

# Stability of anti-tuberculosis actives in the Pheroid™ delivery system

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Dissertation submitted in partial fulfillment of the  
requirements for the degree Magister Scientiae in  
the department Pharmaceutics at the North West  
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Potchefstroom  
2010

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# AIMS AND OBJECTIVES

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## **Stability of anti-tuberculosis actives in the Pheroid™ delivery system**

Tuberculosis (TB) is an infectious disease being caused by *Mycobacterium tuberculosis*, which is carried by air when infected people are sneezing or coughing.

More than 9 million cases of TB were documented worldwide in 2007, during which South Africa ranked 5<sup>th</sup> (WHO, 2009:1). Besides these alarming figures, a more recent crisis regarding the TB pandemic has been the development of drug resistance. Initially it was multi-drug resistance (MDR), but in recent years a new occurrence, i.e. extreme drug resistance (XDR), has made the fight against TB even more difficult.

As the main problem of resistance is caused by poor patient compliance, the need has arisen to find therapies with shorter treatment times, as well as longer dosage intervals. This study was part of a project that is striving towards the possible optimisation of drug delivery in the current treatment of TB, by using Pheroid™ technology as the drug delivery system. This is due to the fact that the Pheroid™ structure is reported to amplify the absorption and improve the bioavailability of certain drugs, with fewer side-effects, and with the potential of helping against drug resistance (Grobler, 2004:3).

Two formulations were thus prepared, one with the ethambutol-isoniazid combination (Pyriftol IE) and another with the rifampicin-pyrazinamide (Pyriftol RP) combination, each in the pro-Pheroid system.

The aim of this study was directed towards the stability and testing of the active ingredients within the pro-Pheroid system.

With regards to the above factors, the following objectives were the foundation of this study:

- To formulate dosage forms, which incorporate the active pharmaceutical ingredients into the pro-Pheroid delivery system.
- To evaluate the effectiveness of published high pressure liquid chromatography (HPLC) methods in analysing the tuberculostatic active contents in these pro-Pheroid formulations.
- To evaluate the stability of the four tuberculostatic actives in the pro-Pheroid delivery system, during a stability testing programme, over a period of three months, at various controlled storage conditions.
- To determine the suitability of the pro-Pheroid structure for inclusion of the tuberculostatic actives, used in the formulations.

# ABSTRACT

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Tuberculosis poses a serious health threat worldwide, with 9.27 million cases being reported in 2007 (WHO, 2009:1). Africa represented 31% of this figure, with South Africa ranking 5<sup>th</sup> globally. Despite an already high mortality rate of just under 2 million during 2007, resistance has also become a significant factor to reckon with in combating this disease. Poor patient compliance is reported to be the major attributing factor to formed resistance, which has necessitated the consideration of shorter treatment times.

Pheroid™ technology is a new drug delivery system that is reported to improve absorption and enhance bioavailability. It was anticipated that its advantages could be utilised in helping to fight drug resistance. The Pheroid™ consists of two liquid phases (an oil- and an aqueous based phase) and a dispersed gas phase, which are allied with the dispersed fatty acid phase. Pro-Pheroid production is equal to that of the Pheroid™, with the exception that the aqueous phase is omitted and that the active ingredients are included in the oil phase.

This study was performed to establish the stabilities of four tuberculostatic active pharmaceutical ingredients (APIs), i.e. rifampicin, isoniazid, ethambutol and pyrazinamide, individually, as well as in combination, when incorporated into the pro-Pheroid delivery structure. For analysis, high pressure liquid chromatography was utilised, by using methods that were adjusted and validated according to ICH guidelines. The two combination products being formulated during this study were ethambutol-isoniazid in pro-Pheroid (Pyriftol IE), and pyrazinamide-rifampicin in pro-Pheroid (Pyriftol RP).

These formulations were stored at stability conditions of 5°C, 25°C+60% RH, 30°C+65% RH and 40°C+75% RH, over a period of three months.

The main problem with analysing Pyriftol RP was homogeneity, as this sample was very viscous. This may have caused inconsistencies during sampling of the APIs, possibly leading to the variable results obtained. Despite the low initial



assay values obtained for pyrazinamide, the assay results for the four conditions over the three-month period remained stable. Most of the results complied with the required specifications of 90% – 110%.

The initial results for rifampicin were within specification. Highly variable results were obtained for all the other stability intervals and no significant conclusion could be made from the results.

For ethambutol, the initial result was 100%, with slightly higher results after one month. The 5°C and 40°C+75% RH results were out of specification, with values higher than 110%. For months 2 and 3, results were significantly lower than the initial results, with no value complying with specifications. The analytical technique may have caused the high variability, since a copper reaction on the column was required to determine the ethambutol contents.

The initial assay results for the isoniazid sample was 85.3%. This low initial result could have been due to consistency problems during manufacturing. Throughout the stability studies, the assay values were relatively stable, but all were below the lower acceptance criterion. The results for the 5°C samples were inconsistent throughout the study.

Five previous studies had confirmed the analytical problems being experienced with the Pheroid™ / pro-Pheroid delivery system, irrespective of the pharmaceutical active ingredient being used in these formulations. This study also emphasised the analytical, and probably also the formulation challenges within this delivery system.

For future studies to be undertaken, it is advised that the development of an analytical method, suitable for analysis of this complex and unstable delivery system, be prioritised.

# UITTREKSEL

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Tuberkulose hou wêreldwyd 'n ernstige gesondheidsrisiko in, met 9.27 miljoen gevalle wat in 2007 gerapporteer is (WHO, 2009:1). 31% hiervan was in Afrika, met Suid-Afrika gelys as 5<sup>de</sup> ter wêreld. Ten spyte van die hoë sterftesyfer van ongeveer 2 miljoen persone vir 2007, het weerstandigheid ook 'n faktor geword om mee rekening te hou in die stryd teen hierdie siektetoestand (WHO, 2009:3). Die grootste oorsaak van weerstandigheid word aan swak pasiëntmeewerkendheid toegeskryf, wat daartoe aanleiding gegee het dat korter behandelingstydperke oorweeg moet word.

Pheroid™ tegnologie, 'n nuwe afleweringstelsel, bevorder volgens navorsing gedoen, die absorpsie van geneesmiddels en verbeter biobeskikbaarheid. Die toepassing van hierdie voordele is oorweeg in die stryd om weerstandigheid teenoor geneesmiddels te help beveg. Die Pheroid™ is vergelykbaar met 'n emulsie, behalwe dat dit uit twee vloeistoffases (een met 'n oliegebaseerde - en een met 'n waterige basis), tesame met 'n gasfase wat met die vetsuurfase gedispergeer is, bestaan. Pro-Pheroid produksie is aan dié van die Pheroid™ gelykstaande, met die verskil dat die water gebaseerde fase uitgelaat word en die aktiewe bestanddele dus in die oliegebaseerde fase ingesluit word.

Hierdie studie is gedoen om die stabiliteit van vier anti-tuberkulose aktiewe farmaseutiese bestanddele (AFBe), nl. rifampisien, isoniasied, etambutol en pirasienamied, individueel, sowel as in kombinasie, in die pro-Pheroid afleweringstelsel te bestudeer en te bevestig. Analise is met hoë-druk vloeistof chromatografie uitgevoer, deur van aangepaste metodes, wat volgens ICH riglyne gevalideer is, gebruik te maak.

Die twee kombinasie formulerings wat in hierdie studie gebruik is, was etambutol-isoniasied (Pyrritol IE) en rifampisien-pirasienamied (Pyrritol RP), elk in die pro-Pheroid afleweringstelsel.

Hierdie formulerings is vir 'n tydperk van drie maande by gekontroleerde kondisies gestoor, nl. 5°C, 25°C+60% RH, 30°C+65% RH en 40°C+75% RH.

Die grootste probleem tydens die analise van Pyriftol RP was homogenisiteit, aangesien die preparaat taamlik viskeus was. Dit kon tot wisselende monsterneming van die AFBe aanleiding gegee het, wat dus moontlik 'n rol in die wisselvallige data verkry, kon gespeel het. Ten spyte van lae aanvangsresultate vir pirasienamied, was die resultate vir die vier stoorkondisies oor die drie-maande periode stabiel. Meeste van die resultate het aan die spesifikasies van 90% – 110% voldoen.

Aanvanklike resultate vir rifampisien was binne spesifikasie. Groot wisseling is egter vir alle verdere resultate tydens die res van die stabiliteitstudie verkry. Geen definitiewe uitsluitel kon dus oor die resultate gemaak word nie.

Etambutol se aanvanklike resultaat was 100%, met effense hoër resultate na 'n maand. Die 5°C en 40°C+75% RH resultate was buite spesifikasie, met waardes hoër as 110%. Maande 2 en 3 se resultate was beduidend laer as die aanvanklike waarde, met geen van hierdie waardes binne spesifikasie nie. Die analitiese tegniek mag vir die variasie verantwoordelik gewees het, aangesien 'n koperreaksie in die kolom moes plaasvind, ten einde die etambutol inhoud te kon bepaal.

Isoniasied se aanvanklike resultate was 85.3%. Die lae waarde kon die gevolg van bestendighedsprobleme tydens vervaardiging gewees het. Die resultate was deurgaans konstant, maar laer as die aanvaardingskriteria. Die resultate van die 5°C monsters was teenstrydig dwarsdeur die studie.

Vyf vorige studies het die analitiese probleme wat met die Pheroid™ / pro-Pheroid afleweringstelsel bevind is, bevestig, ongeag die farmaseutiese aktiewe bestanddeel wat in hierdie formulerings gebruik is. Hierdie studie het dus die analitiese, en waarskynlik ook die formuleringsuitdagings van hierdie Pheroid™ / pro-Pheroid afleweringstelsel, gestaaf.

Dit word aanbeveel dat die ontwikkeling van 'n toepaslike analitiese metode vir die analise van hierdie komplekse en onstabiele afleweringstelsel prioriteit behoort te geniet, tydens enige toekomstige studies.

# CHAPTER 1

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## INTRODUCTION

### 1.1 HISTORY OF TUBERCULOSIS

The first evidence of spinal tuberculosis (TB), as noted from fossil bones, dates as far back as 8,000 BC. Hippocrates was the first researcher who reported his findings on tubercles (phymata), as observed in the tissues of cattle, sheep and pigs, since human autopsies were not performed in Greek culture at the time (Herzog, 1998:5).

The next available records on TB was by Caelius Aurelianus, a physician from Rome, who reported his observations during the 5<sup>th</sup> century AD as follows: “The patients suffer from a latent fever that begins towards the evening and vanishes again at the break of day. It is accompanied by violent coughing, which expels thin purulent sputum. The patient speaks with a hoarse voice, breathes with difficulty and has hectically flushed cheeks. The skin on the rest of the body is ashen in shade. The eyes have a weary expression; the patient is gaunt in appearance but often displays astonishing physical or mental activity. In many cases, wheezes are to be heard in the chest, and when the disease spreads, sweating is seen on the upper parts of the chest. The patients lose their appetite or suffer hunger pangs. They are often also very thirsty. The ends of the fingers swell and the fingernails curve greatly.” (Herzog, 1998:5).

During the Renaissance in Northern Italy, Fracastorius from Verona (Girolamo Fracastoro, 1478–1553), a pioneer in contagious diseases, wrote that phthisis had been spreading as an invisible ‘virus’, and that it could subsist for two years in the clothing of a consumptive. He also commented that the illness was connected to excretion, due to pulmonary sores (Herzog, 1998:6).

The 17<sup>th</sup> century hailed the period of specific patho-anatomical explaining of ingestion. The first was by the Dutch Sylvius de la Boë (1617–1655), who

referred to tubercles as unvarying and distinguishing lesions in the lungs and other organs of consumptives, together with their tendency to cavities and sores. Richard Morton (1637–1698) expressed his findings a decade later in London, claiming that the sickness has three phases, i.e. commencing as inflammation, followed by tubercle development, and eventually advancing to ulcers and thus to phthisis. He also added that there had been cases where advancement was extremely slow, allowing continued life (Herzog, 1998:6).

Subsequently, the leading anatomist of his time, Giovanni Battista Morgagni from Padua (1682–1771), was so convinced about the consumption being infectious that he declined from executing autopsies on consumptives for fear of infectivity. Italian medical journals of the time supported his beliefs. The Republic of Lucca followed by an official decree, specifying that “in future, human health must no longer be endangered by objects remaining after the death of a consumptive person”. In addition, names of patients with infection were to be conveyed to governments, so that sterilisation methods could be pursued (Herzog, 2008:6-7).

Following the above legislation, the British Benjamin Marten (1704–1722) was the first to suggest that the illness may be instigated by miniature organisms. This has led to the observation that the amount of time spent with an infected person could be dismissed, although distance was noted as playing a significant role. Phillipp Klencke (1813–1881) proved in 1843 that when close enough to a patient to inhale expired air, the illness may be fabricated in the healthy person (Herzog, 2008:7).

“Blood or sputum from tuberculous rabbits, injected into other laboratory animals in controlled experiments, produced full-blown tuberculosis, whereas analogous transfer of cancerous or fibrotic tissue had no effect on the recipients.” These findings by Villemin (1865, cited in Herzog, 2008:8) led to the conclusion that the disease had to be caused by a particular airborne microorganism.

Robert Koch (1843–1910) then led the way in illustrating slender rods, the so-called tubercle bacilli (*Mycobacterium tuberculosis*) within tuberculous tissue, by applying unique discolouration methods. This technique was only significant in detecting people infected with TB and was mastered by the invention of X-rays by Wilhelm Conrad von Röntgen (1845–1923). Together with swift progress in radiology, the change, path and seriousness of TB could since then be examined truthfully (Herzog, 2008:8-9).

## **1.2 EPIDEMIOLOGY**

Millions of people have died as a result of the limited knowledge of tuberculosis. Extensive research over the last decades, especially, has helped create our current understanding of how the disease develops, spreads and how it should be managed.

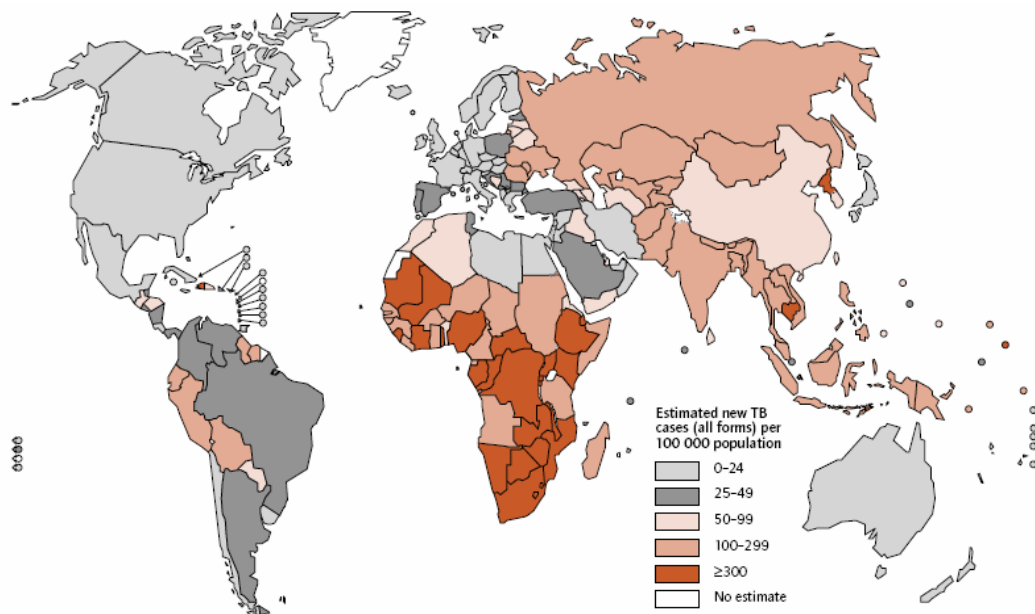
The following information and data were compiled by the World Health Organization (WHO), after investigating 196 nations and regions, representing 99.6% of the probable number of TB cases globally, as well as 99.7% of the world's inhabitants (WHO, 2009a: 1).

Worldwide, an anticipated 9.27 million cases of TB were documented in 2007. This number showed an escalation from previous years. 31% of these cases were reported in Africa, with South Africa being 5<sup>th</sup> worldwide, having 0.46 million cases (WHO, 2009a: 1).

During 2007, TB was reported amongst 13.7 million people and with regards to mortality, a likelihood of 1.3 million fatalities were reported amongst HIV negative cases of TB. An extra 456,000 fatalities were reported amongst patients that were HIV positive, although the latter were categorised as HIV deaths in the International Statistical Classification of Diseases, also known as ICD-10 (WHO, 2009a: 1-2).

Not forgetting resistance, a probable number of 0.5 million cases of multi drug resistant TB (MDR-TB) was reported, with 16,000 being from South Africa. In

addition, at least one case of extensively drug resistant TB (XDR-TB) was reported by 55 nations and regions each at the end of 2008 (WHO, 2009a: 2).

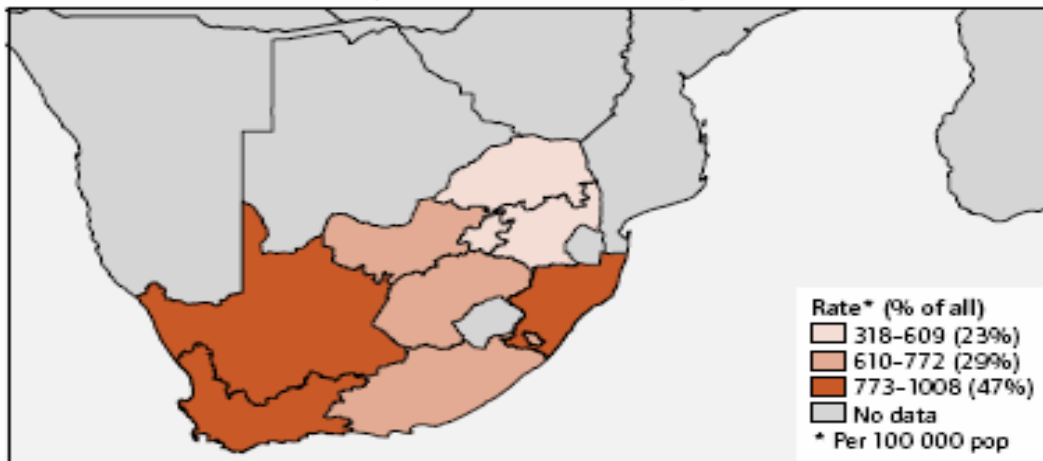


**FIGURE 1.1: A global view of estimated new TB cases (all forms) in 2007** (WHO, 2009a: 8).

In general, the number of TB cases is rising due to escalating populations. Despite this, the occurrence of TB has decreased per capita in five out of the six WHO regions, except in Europe, where a plateau has been reached (WHO, 2009a: 32).

In some respects, South Africa has achieved little success in treatment and cure, as fatalities from TB are on the rise. This may be connected to the high HIV infection rate in this country. South Africa is also known for the highest number of MDR-TB and XDR-TB cases reported in the region (WHO, 2009a: 145).

**TB notification rate (new and relapse), 2007**



**FIGURE 1.2: Number of cases of TB infection in South Africa in 2007**  
(WHO, 2009a: 145).

### 1.3 TRANSMISSION AND PATHOGENESIS

TB is instigated by a life form, called *Mycobacterium tuberculosis*, also known as tubercle bacilli, and is transferred among people by air. A healthy person may thus inhale air filled with droplet nuclei, when close to enough to an individual having infectious TB who is sneezing or coughing, thus leading to infection of the healthy individual. The disease only develops once the tubercle bacilli reproduce in the small air sacs of the lungs, after which a minor amount penetrates the bloodstream and spreads throughout the whole body. All the while the body's immune system would manage fighting the bacilli (Global Tuberculosis Institute, 2009b).

Individuals with latent TB infection (LTBI), but not the disease itself, do not have TB symptoms and cannot spread the illness. The illness usually affects the lungs (pulmonary TB), but it can also appear in other locations in the body (extrapulmonary TB). Furthermore, miliary TB arises once tubercle bacilli penetrate the bloodstream and are passed throughout the body, where they develop and trigger the illness in several places (Global Tuberculosis Institute, 2009b).



## 1.4 VACCINATION

Worldwide, where tuberculosis is commonplace in some areas, it is recommended for babies to be vaccinated with bacille calmette guerin (BCG), in order to try and prevent the disease. This vaccine is prepared by frail live bacteria, related to TB bacteria (*Mycobacterium tuberculosis*), which prevent the TB bacteria from increasing inside the body, hence stopping the TB from progressing (WHO, 2009b).

## 1.5 DIAGNOSIS

The tuberculin skin test is applied to conclude whether an individual has latent TB infection. The Mantoux tuberculin skin test has until recently been the most reliable and has been recommended as the only kind to be utilised (Global Tuberculosis Institute, 2009a).

For an affirmative response to the Mantoux test, one needs to consider the size of the response, the person's health risk factors and whether the person is exposed to infectious TB at work, in the community, or at home. It is advised that if someone, who is exposed to infectious TB, tests negative to the tuberculin skin test, that individual should be retested in 10 weeks' time (Global Tuberculosis Institute, 2009a).

However, a new serological test, QuantiFERON-TB Gold<sup>®</sup>, now available in South Africa, has recently been permitted by the FDA and has become the preferred test over the skin test, generally because it is more detailed (Global Tuberculosis Institute, 2009a).

Identifying the TB illness consists of four stages, i.e. medical background, tuberculin skin test, X-ray and bacteriologic assessment (Global Tuberculosis Institute, 2009a).

The background check involves asking the patient if (s)he has been in contact with a person having infectious TB, whether the patient has any symptoms,

whether the patient has had latent TB infection or has had the illness before, and inquiring about the patient's risk factors for developing the illness. Symptoms for pulmonary TB may consist of:

- Coughing;
- Pain in the chest when breathing or coughing; and
- Coughing up sputum or blood (Global Tuberculosis Institute, 2009a).

The universal signs of TB illness (pulmonary or extrapulmonary), usually when consistently occurring over 2 - 4 weeks, may involve:

- Weight loss;
- Fatigue;
- Malaise;
- Fever; and
- Night sweats.

Signs of TB illness are determined by the part of the body being affected by the illness (Global Tuberculosis Institute, 2009a).

Patients with signs of TB illness can be given a tuberculin skin test, or the QuantiFERON TB Gold test<sup>®</sup>, although it is advised that they should be thoroughly assessed for the disease, irrespective of the outcome of these tests (Global Tuberculosis Institute, 2009a).

Chest X-rays are then utilised to eliminate the prospect of pulmonary TB illness in an individual having tested affirmatively for the tuberculin skin test, whilst also verifying lung anomalies in individuals showing signs of TB illness. These findings cannot approve, nor dismiss the possibility of a patient having TB illness (Global Tuberculosis Institute, 2009a).

The conclusive phase, a bacteriological examination, consists of getting a sputum specimen from individuals with probable pulmonary TB illness, to be studied under a microscope for the existence of acid-fast bacilli (AFB). Any observed AFB is counted and these patients, with positive AFB smears, are regarded as infectious. The specimen is also cultivated, or matured, to test for the presence of *M. tuberculosis*, in which case the occurrence of the TB illness would be confirmed. Different specimens are acquired from individuals with possible extrapulmonary TB illness (Global Tuberculosis Institute, 2009a).

After cultivation of these specimens, drug receptiveness could be tested, as these findings can be very helpful to healthcare workers in their decision regarding the proper drugs to be used for therapy (Global Tuberculosis Institute, 2009a).

## 1.6 DRUG RESISTANT TUBERCULOSIS

Resistance, generally associated with the lungs, means that TB emitting bacilli are opposed to one or more antituberculosis drugs / active pharmaceutical ingredients (APIs). Table 1.1 describes the different known TB resistances.

**TABLE 1.1: Types of drug resistant TB (WHO, 1997:5)**

TYPE	DESCRIPTION
Primary resistance	Where bacterial resistance is detected in individuals, who do not have a history of treatment.
Initial resistance	Where a health worker, after review, is suspicious that an individual may have a history of therapy. It can be regarded as a combination of primary and unidentified acquired resistance.
Acquired resistance	Where any kind of documentation exists that indicates prior therapy of at least one month and longer.

Normally, the WHO customary first-line therapy (6 - 8 months) would conquer the possibility of collapse, because of primary resistance in first-time diagnosed individuals, which minimises the chances of catastrophe as a result of acquired resistance in people with a history of therapy (WHO, 1997:5).

*Failure of retreatment* is associated with an individual having TB emitting bacilli, following a directly observed, 5-month chemotherapy course, or subsequent to the finishing of an 8-month retreatment regime, where both were overseen by a qualified health worker (WHO, 1997:5).

The above retreatment regime is made up of three primary preparations, i.e. ethambutol, isoniazid and rifampicin, all complemented by pyrazinamide for the duration of the first three months, and by streptomycin throughout the first two months (2SHRZE / 1HRZE / 5HRE) [where streptomycin is (S), isoniazid is (H), rifampicin is (R), pyrazinamide is (Z), and ethambutol is (E)]. If any bacilli are present, following five months of appropriate management of this regime in an individual, the patient is normally resistant to a minimum of one and sometimes two of the major bactericidal preparations prescribed (isoniazid and / or rifampicin) (WHO, 1997:5).

Another form exists, *chronic case*, when an entire directly overseen WHO retreatment regime fails. This occurs after at least two attempts at chemotherapy, and occasionally even more than two attempts, irrespective of whether any of them was finished or not. These occurrences are typically, although not definitely, emitters of resistant bacilli (the degree of acquired resistance is extremely elevated in this class of patients), and frequently emitters of multi drug resistant (MDR) bacilli (WHO, 1997:5).

MDR bacilli, currently the most serious type of bacterial resistance, are resistant to no less than isoniazid and rifampicin, the two core anti-tuberculosis drugs currently available. This has made MDR-TB a significant cause for panic in TB management in several countries. Just like other types of drug resistance, MDR-TB is the result of human error, due to at least one of the following:

- Recommendation of chemotherapy;
- Organising of medicine stock;
- Administration of occurrences; and
- Procedure of medicine distribution to the patient (WHO, 1997:6).

Finally, the most serious form of TB comprises extensively drug resistant tuberculosis (XDR-TB), which is classified as TB that is resistant to isoniazid and rifampicin, but also to fluoroquinolones, as well as to one out of three injectable second-line preparations. It usually presents itself in the event of abuse and poor management of second-line treatment (Sweetman, 2009).

## **1.7 TREATMENT**

Directly observed therapy short course (DOTS) is a managing policy for tuberculosis, as developed by the World Health Organization (WHO). It consists of diagnostic measures, directly overseen treatment, certified sufficient medicine distribution and frequent assessment of the TB management plan (Sweetman, 2009).

Multi drug therapy for 6 - 8 months is necessary to heal TB in infected patients, to decrease the chances of setback and to avoid the return of drug resistant illness. This consists of a thorough preliminary stage of 2 - 3 months, intended for prompt sputum alteration, after which there is a maintenance stage of 4 - 6 months for eradication of remaining bacilli and to avoid setback. The option of treatment is determined by neighbourhood habits of drug resistance and the accessibility to medicine, whilst the WHO further advises that therapy procedures should be based on the brutality of the illness and the records of past TB therapy (Sweetman, 2009).

First-time instances of both extra- and pulmonary tuberculosis are treated with an opening stage of rifampicin (R), isoniazid (H), pyrazinamide (Z), and ethambutol (E) for two months, pursued by a maintenance stage of rifampicin

(R) and isoniazid (H) for four months. This therapy is referred to as RHZE2 / RH4. It is advised that pregnant women and children be given the same therapy as that of non-pregnant adults, with the exception that the dosages for children be altered in accordance to their age, or body-weight (Sweetman, 2009).

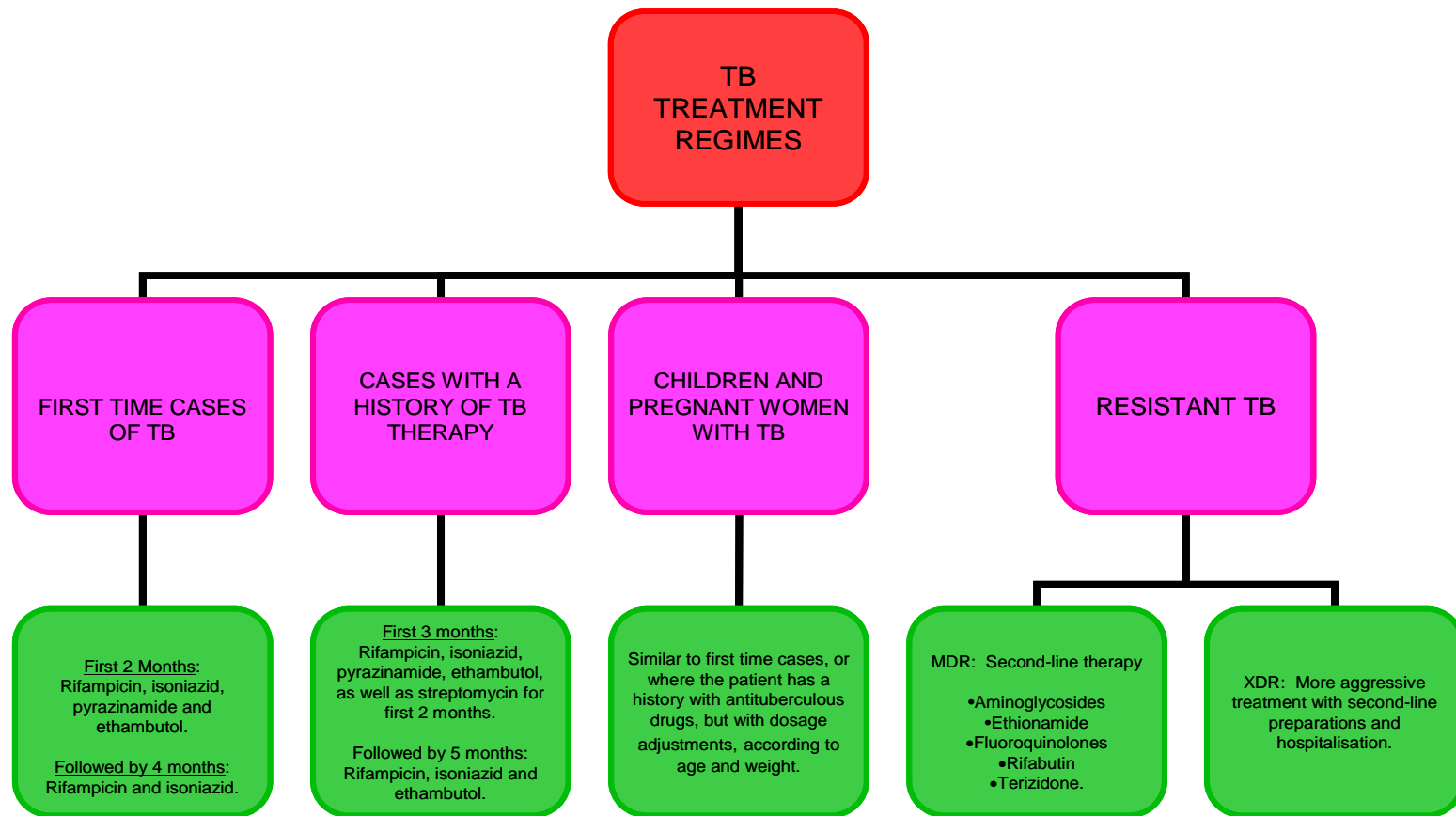
Ethambutol is not advised for treating children, due to the belief of complications in identifying ocular toxicity. Similarly, it is better to avoid fluoroquinolones, aminoglycosides, ethionamide and prothionamide when treating pregnant women. During therapy, liver enzymes and signs of drug induced hepatitis ought to be closely monitored. After pregnancy, women are allowed to breast-feed, even whilst using medicine, since the amount of medicine in breast milk is too insignificant to cure or avoid tuberculosis in babies (Sweetman, 2009).

In first-line therapy, during the maintenance stage, rifampicin may be replaced by ethambutol to reduce costs, even though the time frame is then prolonged to eight months. However, the possibility of setback increases (RHZE2 / HE6). Ethambutol may then be replaced by streptomycin (S) in case of tuberculous meningitis (RHZS2), which is recommended to be followed by a maintenance stage of 6 - 10 months with rifampicin and isoniazid (Sweetman, 2009).

The preliminary stage, for people having a history of tuberculosis therapy, include: rifampicin, isoniazid, pyrazinamide and ethambutol for three months, together with streptomycin during the first two months. The maintenance stage would then consist of rifampicin, isoniazid and ethambutol for a period of five months (RHZES2 / RHZE1 / RHE5), as per the guidelines proposed by Sweetman (2009).

Since drug resistance is suspected when an individual presents affirmative results, following a monitored short course in chemotherapy, a launch of second-line medicine should be implemented (Sweetman, 2009). This would include preparations, such as rifabutin, ethionamide, fluoroquinolones, aminoglycosides and terizidone. Although cycloserine may be prescribed, it is not registered for use in South Africa (Gibbon, 2005: 297).

Figure 1.3 summarises the treatment regimes of TB, as discussed in par. 1.7.



**FIGURE 1.3:** Summary of treatment regimes.



## **1.8 CONCLUSION**

In this chapter the origin of tuberculosis was introduced, how it affects the infected individual and how to manage it. Despite significant developments in understanding the disease and how to cure it, various healthcare disciplines are faced with serious challenges, due to the occurrence of resistance towards available therapies / drugs available.

Resistance is mainly caused by the fact that patients deviate from their prescribed therapies. The need for shorter courses of treatment is thus suggested as a means of ensuring compliance to prescribed treatment.

The anti-tuberculosis formulation being investigated during this study aimed at establishing a dosage form that would enable a shorter period of therapy. However, this study specifically focused on the stability of the formulation being developed and HPLC testing thereof.

In the next chapter the active pharmaceutical ingredients, used in the developed formulation, are discussed.

# CHAPTER 2

---

## TUBERCULOSTATICS

### 2.1 PREFACE

Many treatment regimens for tuberculosis are available on the market today, but for the purpose of this study the focus was on first-line medication.

First-line medication comprises of a) ethambutol, b) isoniazid, c) pyrazinamide and d) rifampicin. They are prescribed as fixed-dose, multi drug treatments, aiming at avoiding the emergence of resistance to TB medication.

In the following paragraphs, the attributes and features of these drugs, as well as reported occurrences of polymorphism are discussed.

### 2.2 ETHAMBUTOL

#### 2.2.1 Introduction

Usually ethambutol is in the form of a salt, i.e. ethambutol hydrochloride (HCl), when used as an active pharmaceutical ingredient (API). It is prescribed as a key ingredient, not only because of its tuberculostatic characteristics, but also due to its inhibiting effects on the appearance of resistant bacilli (Becker *et al.*, 2008a:1351).

Ethambutol is predominantly bacteriostatic, but it can also be bactericidal when assigned in elevated dosages. This may be due to the fact that ethambutol seems to work by restraining mycobacterial RNA creation (Gibbon, 2010:316).

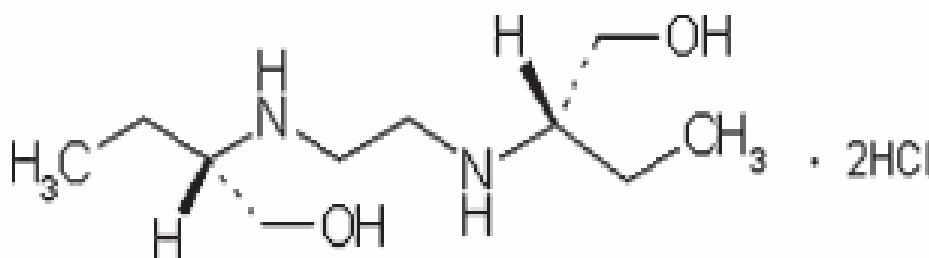
However, severe adverse drug reactions (ADR) may occur, such as visual problems and even irreversible blindness, when using ethambutol. This occurs, because of drug induced optic neuritis. It is thus important to know that toxicity is dose dependant, and that the first step in treatment, when ADR is observed,

would be to suspend the use of ethambutol. It is also advised that patients should be informed on how to identify and convey visual changes, whilst patients with existing ocular defects should be monitored closely (Becker *et al.*, 2008a:1351-1352).

### 2.2.2 Physical and chemical attributes

The International Pharmacopoeia recommends that ethambutol dihydrochloride, which is an unscented, white, crystalline powder, should be stored in an airtight bottle due to the fact that it is hygroscopic (Ph.Int., 2008).

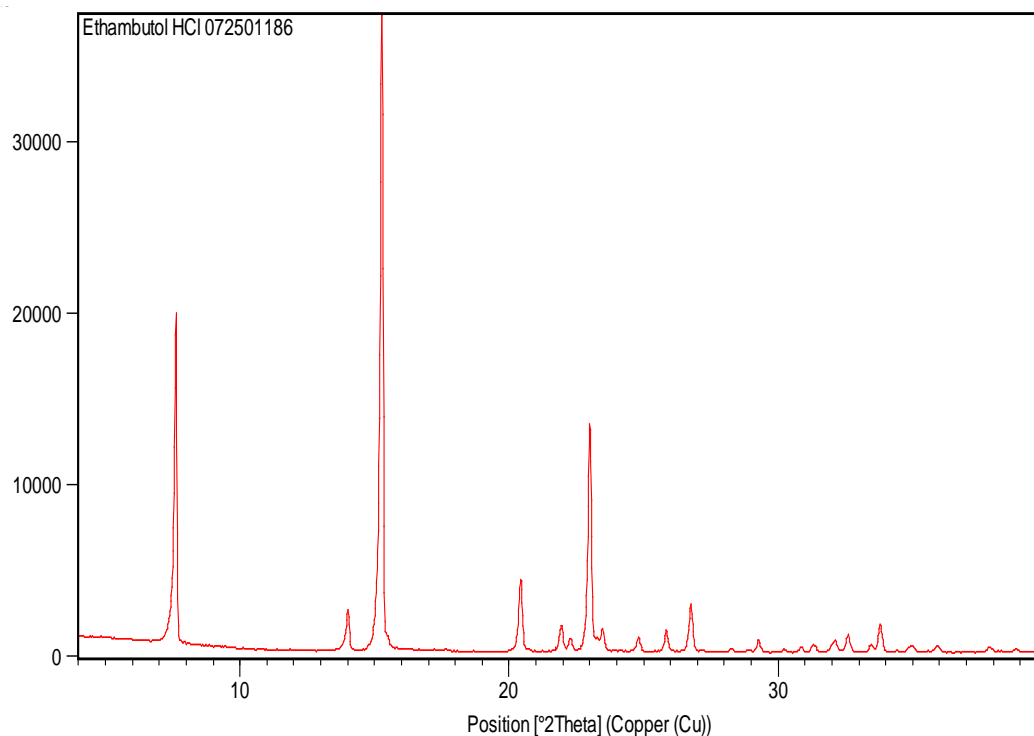
The chemical structure of ethambutol dihydrochloride is illustrated by figure 2.1.



**FIGURE 2.1:** Chemical structure of ethambutol dihydrochloride (Becker *et al.*, 2008a:1351).

The relative molecular mass of ethambutol dihydrochloride is 277.2 g/mol and it is a weak base. Ethambutol dihydrochloride is promptly soluble, with a minimum of 1 g being soluble in 1 ml of water at room temperature (Becker *et al.*, 2008a:1352).

Four polymorphic forms have been reported for ethambutol dihydrochloride, whilst only polymorphic form II (see figure 2.2) is currently being utilised in pharmaceutical drug preparations (Becker *et al.*, 2008a:1352).



**FIGURE 2.2: XRPD pattern of ethambutol dihydrochloride, used in this study, representing polymorphic form II.**

## 2.3 ISONIAZID

### 2.3.1 Introduction

Isoniazid is also known as isonicotinic acid hydrazide. It is normally prescribed together with one or more of the other three first-line APIs for the treatment of TB, whilst it is prescribed on its own for the prevention of TB in the aged and high probability areas. Furthermore, it can be prescribed as part of therapy for leprosy (Becker *et al.*, 2007:523).

The meticulous method of action of isoniazid is based on the stalling of mycolic acid synthesis (exclusive to mycobacterial cell walls), as per Gibbon (2010:316). This may also be the reason for isoniazid being bactericidal against both intra- and extracellular mycobacteria (Gibbon, 2005:293).

However, hepatotoxicity, where isoniazid is used for treatment, is being reported worldwide. This may be due to its restraining of explicit microsomal

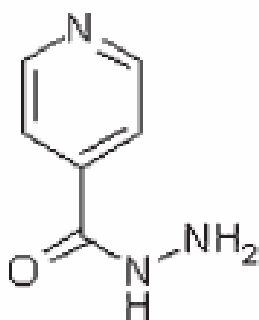
cytochrome P450 enzymes, whilst the toxic effects are elevated when abusing alcohol (Gibbon, 2010:317). The WHO advises that patients who presents the probability of acquiring peripheral neuropathy (usually undernourished people), ought to be given an additional dose of 10 mg of pyridoxine per day (Becker *et al.*, 2007:523).

Patients should also be advised that assimilation would be most advantageous, when isoniazid is ingested before eating (Gibbon, 2010:317).

### 2.3.2 Physical and chemical attributes

Isoniazid can be in the form of colourless crystals, or a white, crystalline powder. It is unscented and should be stored in an airtight amber bottle as air and light may influence the stability of the sample (Ph.Int., 2008).

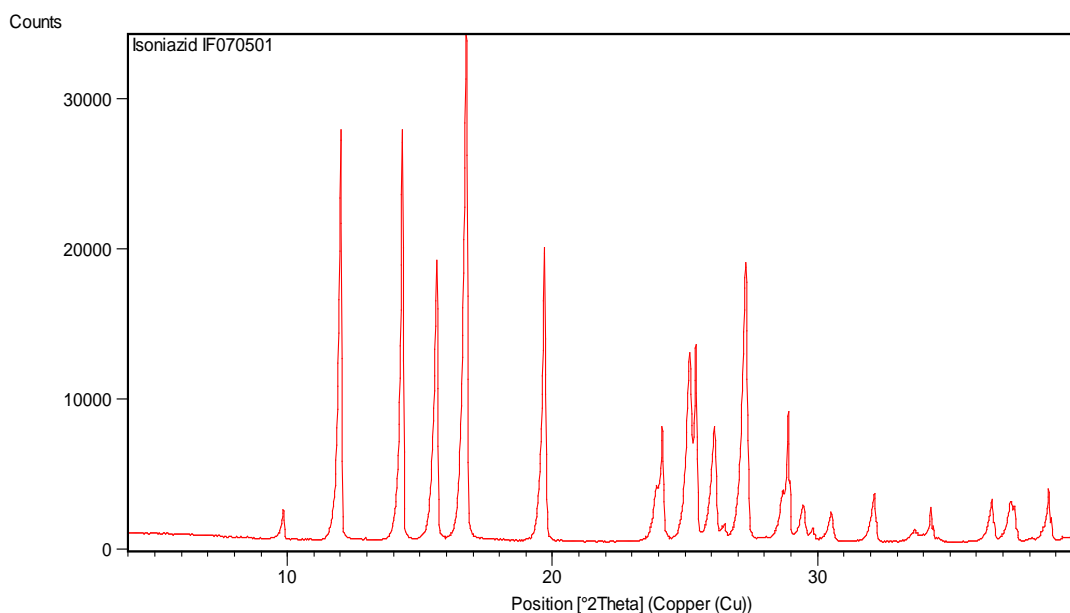
The chemical structure of isoniazid is shown in figure 2.3.



**FIGURE 2.3: Chemical structure of isoniazid** (Becker *et al.*, 2007:523).

The isoniazid molecule tends to be acidic and at room temperature it is soluble to the degree of 125 mg/ml in water. Furthermore, it has a relative molecular mass of 137.1 g/mol (Becker *et al.*, 2007:524).

Nothing on polymorphism was found in the literature, whereas Becker *et al.* (2007:523-524) report an orthorhombic crystal structure for isoniazid. The X-ray powder diffractogram (XRPD) pattern of the isoniazid raw material, used in this study, is shown in figure 2.4.



**FIGURE 2.4: XRPD pattern of isoniazid, used in this study.**

## 2.4 PYRAZINAMIDE

### 2.4.1 Introduction

The reason for the inclusion of pyrazinamide in the original stage only, is because of its bactericidal ability towards lengthy metabolising bacilli. This causes a decline in occurrence of bacteriological setback, once the chemotherapy treatment is finished (Becker *et al.*, 2008b:3710).

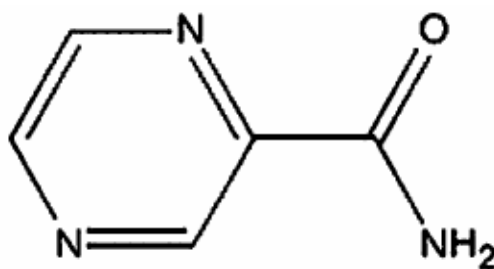
Pyrazinamide, part of the nicotinamide family, is mycobactericidal for intracellular mycobacteria in an acidic medium. Moreover, pyrazinamide is extremely precise against *M. tuberculosis* (Gibbon, 2010:316).

However, the use of pyrazinamide should be monitored closely for signs of drug induced hepatitis. The poisonous properties of pyrazinamide are associated with the prescribed amount and period of therapy, but can arise at any stage during treatment. These negative consequences seem to occur more in some subpopulations, like females, the aged, people with an asian legacy, individuals with renal complications and HIV patients (Becker *et al.* 2008b:3710).

### 2.4.2 Physical and chemical attributes

Pyrazinamide is an unscented, white, or almost white, crystalline powder and should be kept in an airtight bottle.

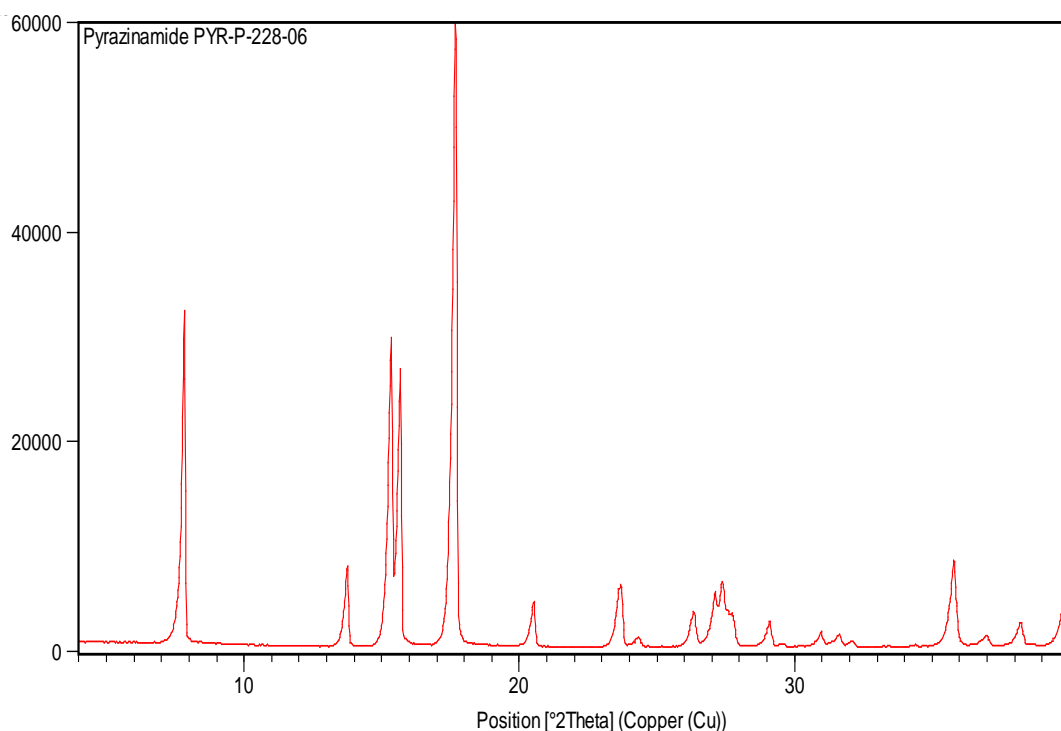
The chemical structure is shown in figure 2.5.



**FIGURE 2.5: Chemical structure of pyrazinamide** (Becker *et al.*, 2008b:3710).

Chemically, pyrazinamide is known as pyrazinecarboxamide, having a relative molecular mass of 123.1 g/mol. It is moderately soluble in water and to some extent in ethanol (Ph.Int., 2008). According to Becker *et al.* (2008b:3711), pyrazinamide is an extremely weak base.

Research revealed that pyrazinamide presents itself in four polymorphic shapes, each having different crystal arrangements, i.e.  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -pyrazinamide. The different forms are determined by the solvent and temperature at the time of manufacture (Becker *et al.*, 2008b:3711). According to the XRPD traces (figure 2.6), the raw material being used in this study, represented the commercially available form, i.e. the  $\alpha$ -form (Castro *et al.*, 2010:276).



**FIGURE 2.6: XRPD pattern of the pyrazinamide (form  $\alpha$ ), used in this study.**

## 2.5 RIFAMPICIN

### 2.5.1 Introduction

Rifampicin is effective against a selection of gram positive, gram negative bacteria and the entire populations of tuberculosis bacilli, as well as other mycobacteria. It thus is the most important API within the blend of antituberculosis therapies, as well as against leprosy, as suggested by the WHO (Becker *et al.*, 2009:2253).

To stop the infection, rifampicin prevents the making of bacterial RNA, by hindering the enzyme, DNA dependant RNA polymerase. In addition, this means that rifampicin improves the metabolism of drugs, especially contraceptives (Gibbon, 2005:293-294).

No serious negative consequences, when using rifampicin, have been reported by patients (Becker *et al.*, 2009:2253). Patients should, however, be informed



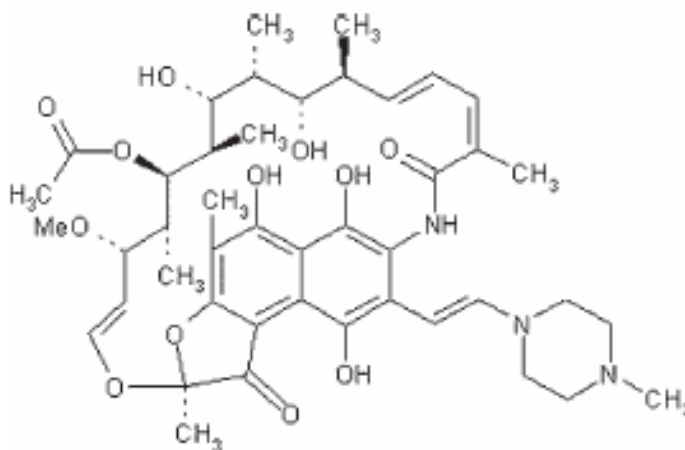
that bodily fluids, such as urine and tears, may turn red-orange to red-brown while on treatment and that contact lenses can permanently stain (Gibbon, 2010:316).

The fact remains that both isoniazid and rifampicin are hepatotoxic. It has been documented that rifampicin induces the hydrolysis pathway of isoniazid, resulting in a hepatotoxic metabolite hydrazine. This interaction is dose related (Askgaard *et al.*, 1995:213).

### 2.5.2 Physical and chemical attributes

Rifampicin is a brick-red to red-brown, crystalline powder. It is unscented or very slightly scented and should be kept in an airtight bottle, away from light. Regarding solubility rifampicin is “very slightly soluble in water, soluble in methanol R; slightly soluble in acetone R, ethanol (~750g/l) TS, and ether R”. (Ph.Int., 2008)

The chemical structure of rifampicin is shown in figure 2.7 (Ph.Int., 2008).



**FIGURE 2.7: Chemical structure of rifampicin** (Becker *et al.*, 2009:2253).

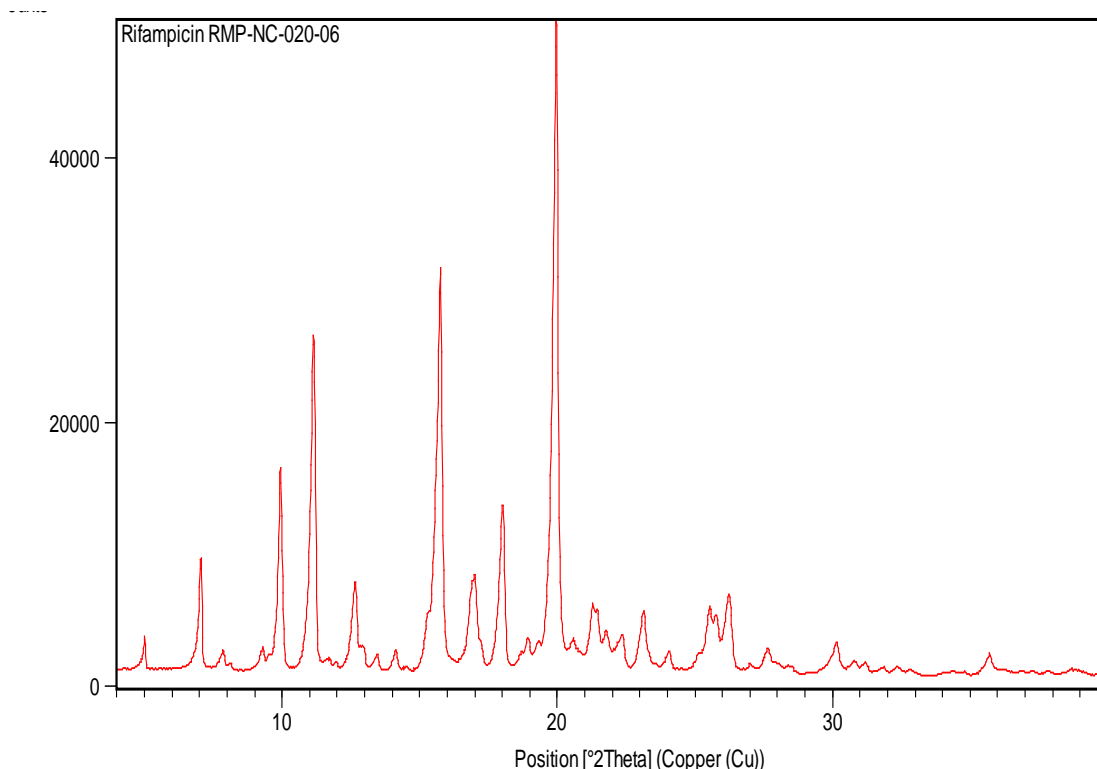
Rifampicin subsists in two crystalline anhydrous types, i.e. forms I and II, as well as in two amorphous forms. A monohydrate, a dihydrate and a pentahydrate also exist. Manufacturers of generic rifampicin in South Africa either include raw

materials of crystalline form II, or a combination of crystalline form II and the amorphous form. Despite all the available information regarding different crystalline types, the pharmacopoeias do not prescribe any specific polymorph (Becker *et al.*, 2009:3; Henwood *et al.*, 2000:408).

With regards to its stability, rifampicin is the most stable as a solid and should be kept in an airtight bottle at room temperature, also shielded against humidity, light and oxygen. It decays rapidly in an acidic liquid, but relatively slowly under neutral situations (Becker *et al.*, 2009:2254).

Based on its stability, together with the fact that rifampicin is an amphoteric molecule, solubility data varies for each polymorphic form, thus making it very difficult to make assumptions regarding its solubility (Becker *et al.*, 2009:2254).

The X-ray powder diffractogram (XRPD) pattern of the rifampicin raw material, used in this study, is shown in figure 2.8.



**FIGURE 2.8: XRPD pattern of rifampicin, used in this study.**

## **2.6 CONCLUSION**

The physicochemical properties of the four pharmaceutical active ingredients, used in first-line antituberculosis medications, i.e. ethambutol, isoniazid, pyrazinamide and rifampicin, were discussed.

The literature was further searched for any existing reports on the occurrence of polymorphism among these APIs, since it was important to ensure that the correct form(s) of each was used in this formulation study.

In the next chapter, the innovative Pheroid™ technology is introduced, which was used as the drug delivery system of the APIs in the developed antituberculosis formulations during this study.

The Pheroid™ technology as a drug delivery system poses the possibility of managing drug delivery more specifically and controlled, which could then lead to a shorter period of tuberculostatic therapy overall.

# CHAPTER 3

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## PHEROID™ TECHNOLOGY

### 3.1 PREFACE

In this chapter, the Pheroid™ technology is introduced. It is a drug delivery system, formerly known as the Emzaloid™ technology. “The Pheroid™ delivery system is a colloidal system that contains unique and stable lipid-based, submicron- and micron-sized structures, called Pheroids™, uniformly distributed in a dispersion medium that may be adapted to fit the indication.” (Grobler *et al.*, 2008:5).

The dispersed phase may be influenced when considering morphology, structure, size and purpose. Even though the particularly minute atoms that are suspended in the continuous phase, normally found in a colloidal system, range between 1 - 100 nm in diameter, the Pheroid™ is usually formulated to possess a diameter of 200 nm to 2 µm (Grobler *et al.*, 2008:5).

Pheroids™ consist of ethylated and pegylated, polyunsaturated fatty acids, together with the omega-3 and -6 fatty acids, except for arachidonic acid. These fatty acids are arranged in the cis-formation, similar to the orientation of fatty acids found in human beings. Pheroids™ also have a lipid bilayer, although it does not contain any phospholipids, nor cholesterol. The formation of Pheroids™ is brought on by a self-assembly process that is like low-energy emulsions, as well as microemulsions, plus there is no need for lyophilisation, nor hydrations of the lipid components (Grobler *et al.*, 2008:6, 9).

As with emulsions, the Pheroids™ are dispersed within a dispersion medium, with the exception of an extra gas phase, which is associated with the fatty acid dispersed phase. The exact relation of pegylated to ethylated fatty acids being utilised in the accumulation of the Pheroids™, builds on the reservoir qualities of the polymeric microspheres (Grobler *et al.*, 2008:9).

When examining the gas phase, it is nitrous oxide (N<sub>2</sub>O) being scattered within the dispersed phase all the way through the continuous phase. This gas acts in this phase by helping with the miscibility of the fatty acids in the dispersal medium, by helping with the self-assembly procedure of the Pheroids™, as well as by adding to the stability of the formed Pheroids™ (Grobler *et al.*, 2008:10).

With regards to the advantages of the Pheroids™, the fact that they are polyphilic is the most significant. This means that medicines, possessing a variety of solubilities, together with those being insoluble, are able to be entrapped. Other advantages of the Pheroids™ include:

- Enhancement of delivery of active ingredients;
- Delay in onset of action;
- Decreasing the minimal effective concentration;
- Enhancing therapeutic efficacy;
- A decrease in cytotoxicity;
- Penetration of maximum known barriers in the body, as well as in cells;
- Aptitude to directly aim at therapy regions;
- Absence of immunological responses;
- Skill to transport genes to cell nuclei; and
- Lowering the prevalence of drug resistance (Grobler, 2004:3).

### **3.2 PLANNING OF PHEROIDS™**

The drafting of the Pheroid™ permits exploitation of its structural, as well as functional attributes. Several aspects are used to achieve such changes, such as altering the fatty acid composition or concentrations, altering / the inclusion of the cryo-protectants, whilst charge-inducing agents could also be included. Another option would be to insert non-fatty acids or phospholipids, such as cholesterol, for example, or to alter the hydration medium. Even using a

different technique during preparation would bring about a change, which could alter the character and concentration of the active ingredient (Grobler *et al.*, 2008:15).

### **3.3 FORMS OF PHEROIDS™**

Using exploitation of composition and the development procedure, different Pheroid™ forms can be created, for example:

- Lipid-bilayer vesicles;
- Pheroid™ microsponges; and
- The Pheroid™ being produced as a pro-Pheroid (Grobler, 2004:4).

The construction of a pro-Pheroid is based on a procedure where nitrous oxide is being gassed into the oil phase, after which the active ingredient is blended into this oil phase. Consequently, the pro-Pheroid is made up of the oil-based liquid phase being interdispersed within the gas phase. This constitution of the pro-Pheroid acts as a trigger for the formation of the Pheroid™, as soon as the aqueous liquid phase familiarises with the oil phase. In essence, the Pheroid™ is water based in comparison with the pro-Pheroid that is oil based (Padayachee, 2008:11).

### **3.4 CONCLUSION**

Pheroid™ technology is exceptional in the sense that it accommodates every drug type, from hydrophilic to lipophilic. Being polyphilic, Pheroids™ can entrap active ingredients having different solubilities, as well as active compounds that are poorly soluble. This makes Pheroid™ technology a pioneer in the drug delivery industry.

All of the actives used during this study, except for rifampicin, were soluble in water. From the physicochemical data that was discussed in chapter 2, it could

be expected that the delivery system used in this study would be the Pheroid™, i.e. the hydrophilic system. However, the pro-Pheroid was regarded the best choice for this study, due to volume constraints (Nieuwoudt, 2009:41).

Some of the main considerations when developing this pro-Pheroid delivery system included the daily dosage per weight of the formulation in comparison with Rifafour® e-275, as well as future dosage form development for human consumption (Nieuwoudt, 2009:41).

# CHAPTER 4

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## METHOD ADJUSTMENT AND VALIDATION

### 4.1 PREFACE

This study was part of a project being undertaken by a research team within the Drug Research and Development Unit of the Faculty of Health Sciences, North-West University, Potchefstroom Campus, in an attempt to manufacture a new tuberculostatic preparation, by using the new drug delivery system, the pro-Pheroid.

The formulations for this study included ethambutol and isoniazid in pro-Pheroid (Pyriftol IE), as well as a pyrazinamide rifampicin combination in pro-Pheroid (Pyriftol RP). This particular combination was chosen, since previous studies indicated a significant improvement in bactericidal activity, as well as a decline in relapse figures for the pyrazinamide-rifampicin preparation, in comparison with results for the pyrazinamide-rifampicin-isoniazid preparation (Grosset *et al.*, 1992:551).

Pyriftol IE was prepared using the following formulation:

- Vitamin F ethyl ester;
- Cremophor EL;
- dl- $\alpha$ -Tocopherol;
- Butylated hydroxyanisole;
- Butylated hydroxytoluene;
- PEG 400;
- Ethambutol; and
- Isoniazid.



Pyrifitol RP was prepared using the following formulation:

- Vitamin F ethyl ester;
- Cremophor EL;
- dl- $\alpha$ -Tocopherol;
- Butylated hydroxyanisole;
- Butylated hydroxytoluene;
- PEG 400;
- Pyrazinamide;
- Rifampicin; and
- Ascorbyl palmitate.

This specific study was performed with the aim of testing the stability of the mentioned formulations. By visual inspection and handling of the dosage forms, the Pyrifitol IE preparation appeared less viscous than the Pyrifitol RP. The Pyrifitol RP formulation was a paste, mainly because of the large amount of active ingredients, representing more than half of the total preparation, whilst the Pyrifitol IE was more like a cream. In both formulations sediment formed, which became more visible over time.

First, a literature study was required to find methods available for testing the applicable active ingredients, as basis for use in the development of suitable methods of analyses for the formulations in this study. The International Pharmacopoeia (IP) has monographs for the ethambutol-isoniazid-pyrazinamide combination, as well as for rifampicin only (Ph.Int., 2008). Since the wavelength detection of pyrazinamide was at 270 nm and that of rifampicin at 254 nm, it was necessary to determine whether it would be feasible to analyse pyrazinamide by means of the rifampicin monograph assay method. The second major goal was to acquire a shorter retention time for ethambutol for quicker analysis during production.

## 4.2 ADJUSTMENT OF EXISTING METHODS

Generally, the development of a method of analysis is built around previous research, or established literature, and by applying equivalent instrumentation. During the development phases of HPLC methods during this study, the main focus was to choose suitable methods of quantitation, columns, detectors and mobile phases (Swartz & Krull, 1997:25-26).

Usually, the need for modification of a method arises due to several aspects, such as:

- The existing method is time consuming and / or expensive;
- There is no established method to analyse a particular analyte;
- Existing methods may be unreliable and the established method has meager accuracy or precision;
- The methods available were developed for older and less sensitive instruments; and
- Improvements on instrumentation technology (Swartz & Krull, 1997:26).

Steps in a strategy for HPLC method development are as follows:

1. Define method and separation goals.
2. Gather sample and analyte information.
3. Initial method development – test runs for first chromatograms.
4. Method modification and optimisation.
5. Method validation (Dong, 2006:195).

With the above steps in mind, the physicochemical properties of the APIs were investigated, with special focus on wavelength detection. It was experimentally determined that it was possible to analyse pyrazinamide, using the rifampicin monograph assay method, bearing in mind that pyrazinamide should be detected at 270 nm, compared to rifampicin at 254 nm. Alterations made to the

phosphate mobile phase and the preparation of the standard and sample also shortened the retention time for rifampicin from 28 to 12 minutes, with little effect on the pyrazinamide peak, which remained at 2.5 minutes. This resulted in a total runtime of 20 minutes with excellent separation of both peaks.

Alterations made to the buffer: methanol ratio improved the ethambutol retention time from 17 to 4 minutes, when using a standard without isoniazid. In the standard preparation containing isoniazid, the retention time for ethambutol was 13 minutes and for isoniazid 2 minutes. The two actives were completely separated, having a runtime of 20 minutes after alterations were also made to the preparation of the standard and monster samples.

After establishing the mobile phase compositions for these two combination formulations, samples of the mobile phases and the separate actives were analysed to verify any possible interferences. Since the desired findings were acquired, the next step was to validate the methods.

At the time of validation, unfortunately, no pro-Pheroid was available for specificity studies. Validation would entail a procedure to guarantee that the experimental method was truthful, repeatable, as well as fit for the indicated APIs in combination. Even though validation is required by all regulatory procedures, the definite execution is somehow up for own analysis and may vary considerably amongst corporations (Dong, 2006:227).

## **4.2.1 Method for isoniazid and ethambutol in pro-Pheroid**

### **4.2.1.1 Chromatographic conditions**

The analytical procedure was performed under the following isocratic conditions, after modifying the IP method (Ph.Int., 2008):

**Analytical instrument:** Shimadzu Prominence series HPLC, equipped with LC-20AD quaternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software

<b>Column:</b>	Phenomenex Luna C <sub>18</sub> (2), 150 x 4.6 mm, 5 µm
<b>Mobile phase:</b>	Buffer:Methanol (96:4). Buffer prepared from 100.0 g of ammonium acetate, together with 0.4 g of copper acetate, made up to 2,000 ml with water. Adjust pH to 5.0, using glacial acetic acid.
<b>Flow rate:</b>	1.0 ml/min
<b>Injection volume:</b>	10 µl
<b>Detection:</b>	UV at 270 nm
<b>Retention time:</b>	Approximately 2 minutes for isoniazid and approximately 13 minutes for ethambutol
<b>Stop time:</b>	20 minutes
<b>Solvents:</b>	Assay: Methanol (100%) for sample preparation and water for standard preparation.

#### 4.2.1.2 Standard preparation

1. Accurately weigh 1.62 g of ethambutol and 0.52 g of isoniazid and transfer into a 100 ml amber (for the isoniazid) volumetric flask.
2. Dissolve in 50 ml of water by mechanical shaking for 2 minutes.
3. For isoniazid analyses, allow to cool to room temperature and make up to volume with water.
4. For ethambutol analyses, dilute 1 ml to 10 ml with water.

#### 4.2.1.3 Sample preparation

Samples of each formula were stored at the following stability conditions: 30°C ; 25°C+60% RH; 30°C+65% RH and 40°C+75% RH for 3 months. Samples were prepared in duplicate for analysis as follows:

1. Accurately weigh 0.5 g of pro-Pheroid™, Pyrifol IE and transfer into a 20 ml amber volumetric flask.
2. Add 10 ml of methanol and mechanically shake for 5 minutes.
3. Allow to cool to room temperature and make up to volume with methanol.
4. Filter a portion of the solution, discarding the first few millilitres.
5. Transfer 4 ml of the filtrate into a 20 ml amber volumetric flask for analysis of isoniazid.
6. Add methanol and mechanically shake for 2 minutes and allow cooling to room temperature.
7. Make up to volume with methanol.
8. For analysis of ethambutol, transfer 1 ml of the solution in (6) into a 10 ml volumetric flask.
9. Make up to volume with methanol and mechanically shake for 2 minutes.
10. Allow to cool to room temperature before analyses.

## 4.2.2 Method for pyrazinamide and rifampicin in pro-Pheroid™

### 4.2.2.1 Chromatographic conditions

The analytical procedure was performed under the following isocratic conditions, after modifying the IP method (Ph.Int., 2008):

**Analytical instrument:** Shimadzu Prominence series HPLC, equipped with LC-20AD quaternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software

**Column:** Phenomenex Luna C<sub>18</sub> (2), 250 x 4.6 mm, 5 µm

**Mobile phase:** Buffer:Methanol (35:65). Buffer prepared from 2.7218 g of potassium phosphate in 2,000 ml with water. Adjust pH to 7.0, using sodium hydroxide.

<b>Flow rate:</b>	1.0 ml/min
<b>Injection volume:</b>	10 µl
<b>Detection:</b>	UV at 254 nm for rifampicin and 270 nm for pyrazinamide simultaneously, using diode array
<b>Retention time:</b>	Approximately 2.5 minutes for pyrazinamide and 12 minutes for rifampicin
<b>Stop time:</b>	20 minutes
<b>Solvents:</b>	Assay: Water for standard preparation of pyrazinamide and methanol (100%) for preparation of the rifampicin standard, as well as for preparation of the samples.

#### **4.2.2.2 Standard preparation**

##### **Rifampicin standard**

1. Accurately weigh 0.59 g of rifampicin and transfer into a 100 ml amber volumetric flask.
2. Dissolve in 50 ml of methanol and mechanically shake for 2 minutes.
3. Allow to cool to room temperature and make up the volume with methanol.

##### **Pyrazinamide standard**

1. Accurately weigh 0.44 g of pyrazinamide and transfer into a 100 ml volumetric flask.
2. Dissolve in 50 ml of water and mechanically shake for 2 minutes.
3. Allow to cool to room temperature and make up the volume with water.
4. Dilute 1 ml to 10 ml with water.

### **Sample preparation**

Samples of each formula were stored at the following stability conditions: 5°C; 25°C+60% RH; 30°C+65% RH and 40°C+75% RH for 3 months. Samples were prepared in duplicate for analysis as follows:

1. Accurately weigh 0.5 g of Pyrifitol RP and transfer into a 20 ml amber volumetric flask.
2. Add 10 ml of methanol and mechanically shake for 5 minutes.
3. Allow to cool to room temperature and make up to volume with methanol.
4. Filter a portion of the solution, discarding the first few millilitres.
5. Transfer 4 ml of the filtrate into a 20 ml amber volumetric flask.
6. Make up to volume with methanol.
7. Mechanically shake for 2 minutes and allow cooling to room temperature.
8. For analysis of pyrazinamide, transfer 1 ml of the solution in (6) into a 10 ml volumetric flask.
9. Make up to volume with methanol and mechanically shake for 2 minutes.
10. Allow to cool to room temperature before analyses.

### **4.3 VALIDATION**

Specific guidelines exist for performing validation studies. Since this study aimed at identifying and measuring APIs in formulation, methods were classified as category one analytical methods. Assay testing in this category is validated by testing compliance in accuracy, precision, specificity, linearity, range and ruggedness (Swartz & Krull, 1997:68-69; ICH Topic Q1 (R1), 1995:4).

### **4.3.1 Accuracy**

Accuracy is defined as “the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value.” It is being evaluated as the percentage of active compound retrieved by assay, which is then weighed against that of a standard reference material (Swartz & Krull, 1997:56).

When verifying method accuracy, the International Conference on Harmonisation (ICH) (ICH Topic Q1 (R1), 1995:11) recommends compiling information from a minimum of nine test runs. This may include a minimum of three concentration levels that are within the particular selection. For assay analysis, specifically, accuracy is evaluated by analysing samples with known quantities of APIs. The data is then documented as the percentage of API retrieved of the identified, additional quantity, or as the variation between the mean and true values, with confidence intermissions being displayed through a control chart (Swartz & Krull, 1997:57).

### **4.3.2 Precision**

Precision “is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples.” Consistent with the ICH guideline (ICH Topic Q1 (R2), 1995:12), precision should be done on three diverse levels, i.e. repeatability, intermediate precision and reproducibility (Swartz & Krull, 1997:57).

Repeatability is the aptitude of the method to produce related findings, when comparing numerous preparations from an indifferent, homogenous sample, when performed by an individual on the same equipment, closely following each other. It can be measured, either by analysing six preparations at 100% concentrations, or three samples at 80, 100 and 120% concentrations of theoretical assay values each (Swartz & Krull, 1997:57).



Changing laboratory analysts, the apparatus and by performing testing on different days, form part of intermediate precision. The inconsistency that may occur in the laboratory, thus assessing the precision that can be anticipated inside the laboratory itself, is determined by intermediate precision (Swartz & Krull, 1997:60).

Reproducibility determines the precision, when experimentation is done in completely different institutions (Swartz & Krull, 1997:60).

### **4.3.3 Specificity**

Specificity is the method's capability to distinguish among the desired active compound(s) and supplementary constituents present in the sample. Specificity is the separation between the active compound(s) and additional elements, like impurities, degradants, or excipients. It is important to illustrate the non-interference of a placebo run and if no placebo is available, to do a run by just injecting the solvent (Swartz & Krull, 1997:60-61).

### **4.3.4 Linearity**

Linearity represents the capacity of the method to obtain experimental findings that are precisely comparative to the concentration of the active compound in a certain range. Usually, it is illustrated as the variance of the slope, when assessing the regression line (Swartz & Krull, 1997:65).

### **4.3.5 Range**

Range explores the selection between the upper and lower levels representing minimum specified concentrations of active compound, which has to be determined with precision, accuracy, and linearity using the method. Routinely, it is articulated in similar units as the findings acquired while using the method. ICH guidelines state that a method validation requires a minimum of five concentration levels, as well as definite minimum specified ranges. For the

intention of assay testing, the minimum specified range is 80 - 120% (Swartz & Krull, 1997:65; ICH Topic Q1 (R1), 1995:10).

#### **4.3.6 Ruggedness**

This data element is known to be the “degree of reproducibility of the results obtained under a variety of conditions”, which is then documented as the percentage relative standard deviation (% RSD). Variances in laboratories, i.e. among analysts, equipment, reagents as well as the time passed between each method testing, would be covered by this RSD value (Swartz & Krull, 1997:67).

### **4.4 VERIFICATION OF VALIDATION PARAMETERS**

Since this study was intended for in-house purposes only, this study only verified range, linearity, specificity, precision and accuracy.

#### **4.4.1 Range**

For the objective of this study, a minimum specified range of 50 - 150% of the theoretical assay value was performed, which complied with the acceptance criteria of 70 - 130% of the declared content (Lobrutto & Patel, 2007:461). This led to experimentation being done on preparations with five different concentration levels, with a minimum of 50% and a maximum of 150% of the active ingredient in the sample. In table 4.1 the ranges and amounts of active ingredients of the four active ingredients are summarised.

**TABLE 4.1: The five concentration levels of the range and actual amounts of active ingredients of each preparation tested**

Target Range (%)	CONCENTRATION (mg/ml)			
	Ethambutol	Isoniazid	Pyrazinamide	Rifampicin
50	0.81	0.26	1.47	0.39
75	1.30	0.42	1.76	0.47
100	1.62	0.52	2.20	0.59
125	1.94	0.62	2.64	0.71
150	2.43	0.78	3.30	0.88

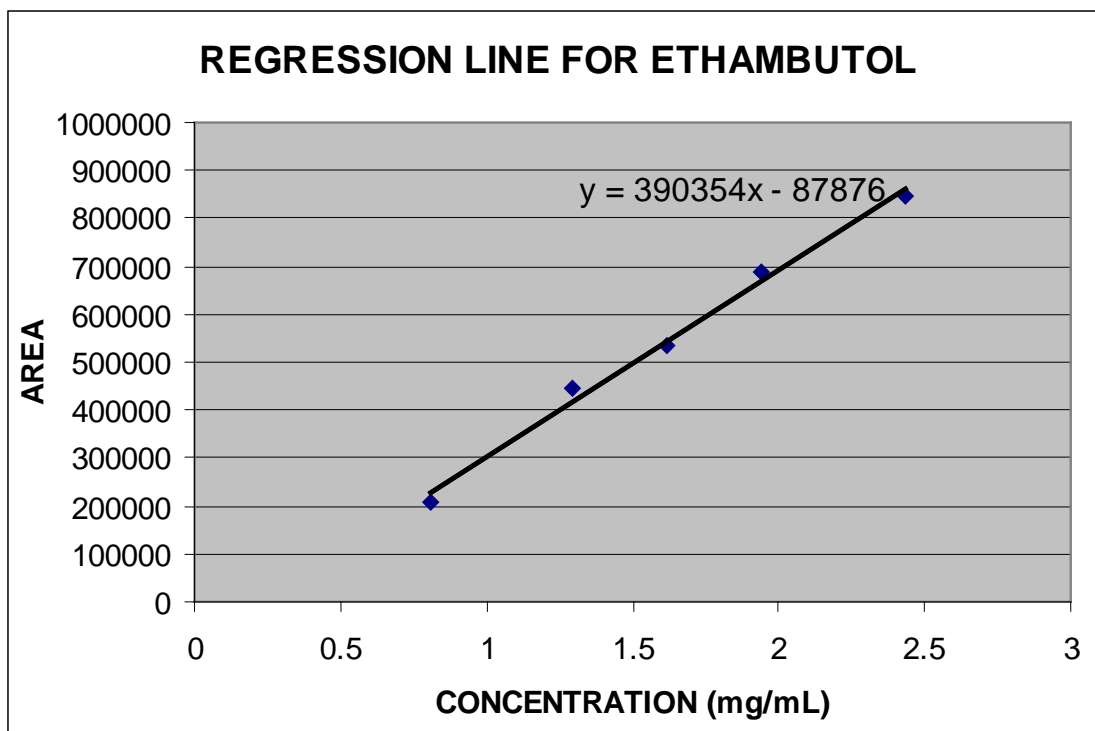
#### 4.4.2 Linearity

The area values used in each range per active ingredient made up a 5-point graph on which linear regression was performed and correlation coefficients were obtained. The acceptance criteria for validation, according to linearity, comprised a correlation coefficient of  $\geq 0.99$ . All the data, as shown in table 4.2, complied (Lobrutto & Patel, 2007:461).

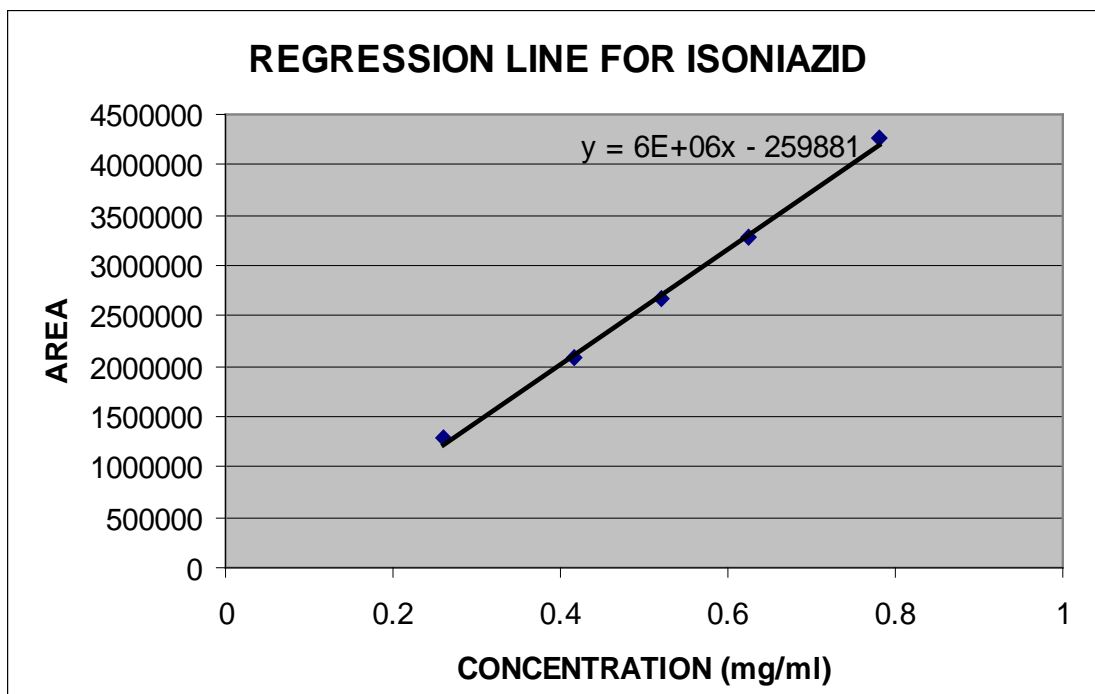
**TABLE 4.2: Correlation coefficients ( $r^2$ ) of the active ingredients after linear regression**

ACTIVE INGREDIENT	CORRELATION COEFFICIENT ( $r^2$ )
Ethambutol	0.99
Isoniazid	1.00
Pyrazinamide	1.00
Rifampicin	0.99

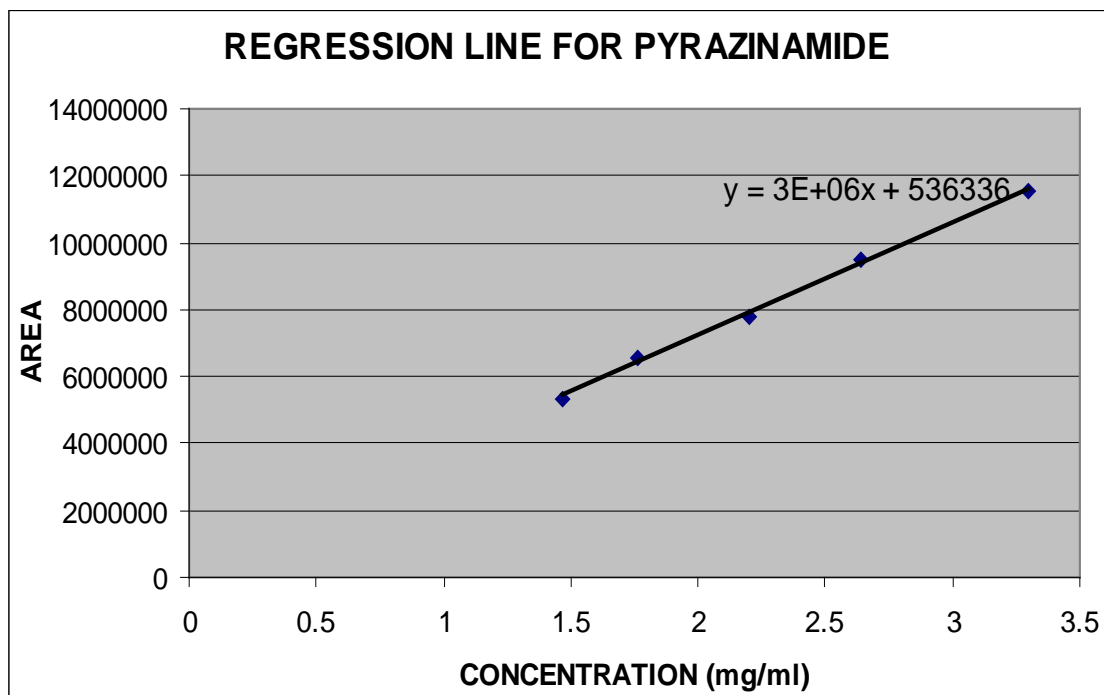
The results are graphically represented in figures 4.1 – 4.4.



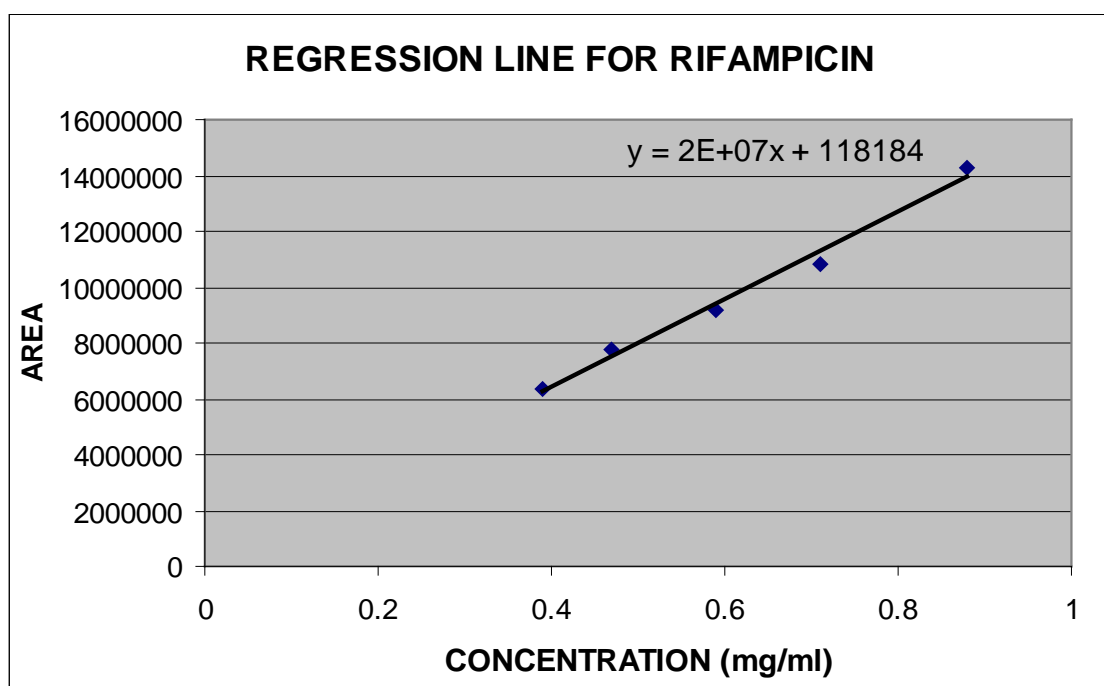
**FIGURE 4.1:** Linear regression graph for ethambutol assay.



**FIGURE 4.2:** Linear regression graph for isoniazid assay.



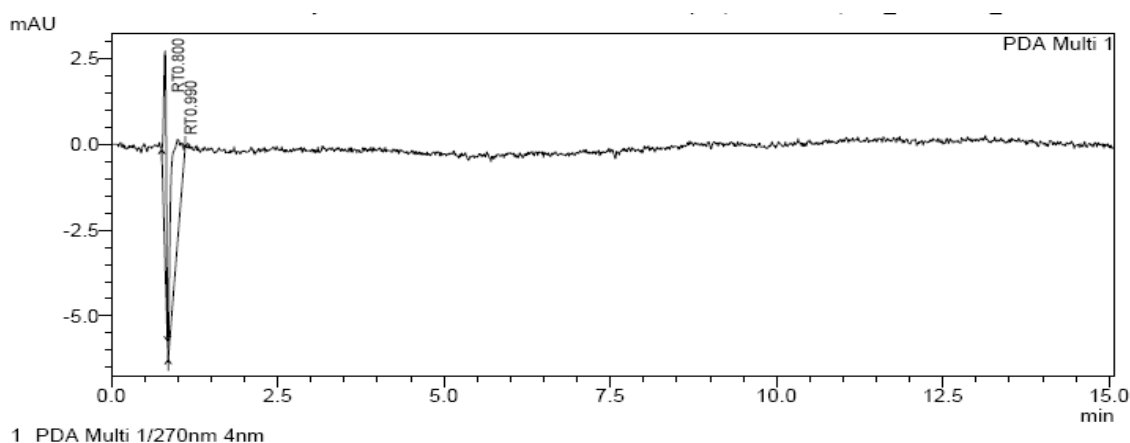
**FIGURE 4.3:** Linear regression graph for pyrazinamide assay.



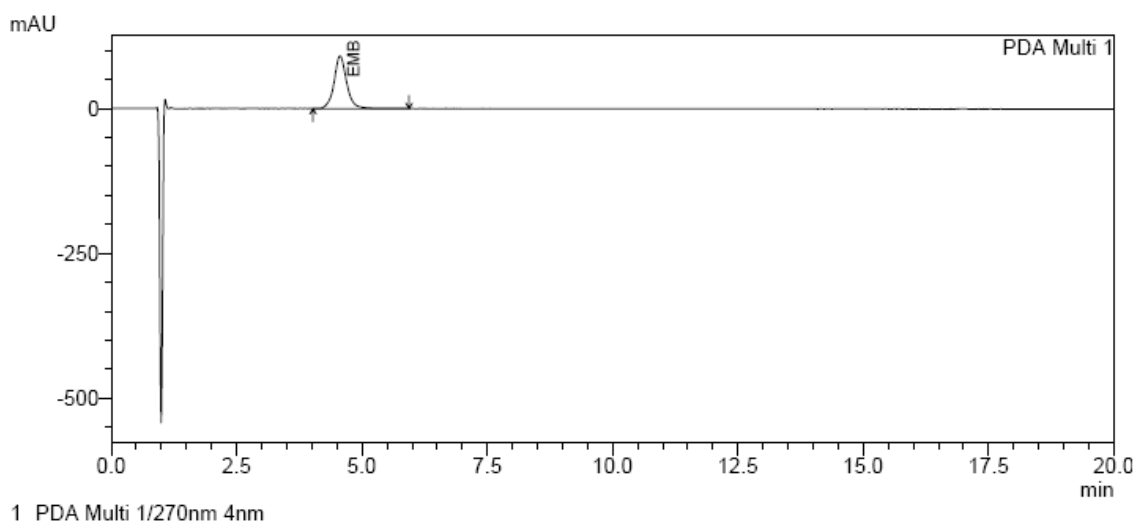
**FIGURE 4.4:** Linear regression graph for rifampicin assay.

### 4.4.3 Specificity

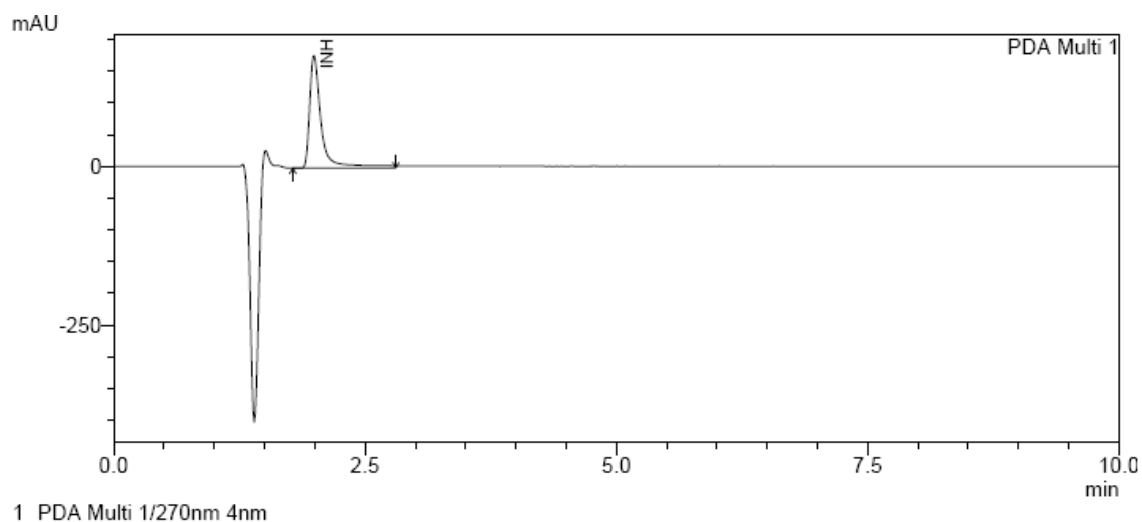
For specificity to be accepted for validation, the peaks should be well separated and not interfere with each other (Lobrutto & Patel, 2007:461). Therefore, chromatograms of the solvents on their own, of each of the active ingredients separately and of both the preparations (with each one including two active ingredients), were examined. Examples of chromatograms obtained are presented in figures 4.5 – 4.12.



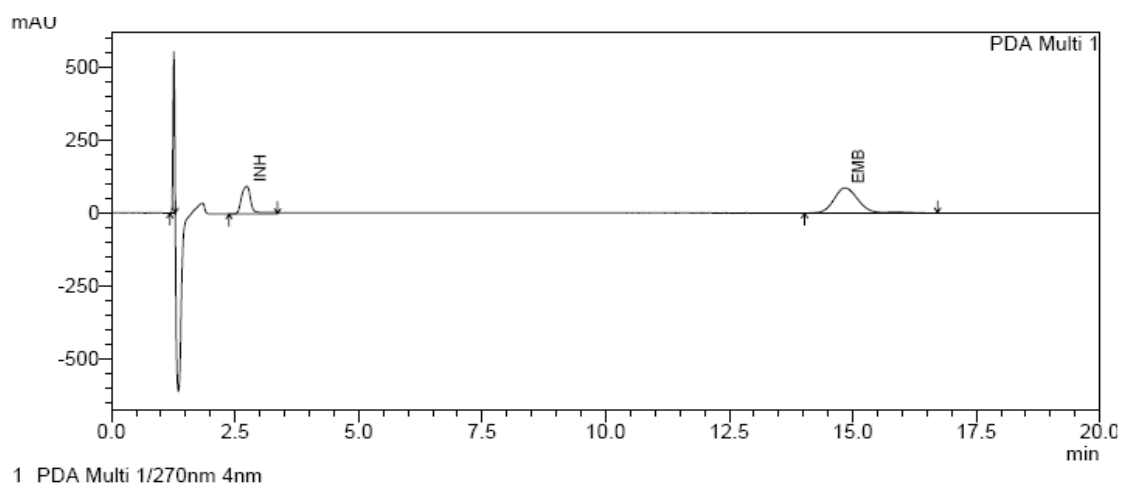
**FIGURE 4.5: Chromatogram with copper mobile phase at 270 nm.**



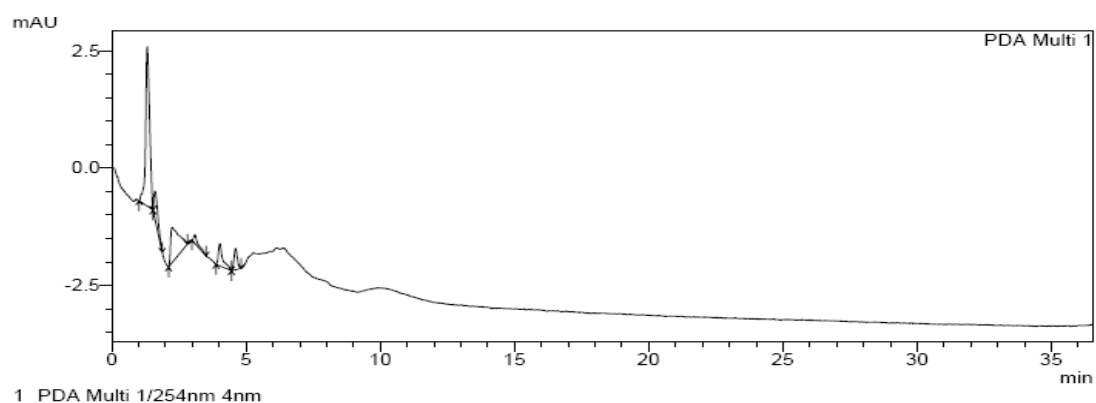
**FIGURE 4.6: Chromatogram for ethambutol at 270 nm.**



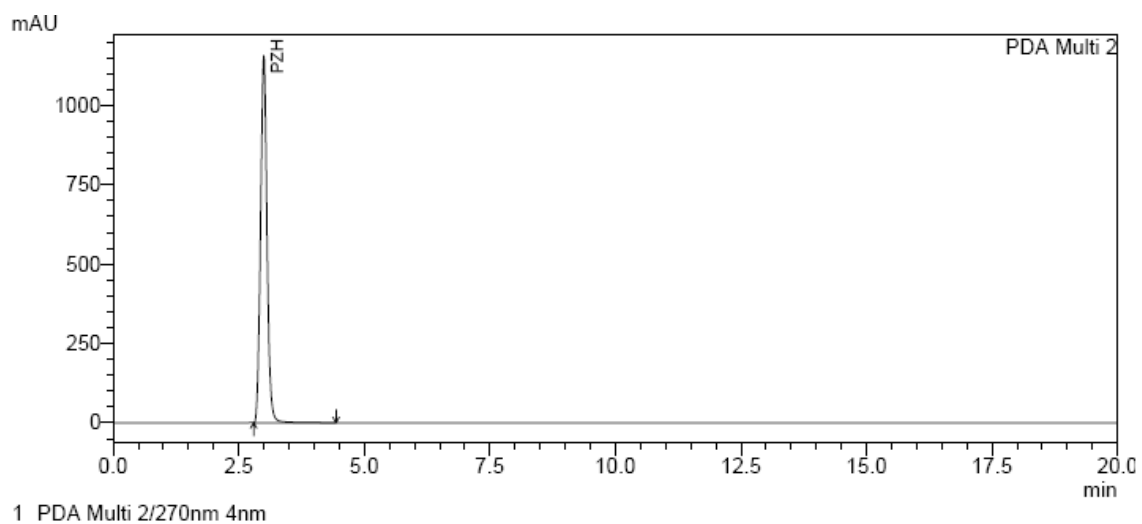
**FIGURE 4.7: Chromatogram for isoniazid at 270 nm.**



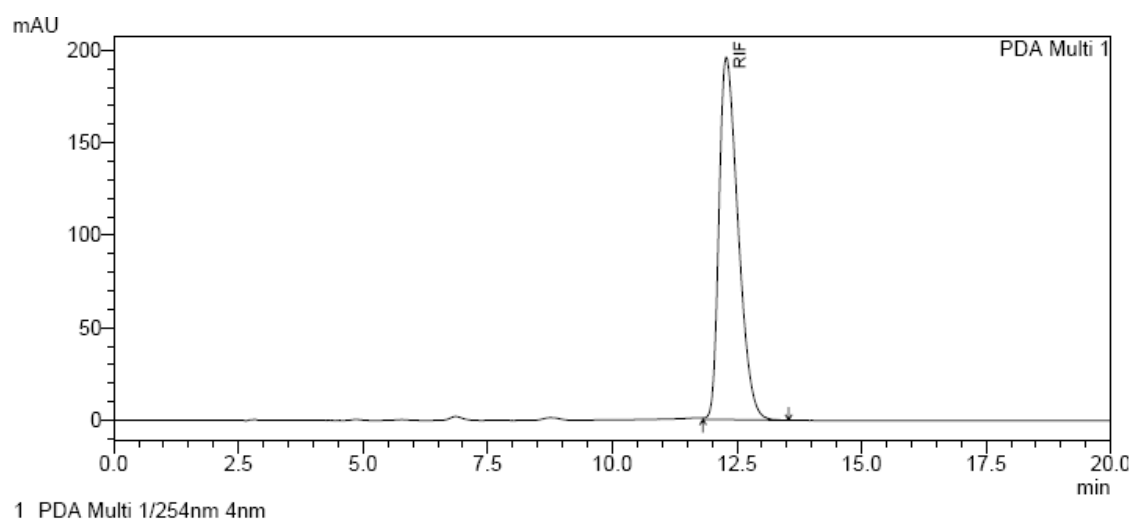
**FIGURE 4.8: Chromatogram with ethambutol and isoniazid at 270 nm.**



**FIGURE 4.9: Chromatogram with phosphate mobile phase at 254 nm.**

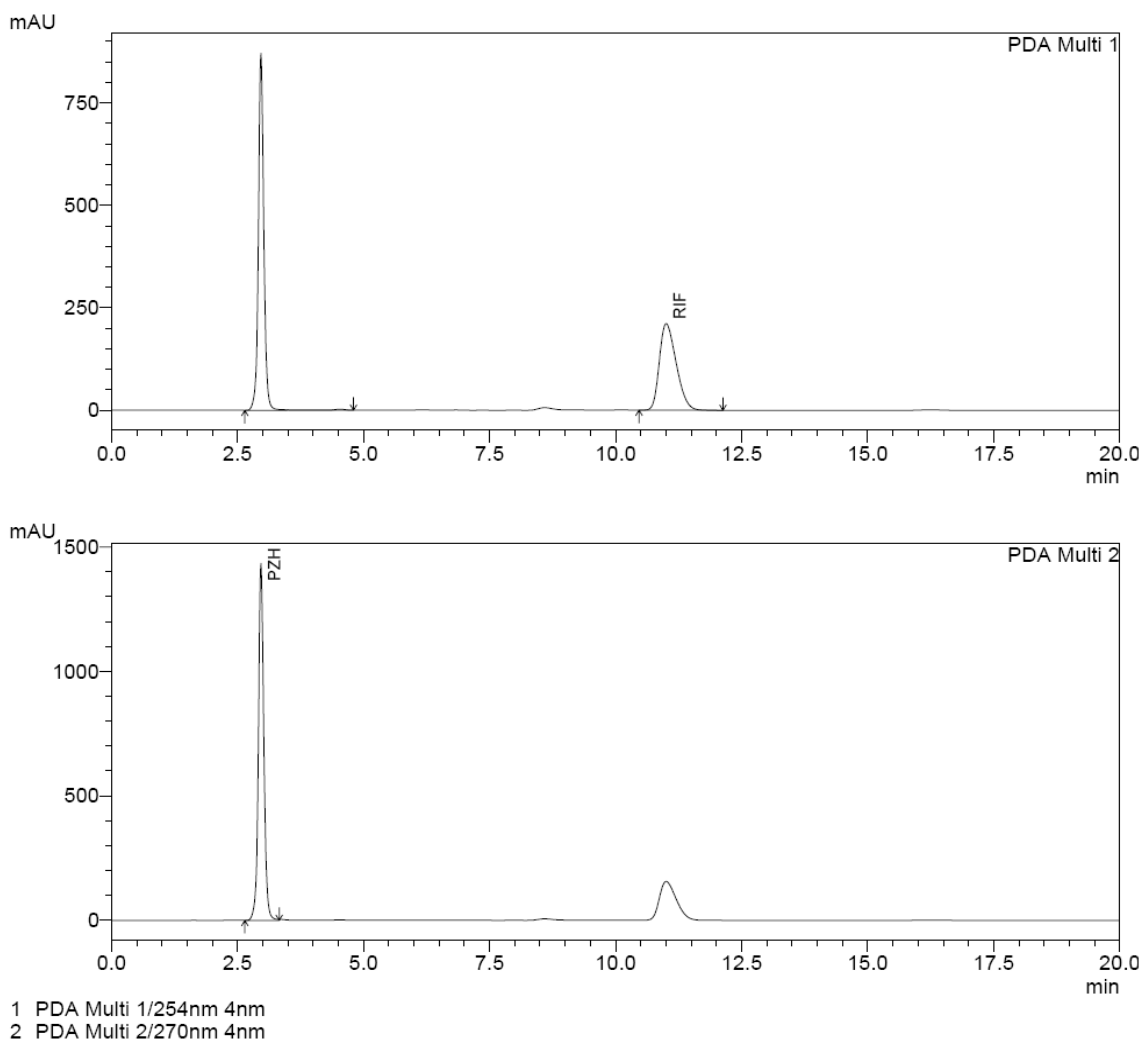


**FIGURE 4.10: Chromatogram for pyrazinamide at 270 nm.**



**FIGURE 4.11: Chromatogram for rifampicin at 254 nm.**





**FIGURE 4.12: Chromatogram for rifampicin (at 254 nm) and pyrazinamide (at 270nm).**

#### 4.4.4 Precision

Acceptable values for precision would be a percentage relative standard deviation (% RSD) of  $\leq 2\%$  (Lobrutto & Patel, 2007:461). For determining precision, a preparation having a 100% concentration was obtained from the manufactured formulation and injected five times and the % RSD of the obtained areas was calculated for each. The results obtained are shown in table 4.3.

**TABLE 4.3: Relative standard deviation values of active ingredients**

ACTIVE INGREDIENT	RELATIVE STANDARD DEVIATION (%)
Ethambutol	3.4
Isoniazid	5.3
Pyrazinamide	3.0
Rifampicin	2.2

The results indicated that repeatability was not achieved as required. Since previous studies indicated interferences from the pro-Pheroid (Kühn, 2008:75-76; Pretorius, 2008:61; Cassim, 2006:145; Van den Berg, 2010:77), it was decided to accept these results.

#### **4.4.5 Accuracy**

According to Lobrutto and Patel (2007:461), the acceptance criteria for accuracy to comply for validation purposes, would be a percentage recovery between 98% - 102%. For this study, control samples, representing a 100% concentration, were prepared and analysed. The concentration per sample was calculated by means of the linear equation obtained during the linearity studies. The recovery percentages are presented in table 4.4.

**TABLE 4.4: % Recovery of the active ingredients**

ACTIVE INGREDIENT	% RECOVERY
Ethambutol	124.1
Isoniazid	97.2
Pyrazinamide	96.4
Rifampicin	87.0

Poor recovery results were obtained for both ethambutol and rifampicin. The nature of the analytical technique for ethambutol was most probably the main reason for the poor results. The instability of rifampicin in solution led to challenges in obtaining proper validation results. Although the recovery values for isoniazid and pyrazinamide were lower than expected, it was accepted for this particular study.

## **4.5 CONCLUSION**

The validation results indicated that the methods were suitable for preliminary studies only.

As this project was for trial formulations only, and since the poor results being obtained were confirmative of those of previous studies on pro-Pheroid formulations, it was decided not to continue with further modification of these methods.

# CHAPTER 5

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## STABILITY TESTING OF FORMULATIONS

### 5.1 INTRODUCTION

Fixed dose combinations of two, three or four active pharmaceutical ingredients are the preferred dosage forms for enhancing better patient compliance in the treatment of tuberculosis. Rifampicin and isoniazid as combination led to poor bioavailability of rifampicin (Mouton, 1979:447). Henwood *et al.* (2000:408) also reported different dissolution rates for rifampicin, due to differences in particle sizes and crystalline forms.

Drug decomposition of rifampicin, isoniazid and pyrazinamide had been investigated under different conditions and the order of decomposition was rifampicin > isoniazid > pyrazinamide (Singh *et al.*, 2001:8). Pyrazinamide is known to be a very stable drug. Unfortunately, very little information is available in the literature regarding the stability of ethambutol.

Stability information of a formulation is required to determine its shelf-life and thus to ensure that its efficacy is consistent up to the expiry date, that the preparation meets its acceptance criteria and remains stable throughout the claimed shelf-life period.

Stability testing is essential for phases I, II and III clinical trials and as a study progresses, it is common for adjustments to be made with regards to the method of preparation and even to the dosage form itself (Grimm, 2007:387).

The aims of stability testing during phases I and II are to assess the stability of the experimental preparations, to acquire supplementary knowledge in order to finalise a preparation and to decide on the most suitable packaging (Grimm, 2007:387).

The more scientific and extensive the stability program, the more trustworthy the results will be. The stability tests during this study were performed on experimental batches only.

In this chapter the outcomes of the performed stability studies are discussed.

## **5.2 REASONS FOR STABILITY TESTING**

### **5.2.1 Ensuring welfare of the patient**

Clinically, stability is not always regarded a necessity. Generally, when the preparation does not degrade into toxic decomposition products, nor is regarded as a narrow therapeutic ratio, it is not expected that patients would experience any harm. Nevertheless, there are documented cases, where instability had resulted in severe clinical effects (Rhodes, 2007:11).

For instance, early in the 1980's, a packaging stability predicament with nitroglycerine tablets regrettably led to some tablets being accessible in the Midwest of USA, with potency values of less than 10% of the label claim. As nitroglycerine is used as an emergency therapy for angina, a very severe cardiac circumstance suspicion arose, in which this batch could have been the cause of deaths at that time. Stability testing is therefore very important in ensuring that the preparation would be safe for use until being used by the patient, or until expiry (Rhodes, 2007:11).

### **5.2.2 Protecting the manufacturer's reputation**

Stability is insurance to the manufacturer that the product would surely preserve suitability for use, with respect to all functionality appropriate features, for as long as it is on the market (Rhodes, 2007:11).

### **5.2.3 Requirements of regulatory agencies**

Depending on where a company is operating, it has to abide to specific requirements / set rules (of which stability would be one) by regulatory authorities, in order for a company to be able to manufacture and market its products (Rhodes, 2007:11).

### **5.2.4 Providing a database for future preparations**

Stability test results can be an advantage when manufacturing similar preparations, and / or when using the same active ingredient, or drug delivery system in the future (Rhodes, 2007:11).

## **5.3 PROBLEMS ATTRIBUTABLE TO INSTABILITY**

- Decrease in concentration of the active;
- Increase in concentration of the active;
- Alteration in bioavailability;
- Loss of content uniformity;
- Decline of microbiological status; and
- Formation of toxic degradation products (Rhodes, 2007:3-7).

All the above factors signify the importance of stability testing of pharmaceutical products in reducing the risk of distributing unsafe, or inefficient medication.

## **5.4 STABILITY PROGRAMME**

Stability testing was performed on four tuberculostatic APIs in pro-Pheroid preparations. The formulations being tested were an ethambutol-isoniazid (Pyriftol IE) combination product, and a pyrazinamide-rifampicin (Pyriftol RP)

combination product, with pro-Pheroid as the drug delivery system in both formulations.

The testing was done in accordance with the International Conference on Harmonisation (ICH) principles. The stability testing of these formulations was carried out over a period of three months, in accordance with the guidelines for the registration of pharmaceuticals for human use (ICH Q1A (R2), 2003:3).

#### **5.4.1 Storage conditions**

Throughout the stability testing period, strictly monitored storage facilities were utilised. The preparations were stored at four different temperature and humidity specifications, in accordance with ICH principles:

- 5°C;
- 25°C + 60% RH;
- 30°C + 65% RH; and
- 40°C + 75% RH.

#### **5.4.2 Stability timetable**

A batch of each of the formulations was manufactured and a test sample of each analysed immediately for initial test results. The remainder of each batch was split evenly to ensure sufficient samples for testing at all the applicable storage conditions.

At months 1, 2 and 3, the samples were removed from the storage chambers and allowed to reach room temperature in the laboratory, before assay testing was performed. The methods, as described in chapter 4, were used for assay testing. Each sample was analysed in triplicate.

### 5.4.3 Assay results

Sediments formed after formulation in both the Pyrifitol IE and the Pyrifitol RP formulations, becoming more evident as the stability period progressed. Samples had to be remixed to obtain homogeneity. The high viscosity, however, caused mixing problems, which may have contributed to some of the poor results being obtained, as well to the variability in the assay results. The acceptance criteria for all the products were taken as 90 – 110%.

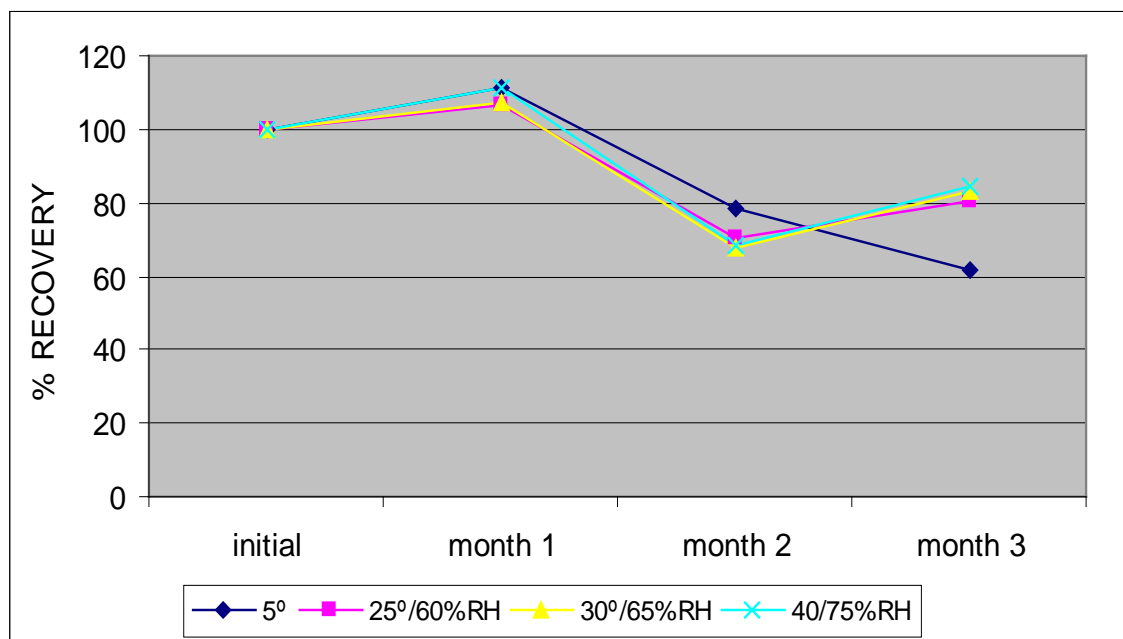
#### 5.4.3.1 Pyrifitol IE (ethambutol-isoniazid in pro-Pheroid)

For Pyrifitol IE, 81.06 g of ethambutol and 25.78 g of isoniazid were used to prepare a 250 g preparation. The assay results are summarised in table 5.1, whereas figures 5.1 and 5.2 represent the % recovery over the duration of the stability period. The percentage recovery was calculated with relation to the theoretical contents of each formulation.

**TABLE 5.1: Percentage recovery of Pyrifitol IE**

ETHAMBUTOL IN PRO-PHEROID				
Initial	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
100.0 (7.6)	5°C	111.6 (4.8)	78.3 (42.0)	61.8 (51.9)
	25°C + 60% RH	106.3 (7.2)	70.5 (11.6)	80.6 (4.3)
	30°C + 65% RH	107.5 (5.6)	68.0 (24.2)	82.9 (3.7)
	40°C + 75% RH	111.2 (3.0)	68.7 (10.9)	84.8 (10.0)
ISONIAZID IN PRO-PHEROID				
Initial	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
85.3 (1.7)	5°C	100.2 (2.3)	116.7 (2.4)	83.6 (5.5)
	25°C + 60% RH	83.7 (5.3)	79.3 (2.3)	76.1 (4.5)
	30°C + 65% RH	84.5 (4.2)	79.6 (1.1)	77.5 (8.5)
	40°C + 75% RH	78.5 (6.2)	79.2 (5.2)	73.6 (6.6)

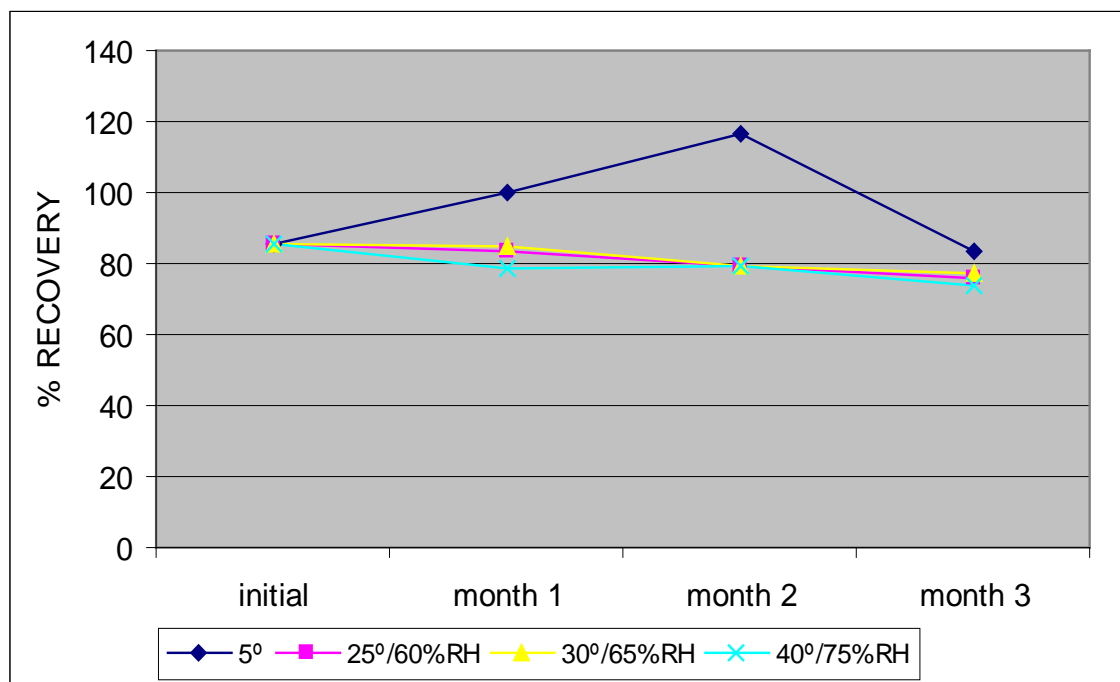




**FIGURE 5.1: % Recovery of ethambutol in pro-Pheroid.**

The initial assay results for ethambutol was 100%, with slightly higher results after one month. The 5°C and 40°C+75% RH results were out of specification, with values higher than 110%. For months 2 and 3, results were significantly lower than the initial results and all the values obtained were out of specification. The analytical technique may have been responsible for the high variability, since a copper reaction was required on the column to determine the ethambutol contents (refer 4.2.1).

The initial assay results for isoniazid was 85.3%. This low initial value could have been due to consistency problems during manufacturing, or because of sedimentation. Throughout the stability studies, the assay values were relatively stable, but all were below the lower acceptance criterion. The results for the 5°C samples were inconsistent throughout the study.



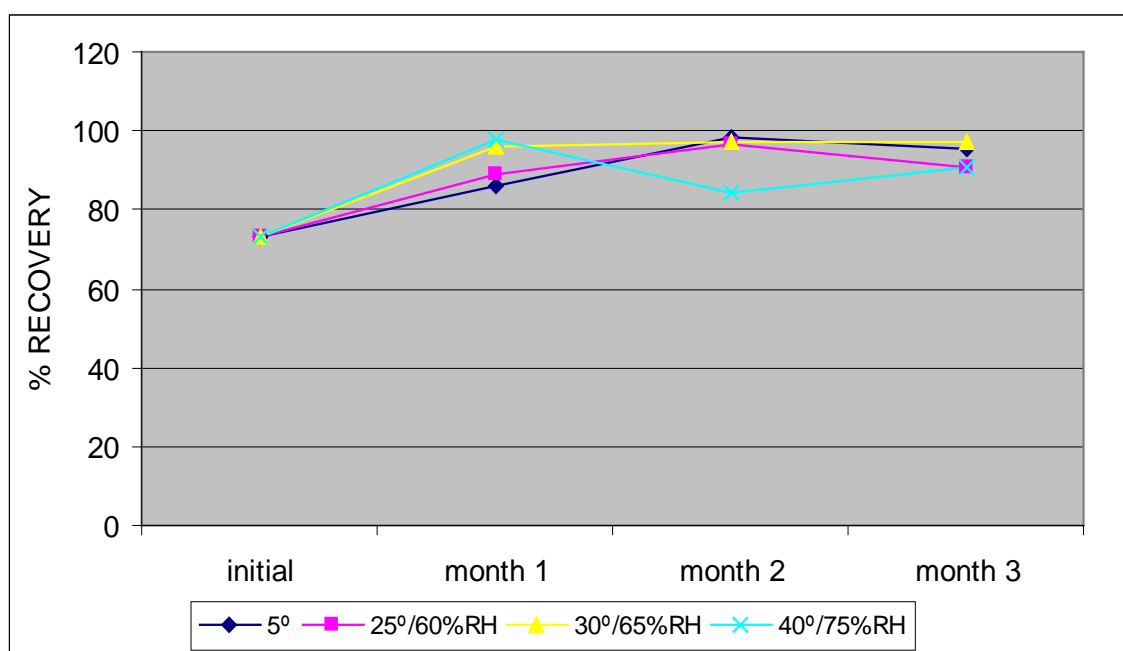
**FIGURE 5.2: % Recovery of isoniazid in pro-Pheroid.**

#### **5.4.3.2 Pyriltol RP (pyrazinamide-rifampicin in pro-Pheroid)**

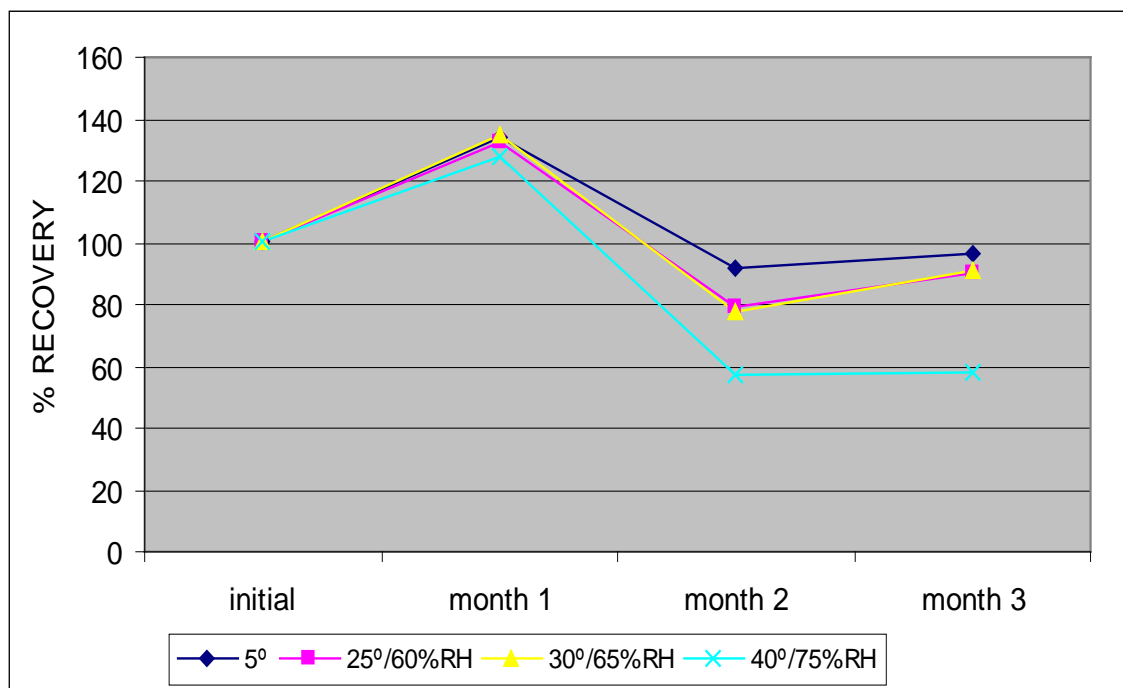
During the manufacturing process of Pyriltol RP, 110.5 g of pyrazinamide, mixed with 29.50 g of rifampicin, were used to prepare 250 g of the formulation. The assay results for pyrazinamide-rifampicin are summarised in table 5.2, with figures 5.3 and 5.4 illustrating the percentage recovery over the duration of the stability period. The percentage recovery was calculated with relation to the theoretical contents of the formulation.

**TABLE 5.2: Percentage recovery of Pyrifitol RP**

PYRAZINAMIDE IN PRO-PHEROID				
Initial	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
73.4 (2.0)	5°C	86.0 (9.2)	98.3 ( 1.0)	95.3 ( 3.7)
	25°C + 60% RH	89.1 (4.9)	96.6 ( 3.3)	90.9 ( 4.6)
	30°C + 65% RH	96.2 (0.8)	97.3 ( 1.6)	97.2 ( 4.2)
	40°C + 75% RH	97.6 (1.6)	84.2 (11.3)	90.5 ( 5.8)
RIFAMPICIN IN PRO-PHEROID				
Initial	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
100.2 (0.6)	5°C	133.9 (6.6)	92.1 ( 2.1)	96.4 ( 4.3)
	25°C + 60% RH	132.9 (3.1)	79.2 ( 3.5)	90.0 ( 4.3)
	30°C + 65% RH	135.1 (1.0)	77.3 ( 4.4)	90.6 ( 5.7)
	40°C + 75% RH	127.6 (5.1)	57.1 ( 5.1)	57.7 (10.0)



**FIGURE 5.3: % Recovery of pyrazinamide in pro-Pheroid.**



**FIGURE 5.4: % Recovery of rifampicin in pro-Pheroid.**

The main problem with analysing Pyrifitol RP was homogeneity, as this sample was very viscous. The homogeneity of the actives in these samples could thus have caused the variable results obtained. Despite the low values obtained for pyrazinamide in the initial assay, the assay values for the four conditions over the three-month period remained stable. Most of the results complied with the specifications.

The initial results for rifampicin were within specification. Highly variable results were, however, obtained for all the other stability intervals and no significant conclusion could be made from the results. The variability of the obtained results could be attributed to the known instability of rifampicin in solution (Seifart *et al.*, 1991:827).

Previous studies utilising pro-Pheroid or Pheroid™ as carrier, have also indicated that high variability was experienced during analyses of the stability samples of the APIs in anti retro viral (ARV) and antimalarial formulations (Cassim, 2006:145; Kühn, 2008:75; Pretorius, 2008:61).

In a study done by Van den Berg (2010:77), the stability of pro-Pheroid without any actives, was evaluated. It was concluded that the Pheroid™ / pro-Pheroid delivery system underwent changes when subjected to stability studies over three months. These changes influenced the HPLC analyses significantly and subsequently led to a decrease in the solubility of the Pheroid™ formulations in the sample solvent (methanol).

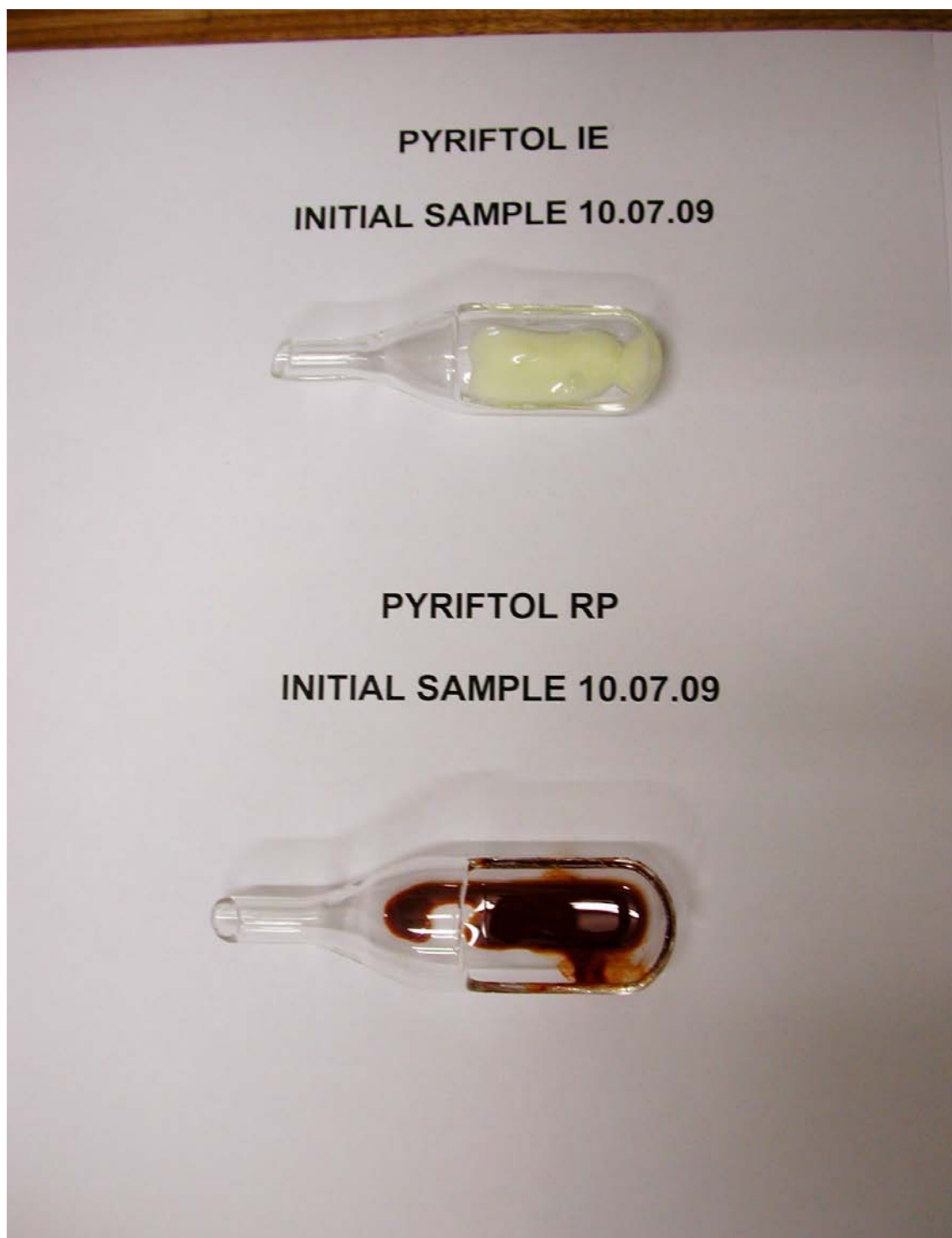
Van den Berg (2010:77) concluded that due to these changes in the chromatography of the Pheroid™ / pro-Pheroid based samples, the HPLC method would have to be adjusted at every testing interval. Such an adjustment would be time consuming when a stability study, comprising a large amount of testing samples, should be analysed. It could also mean that a mini validation would have to be performed every time with a stability take-off. In a research and development context, it is both impractical and costly.

This study was undertaken, before the study of Van den Berg (2010:77) was concluded.

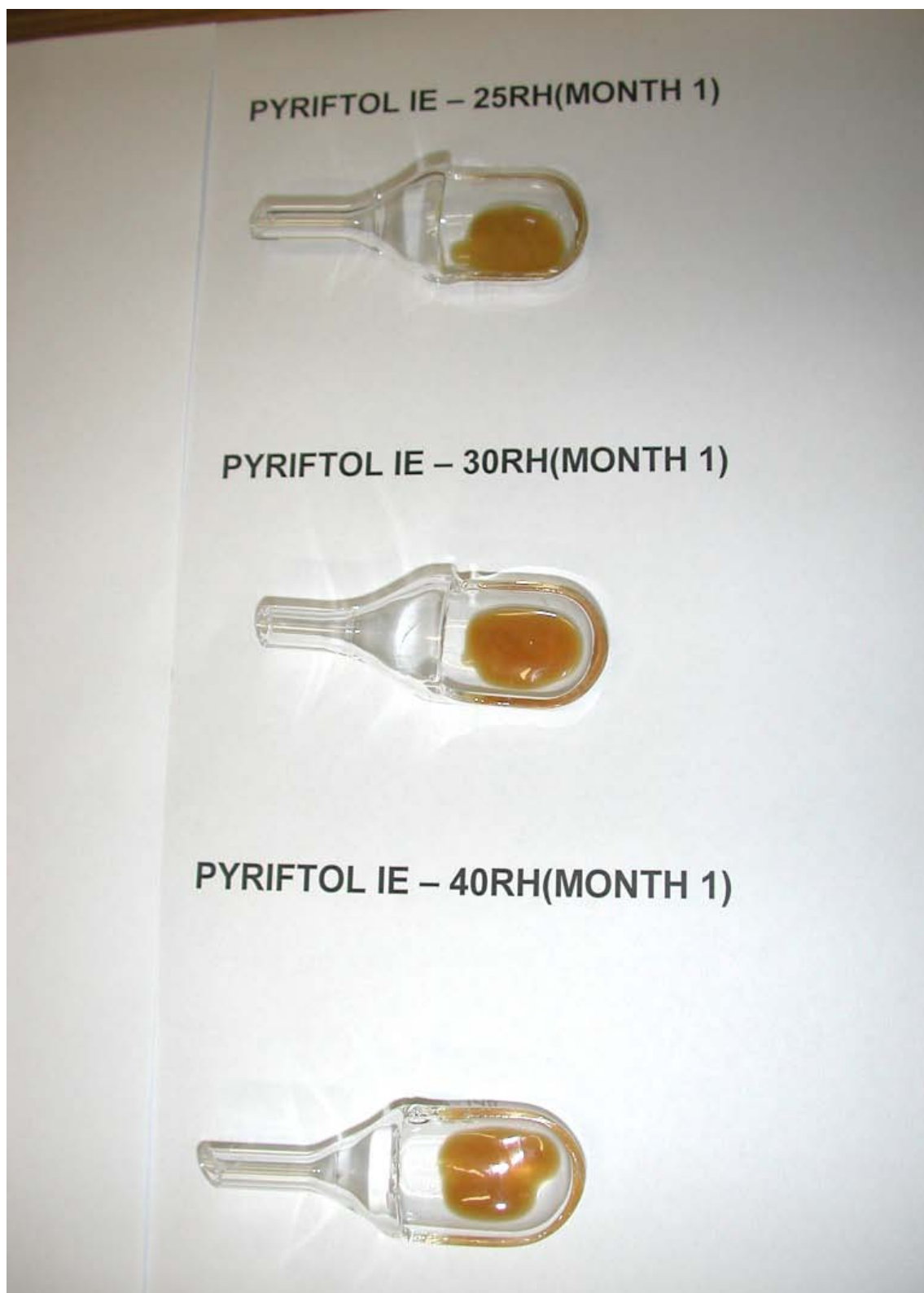
#### **5.4.4 Visual results**

After the first month of the stability study, a change in colour was observed in Pyrifitol IE. Figures 5.5 - 5.8 show the visual changes in the preparation containing isoniazid and ethambutol.

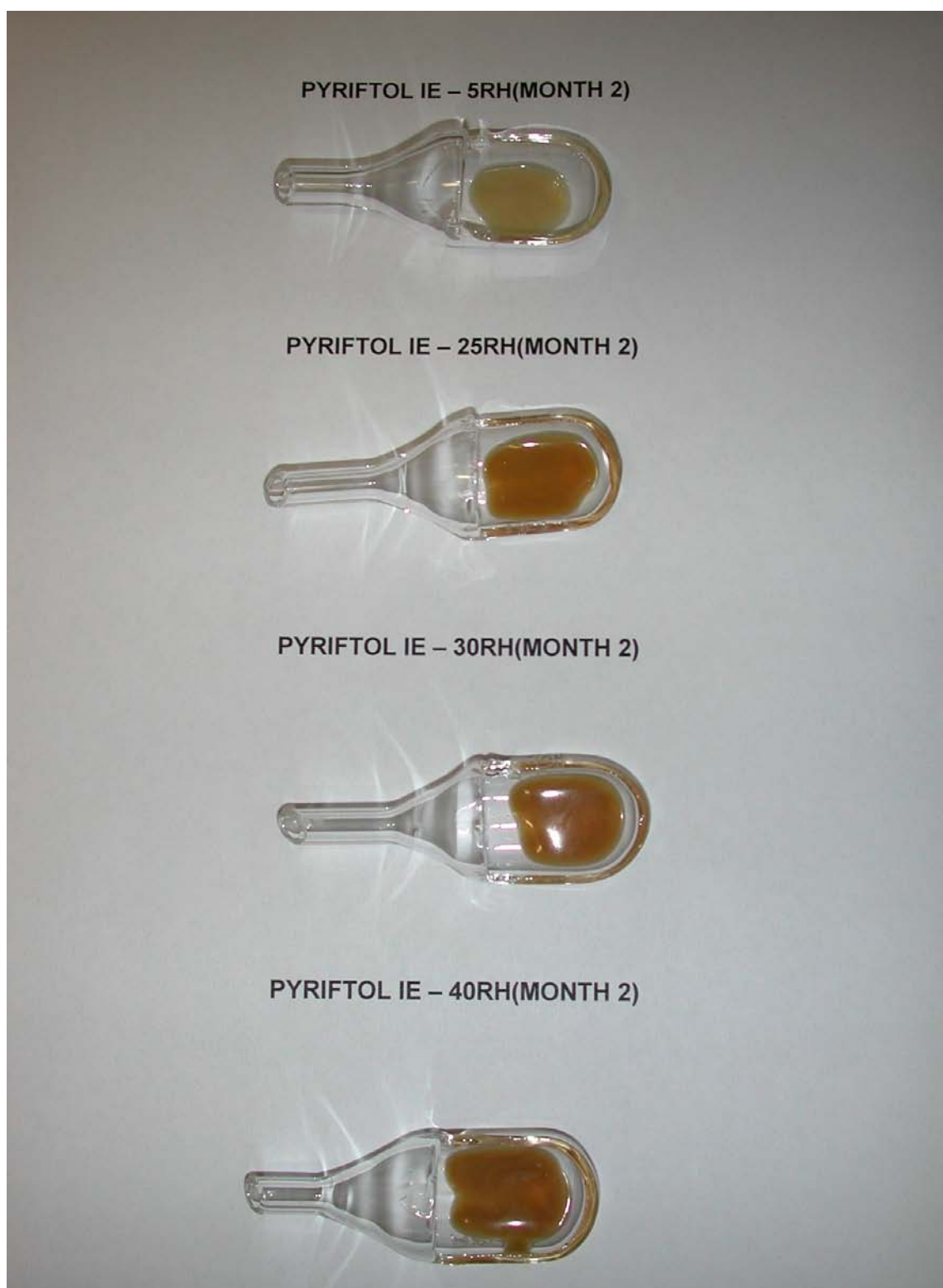
The Pyrifitol RP samples were made dark by the rifampicin, hence colour changes if any, over the three-month stability trial, were difficult to observe.



**FIGURE 5.5: Initial samples of Pyriftol IE and Pyriftol RP.**

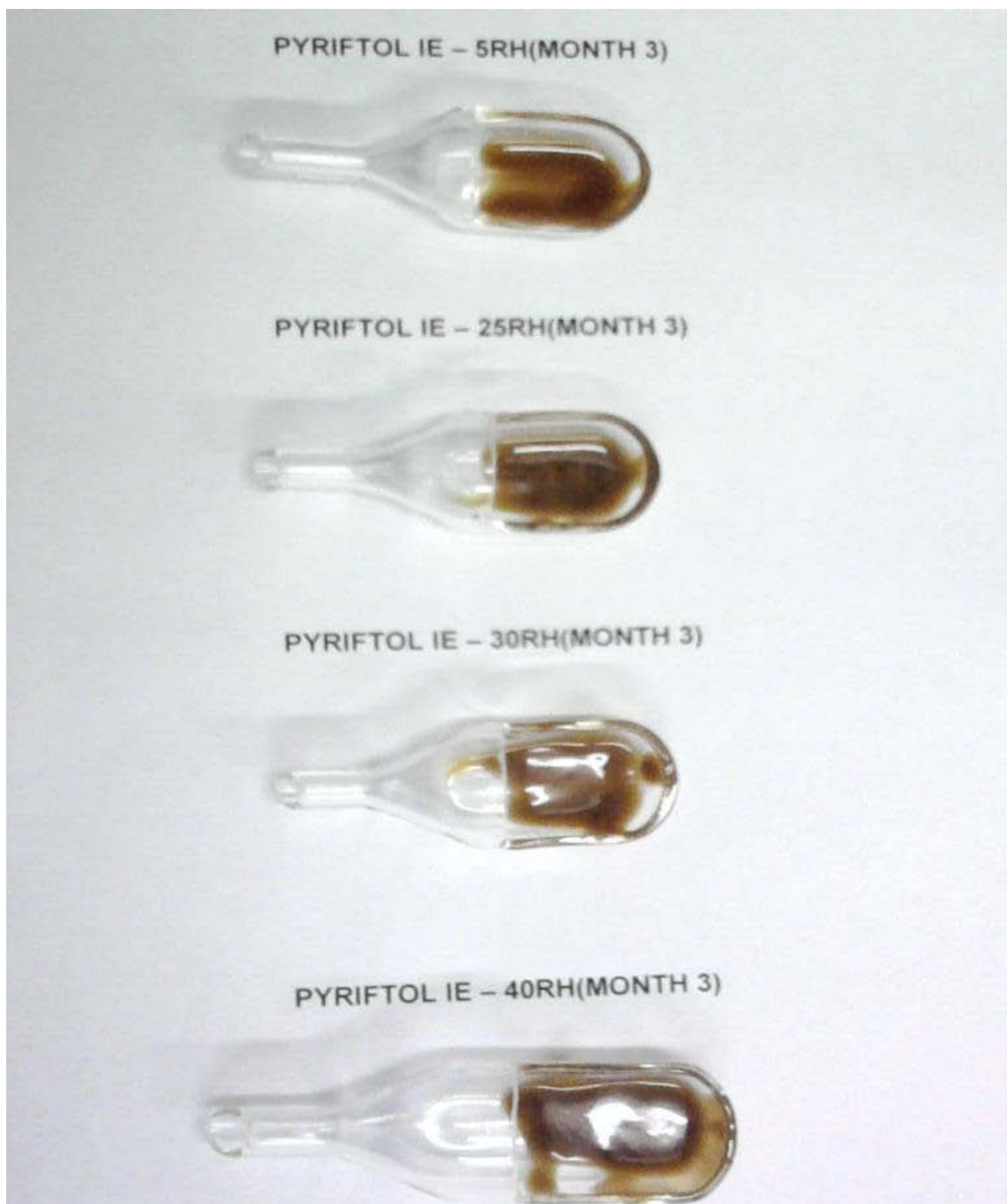


**FIGURE 5.6: Pyrifitol IE samples after one month in the stability storage chambers.**



**FIGURE 5.7: Pyrifitol IE samples after two months in the stability storage chambers.**





**FIGURE 5.8: Pyrifitol IE samples after three months in the stability storage chambers.**

The changes in colour were reported to the trial batch manufacturers. It was not part of this study to determine, nor evaluate the stability of the formulation with regards to visual appearance.

## 5.5 CONCLUSION AND RECOMMENDATIONS

As discussed earlier in paragraph 5.4.3, the assay results for all actives obtained during this study were out of specification. This could have been due to either the degradation of the actives in the formulations over the duration of the stability period, or due to inconsistent sampling, because of the high viscosity of samples, in which sedimentation formed progressively over time.

Isoniazid decomposes, according to Singh *et al.* (2001:8), at a pH of 3.1 to form isonicotinic acid as decomposition product. Under alkaline, as well as aerobic and anaerobic conditions, degradation products are also formed (Singh *et al.*, 2001:8). Isoniazid, however, remained stable throughout this stability study.

Singh *et al.* (2001:8) report that rifampicin decomposes at pH 2 - 3 to form 3-formyl rifamycin. Also, at a pH of 8.2 it decomposes into rifampicin quinone and three other decomposition products. As rifampicin is known to be light sensitive, it should be analysed within two hours from sample preparation (USP, 2010).

Rifampicin did present variable assay results during this study which could be due to the stability issues mentioned earlier in this discussion. Unfortunately, the pH of these formulations was not measured during this study. It would be valuable to see if any degradation took place, and if so, whether it was due to the pH conditions of the pro-Pheroid.

According to the study performed by Singh *et al.* (2001:8), pyrazinamide was found to be stable under wet or dry atmospheres in the solid state, in natural day light and with autoclaving of intravenous infusions. The stability results of this study showed that pyrazinamide was indeed the stable active, despite the low initial value, which could have been due to a formulation, or analytical error.

A stability study done by Kühn (2008:76) on the pro-Pheroid abacavir-efavirenz combination, showed that the stability data was inconclusive, giving inconsistent assay results. From the outcomes of this study, it is also recommended that viscosity and particle size should be optimised to ensure stability of these suspensions. By controlling the viscosity and particle size, the samples would

be more uniform for analysis (Kühn, 2008:76). Cassim (2006:145) also reported inconsistent assay results for the Pheroid™-ARV combinations. For instance, the initial assay results for the nevirapine-Pheroid™ formulation was 104%, followed by 20%, 58% and 17% for months 1, 2, and 3, respectively.

Pretorius (2008:61) did a study during which antimalarials were incorporated within the Pheroid™ and pro-Pheroid systems. Peak interference was again observed in the HPLC analysis, with inconsistent assay values.

All these inconclusive assay results prompted the study that was conducted by Van den Berg (2010:31). The objectives of this particular study were to determine what the contributing factors for the inconsistent results were. Therefore, both the HPLC method performance and Pheroid™ / pro-Pheroid stability were evaluated over a three-month period. Some of the key chromatographic parameters were investigated and this study showed that the Pheroid™ delivery system complicated HPLC analysis. This study, without the API included in the Pheroid™ / pro-Pheroid system, showed that the Pheroid™/ pro-Pheroid delivery system underwent changes when subjected to elevated temperature and humidity, which influenced the HPLC analyses (Van den Berg, 2010:77). Unfortunately, this current study was undertaken before the van den Berg results were published, hence resulting in a repeat of the same findings.

Also, despite the inconsistent HPLC assay values for the Pheroid™ / pro-Pheroid system being reported over the past five years, the TB drugs, in particular rifampicin, also had stability issues when in solution (Singh *et al.*, 2001:16).

This stability study was much more complex, because it had to deal with both the Pheroid™ system and with the anti-tuberculosis actives, which are known to have stability issues of their own.

Five previous studies had confirmed the analytical problems experienced with the Pheroid™ / pro-Pheroid delivery system, irrespective of the pharmaceutical

active ingredients used in the formulations (Kühn, 2008:75-76; Pretorius, 2008:61; Cassim, 2006:145; Van den Berg, 2010:77).

This study further emphasised the analytical and probably the formulation challenges within this delivery system. For future studies to be undertaken, it is advised to prioritise the development of an analytical method suitable of analysing these complex and seemingly unstable Pheroid™ / pro-Pheroid delivery systems.

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## Acknowledgements

- ∞ Prof Wilna, I don't think I would ever be able to thank you enough since you always went above and beyond when I needed assistance. I appreciate it.
- ∞ Anita for all the help and advice. Even though you are generally very busy you always made time, even if it was just to calm me down.
- ∞ Liezl-marié for the preparation of my formulations, without you I would literally not have had sample to analyse.
- ∞ Charlina for storing my samples under the different conditions and making sure the samples were fine when we had record electricity interruptions.
- ∞ Elaine for all the help in the HPLC lab even when I asked your opinion over weekends when you were home.
- ∞ NRF for the financial support throughout this post graduate experience.
- ∞ Julia Handford for making sure my writing is up to standard.
- ∞ All my friends who believed in me and cheered me up after long, stressful days in the lab.
- ∞ Then most importantly, thank you to our heavenly Father who put these wonderful people on my path.

***Our deepest fear is not that we are inadequate.  
Our deepest fear is that we are powerful beyond  
measure....***