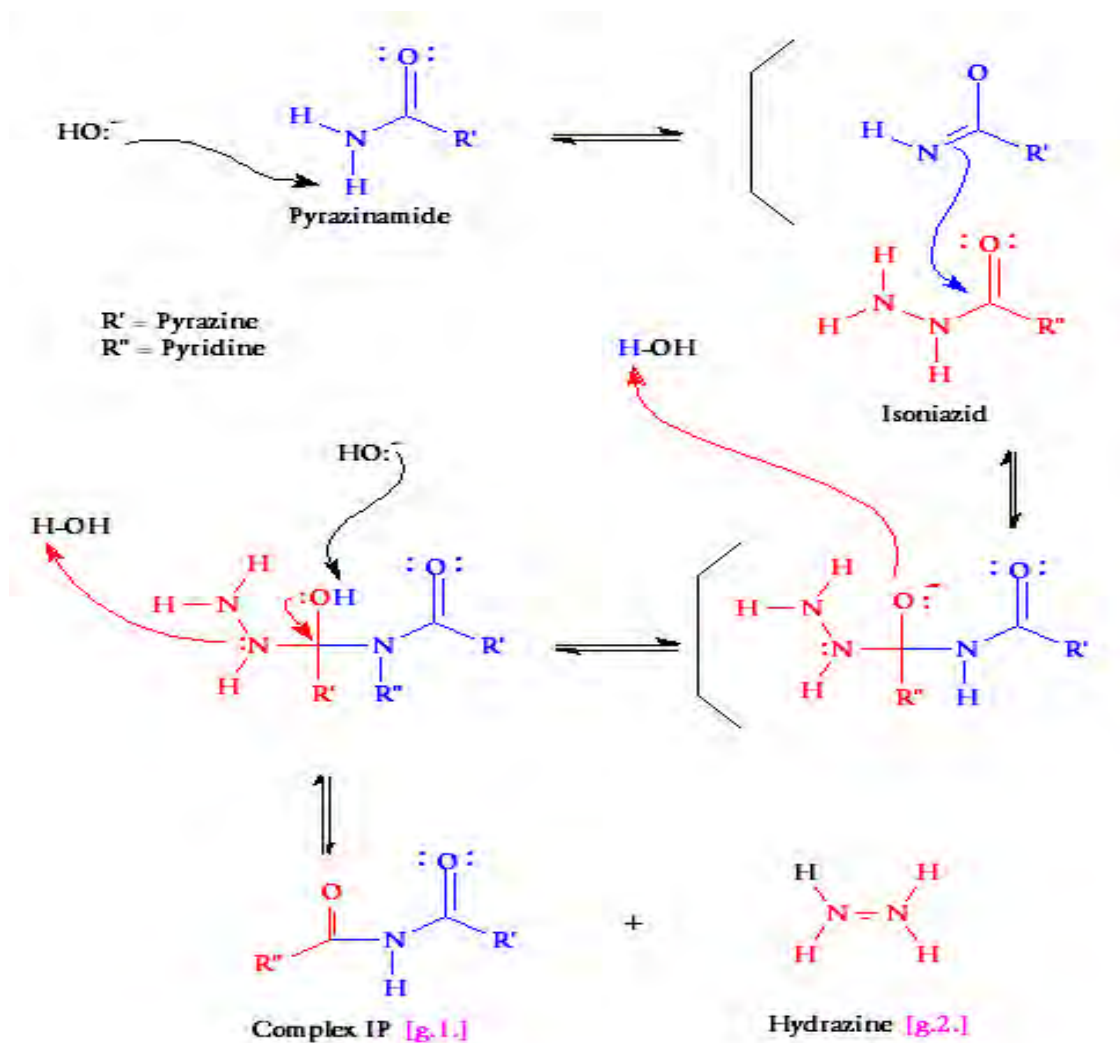


Figure 8.4 Carbonyl Condensation Reaction Between Isoniazid and Pyrazinamide

All of the above mentioned mechanisms served only as a prediction of the interaction between these drugs. Unless the further experiments targeting the specificity of the interaction between the drugs are conducted, the suggestions cannot be conclusive. Nevertheless, evidently rifampicin, isoniazid and pyrazinamide demonstrated the poor aqueous stability due to the hydrolysis and the above suggested drug-drug interactions predominately relied on the presence of water. Hence, further investigation of these drugs within the aqueous or protic solvents should not be recommended.

Tuberculosis, a most health threatening disease can not be effectively controlled unless the patient showed compliance towards the treatment. A “user friendly” formulation containing rifampicin, isoniazid and pyrazinamide would be most beneficial in terms of lowering patient non-compliance and could significantly increase the cure rate of tuberculosis. Although a liquid formulation is most desirable dosage form in terms of the bioavailability and convenience point of view, the solubilities and the stabilities of the drugs are the two main hurdles to a successful liquid formulation. The poor stabilities, as well as solubilities of rifampicin, isoniazid and pyrazinamide were observed throughout all the experiments when conducted within the aqueous medium. Alternate routes should be investigated immediately and hopefully a successful liquid formulation would be created and would be beneficial to the antitubercular programme.

8.2 RECOMMENDATIONS

The following are recommendations for further study:

- < Mixed organic solution are widely used in the pharmaceutical sciences in elixirs and as solvents. Sometimes, these systems could have superior solubilizing and stabilizing effects compared to using the aqueous solvent [Reynolds et al., 1989]. The poor aqueous solubility of rifampicin would imply that rifampicin has a relatively high lipophilicity. Making use of the non protic lipid solvents could effectively enhance the solubility of rifampicin to the desired range. Furthermore, the non protic lipid phase prevents the hydrolysis or protic cleavage within the rifampicin. Hence the stability could be enhanced.
- < Throughout the correlated rifampicin stability investigations, nitrogen enhanced the stability of rifampicin. This implied the occurrence of the scheme I degradation pathway (section 4.1.2.1). Addition of the lipophilic antioxidants in the lipid solvent could further enhance the stability of rifampicin by preventing the formation of rifampicin quinone [a.3.] and it is advisable to store the formulation under nitrogen.

< Isoniazid and pyrazinamide could however be too hydrophilic to dissolve into a lipid phase. Microencapsulation could be an alternative method to achieve the desired concentration as well as stability. Microencapsulation is used in the pharmaceutical field for protecting unstable drugs separating the incompatible substances and masking the unpleasant taste, yet without being concerned about the solubility of the drugs [Turner et al., 1988]. Isoniazid and pyrazinamide could both be coated with aqueous soluble (but lipid inert) polymers and forming microspheres. These microspheres could be suspended in the lipid phase in which rifampicin is dissolved. Any desired concentration and combination of isoniazid or/and pyrazinamide could be readily formulated without recreating a whole new method. But most importantly of all is that these microspheres should prevent the hydrolysis from taking place and further inhibit the interaction between the rifampicin, isoniazid and pyrazinamide.

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