Quality specifications for antituberculosis fixed dose combination products

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Abbreviations

AIDS	= ACQUIRED IMMUNE DEFICIENCY SYNDROME
API	= ACTIVE PHARMACEUTICAL INGREDIENT
BCS	= BIOPHARMACEUTICS CLASSIFICATION SYSTEM
BP	= BRITISH PHARMACOPOEIA
CDC	= CENTRE FOR DISEASE CONTROL
ConD.	= CONCENTRATED
df	= DEGREES OF FREEDOM
DOTS	= DIRECTLY OBSERVED THERAPY SHORT-COURSE
F	= F RATIO
FDA	= UNITED STATES FOOD AND DRUG ADMINISTRATION
FDC	= FIXED DOSE COMBINATION
2FDC	= TWO-DRUG FIXED DOSE COMBINATION
3FDC	= THREE-DRUG FIXED DOSE COMBINATION
4FDC	= FOUR-DRUG FIXED DOSE COMBINATION
FPP	= FINISHED PHARMACEUTICAL PRODUCT
HIV	= HUMAN IMMUNODEFICIENCY VIRUS
HCl	= HYDROCHLORIC ACID
HHS	= UNITED STATES DEPARTMENT OF HEALTH AND HUMAN SERVICES
HPLC	= HIGH PRESSURE LIQUID CHROMATOGRAPHY
ICH	= INTERNATIONAL CONFERENCE ON HARMONIZATION
IntPh	= THE INTERNATIONAL PHARMACOPOEIA
LOD	= LIMIT OF DETECTION
LOQ	= LIMIT OF QUANTITATION
MCC	= MEDICINES CONTROL COUNCIL (SOUTH AFRICA)
MDR-TB	= MULTI-DRUG RESISTANT TUBERCULOSIS
nm	= NANOMETER
μ	= MICRO
M/V	= MASS PER VOLUME
MS	= MEAN SQUARES
PhEur	= EUROPEAN PHARMACOPOEIA
R	= REAGENT
Rf	= FLOW DISTANCE OF SAMPLE ÷ FLOW DISTANCE OF MOBILE PHASE

RH	= RELATIVE HUMIDITY
rpm	= REVOLUTIONS PER MINUTE
SADC	= SOUTHERN AFRICAN DEVELOPMENT COMMUNITY
SS	= SUM OF SQUARES
TB	= TUBERCULOSIS
TLC	= THIN-LAYER CHROMATOGRAPHY
TS	= TEST SOLUTION
UN	= UNITED NATIONS
UNAIDS	= JOINT UNITED NATIONS PROGRAMME ON HIV/AIDS
US	= UNITED STATES
USP	= UNITED STATES PHARMACOPEIA
VS	= VOLUMETRIC SOLUTION
V/V	= VOLUME PER VOLUME
WHO	= WORLD HEALTH ORGANIZATION

ABSTRACT

Quality Specifications for Antituberculosis Fixed Dose Combination Products

Objective: The World Health Organization (WHO) requested the Research Institute for Industrial Pharmacy, at the North-West University, Potchefstroom, South Africa, to develop monographs for antituberculosis products for The International Pharmacopoeia (IntPh). These included monographs for rifampicin capsules; rifampicin tablets; isoniazid and ethambutol hydrochloride tablets; rifampicin and isoniazid tablets; rifampicin, isoniazid and pyrazinamide tablets; and rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride tablets.

Background: Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is an airborne disease that mainly affects the lungs. TB is currently causing a global health emergency and is among the principal killers of HIV infected people. First line antituberculosis medicines include the active pharmaceutical ingredients (APIs) rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin. The WHO recommends the use of fixed dose combination products (FDCs) for the treatment of TB and multi-drug resistant TB. In the development of monographs, it is important to consider the safety, efficacy, and in particular the quality requirements for FDC products, which comprise stability, assay and identification testing, as well as the determination of degradation products / related substances. Comprehensive guidelines on FDCs, as published by the WHO, were applied in this study.

Chemical and physical compatibility of APIs in an FDC, the degradability of each API under conditions of stress when in combination with other APIs, as well as API-excipient incompatibility, are important considerations for FDCs and their specifications. The reaction between isoniazid and rifampicin to form 3-(isonicotinoyl-hydrazinomethyl)rifamycin (commonly known as the isonicotinyl hydrazone, or rif-iso hydrazone) is particularly important, as this could result in a loss of therapeutic activity of rifampicin, that could lead to reduced bioavailability, poor patient compliance and subsequently failure to cure TB.

Experimental procedure: Methods were developed for identity, assay and related substances testing.

Although not requested by the WHO, it was decided to develop a dissolution test for isoniazid and ethambutol hydrochloride tablets, for which the WHO had supplied a number of different brands of products. The aims were to compare the dissolution profiles being generated during this study with those presented in the relevant WHO draft guideline (Chapter 4), and to establish whether the recommended dissolution conditions and specifications were appropriate for control of these FDC products.

General requirements set for HPLC assay methods were: one method for more than one API, easily available columns, the use of an isocratic profile, stability-indicating methods, and the possibility of determining related substances using the same assay method(s).

HPLC methods from available literature were identified, screened and the best alternatives selected for further development. Method development and validation were initially performed on the 4FDC tablets, then adjusted for lower API combinations and eventually for Rifampicin tablets and capsules. The assay method for determining rifampicin was also used for testing rifampicin degradation products.

An infrared spectroscopic method was developed as identity test for Rifampicin tablets and capsules. As an alternative for identifying rifampicin in Rifampicin tablets and capsules a colour -, a TLC - and an HPLC test (based on correlating retention times of Rifampicin in the sample and standard solutions from the assay test results) were developed. For the FDC products, two identity tests (HPLC and TLC) were developed.

Results and discussion: 4FDC products were successfully analysed by employing two methods, i.e. one for analysing ethambutol hydrochloride, isoniazid and pyrazinamide, and a second for analysing rifampicin and its related substances. Ethambutol hydrochloride does not absorb UV light; however, it forms a complex with cupric ions, with maximum absorption at 270 nm. By including Cu(II) acetate in the mobile phase, ethambutol hydrochloride was successfully quantified by UV detection. For the assay of FDC products not containing ethambutol hydrochloride, Cu(II) acetate was omitted from the mobile phase. The validation outcomes confirmed the suitability of these methods.

The mobile phase of the TLC test for the identification of APIs in FDC products, consisted of methanol:strong ammonia solution (100:1.5). The TLC plate could be inspected under UV light (254 nm) after exposing it to iodine vapours. In the absence of UV light (254 nm),

the pyrazinamide spot was only vaguely visible when inspected in daylight after iodine exposure. The spot-intensity of isoniazid, pyrazinamide and ethambutol hydrochloride is concentration dependent and can be used for semi-quantitative determination of these APIs in antituberculosis FDCs.

The dissolution study was conducted according to the conditions as set out in a draft WHO guideline for products containing BCS (Biopharmaceutics Classification System) defined, highly soluble APIs. The study and acceptance criterion were accepted by the WHO and included in the monograph for isoniazid and ethambutol hydrochloride tablets. Disintegration has been included as an alternative to dissolution, with the acceptance criterion based on a correlation between the dissolution profiles and disintegration times, as had been determined for products being supplied by the WHO.

Conclusion: The monographs were compiled according to the format, as required by the WHO, and submitted to the WHO. The monographs were distributed for review within the WHO advisory channels and discussed at WHO consultation and expert committee meetings. Feed-back was considered and the monographs updated accordingly. The monographs were approved for The International Pharmacopoeia during a meeting of the 40th WHO Expert Committee on Specifications for Pharmaceutical Preparations. The monographs have been posted on the WHO website and are to be included in the fourth edition of The International Pharmacopoeia.

OPSOMMING

Kwaliteitspesifikasies vir Vastedosiskombinasieprodukte vir tuberkulose

Doel: Die Wêreldgesondheidsorganisasie (WGO) het die Navorsingsinstituut vir Industriële Farmasie, aan die Noordwes-Universiteit, Potchefstroom, Suid-Afrika, genader om monografieë vir anti-tuberkuloseprodukte te ontwikkel vir Die Internasionale Farmakopie. Dit het monografieë vir rifampisienkapsules; rifampisientablette; isoniasied en etambutolhidrochloriedtablette; rifampisien en isoniasiedtablette; rifampisien, isoniasied en pirasienamiedtablette; en rifampisien, isoniasied, pirasienamied en etambutol-hidrochloriedtablette, behels.

Agtergrond: Tuberkulose (TB), veroorsaak deur *Mycobacterium tuberculosis,* word deur die lug versprei en tas hoofsaaklik die longe aan. TB skep tans 'n wêreldwye gesondheidskrisis en is een van die hoofoorsake van sterftes onder MIV-geïnfekteerde persone. Huidig bestaan die eerstelinie anti-tuberkulosemedisyne uit die aktiewe farmaseutiese bestanddele, naamlik rifampisien, isoniasied, pirasienamied, etambutol en streptomisien. Die WGO beveel die gebruik van vastedosiskombinasieprodukte aan vir die behandeling van TB en multi-geneesmiddelweerstandbiedende TB. Vir die ontwikkeling van monografieë is dit belangrik om die veiligheids-, effektiwiteits-, en in die besonder die kwaliteitsvereistes vir vastedosiskombinasies in ag te neem, wat stabiliteits-, inhouds- en identifikasietoetse insluit, sowel as die bepaling van afbraakprodukte / verwante stowwe. Omvattende riglyne rakende vastedosiskombinasies wat deur die WGO gepubliseer is, is in hierdie studie toegepas.

Die chemiese en fisiese verenigbaarheid van die aktiewe farmaseutiese bestanddele in 'n vastedosiskombinasie; die mate van afbraak van elke aktiewe bestanddeel in die teenwoordigheid van 'n ander onder streskondisies sowel as onverenigbaarheid tussen die aktiewe bestanddeel en hulpstof, is belangrike oorwegings by vastedosiskombinasies en hul spesifikasies. Die reaksie tussen isoniasied en rifampisien om

3-(isonikotinoïelhidrasinometiel)rifamisien (algemeen bekend as die isonikotinielhidrasoon of rif-isohidrasoon) te vorm, is veral van belang aangesien dit tot 'n verlies van terapeutiese aktiwiteit van rifampisien kan lei, wat op sy beurt lei tot laer biobeskikbaarheid en tot swak pasiëntmeewerkendheid kan aanleiding gee en gevolglik die onvermoë om TB te genees.

Eksperimentele prosedure: Metodes is vir die uitvoer van identiteitstoetse, inhoudsbepalings en die bepaling van afbraakprodukte / verwante stowwe ontwikkel.

Alhoewel nie deur die WGO versoek nie, is besluit om 'n dissolusietoets vir isoniasied en etambutolhidrochloriedtablette, waarvoor die WGO 'n aantal verskillende handelsprodukte verskaf het, te ontwikkel. Die doelstellings was om die dissolusieprofiele wat tydens hierdie studie gegenereer is met daardie in die relevante WGO konsepriglyn (Hoofstuk 4) te vergelyk en om vas te stel of die voorgestelde dissolusiekondisies en –spesifikasies geskik was vir die beheer van hierdie vastedosiskombinasieprodukte.

Algemene vereistes wat aan HDVC-inhoudstoetse (HDVC = Hoë Druk Vloeistof Chromatografie) gestel is het ingesluit: een metode vir meer as een aktiewe bestanddeel, maklik verkrygbare kolomme, die gebruik van 'n isokratiese profiel, stabiliteitsaanduidende metodes en die moontlikheid om afbraakprodukte / verwante stowwe met behulp van die inhoudsbepalingstoets(e) te bepaal.

HDVC-metodes is uit beskikbare literatuur geïdentifiseer, gereduseer en die beste moontlikhede geselekteer vir verdere ontwikkeling. Metode-ontwikkeling en -validasie is aanvanklik op 4FDC-tablette (FDC = vastedosiskombinasie) uitgevoer, wat daarna eers na laer kombinasies en uiteindelik na rifampisientablette en –kapsules herlei is. Die inhoudstoets vir die bepaling van rifampisien is ook vir die toetsing van afbraakprodukte van rifampisien gebruik.

'n Infrarooispektroskopiese metode is as identiteitstoets vir rifampisientablette en –kapsules ontwikkel. 'n Kleurtoets, 'n dunlaagtoets en 'n HDVC-toets (gebaseer op ooreenstemmende retensietye van rifampisien in die monster- en standaardoplossings van die inhoudstoetsresultate) is ontwikkel as alternatief om rifampisien in rifampisientablette en -kapsules te identifiseer. HDVC- en dunlaagtoetse is as identiteitstoetse vir vastedosiskombinasieprodukte ontwikkel.

Resultate en Gevolgtrekking: 4FDC-produkte is suksesvol analiseer deur die gebruik van twee metodes, naamlik een vir die analise van etambutolhidrochloried, isoniasied en

pirasienamied, en 'n tweede vir die analise van rifampisien en sy afbraakprodukte. Etambutolhidrochloried absorbeer nie UV-lig nie, maar dit vorm wel 'n kompleks, met maksimum absorpsie by 270 nm met kupri-ione. Deur koper(II)asetaat by die mobiele fase te voeg, kon etambutolhidrochloried suksesvol deur UV-deteksie gekwantifiseer word. Vir die inhoudsbepaling van vastedosiskombinasieprodukte wat nie etambutolhidrochloried bevat nie, is koper(II)asetaat uit die mobiele fase weggelaat. Suksesvolle validasie het die geskiktheid van die metodes bevestig.

Die dunlaagtoets vir die identifikasie van aktiewe bestanddele in vastedosiskombinasieprodukte se mobiele fase het bestaan uit metanol:sterk ammoniakoplossing (100:1.5). Die dunlaagplaat kon in UV-lig (254 nm) ondersoek word nadat die plaat aan jodiumdampe blootgestel is. In die afwesigheid van UV-lig (254 nm) was die pirasienamied-kol dofweg in daglig sigbaar na blootstelling aan jodiumdampe. Die kolintensiteit van isoniasied, pirasienamied en etambutolhidrochloried is konsentrasie-afhanklik en kan vir die semi-kwantitatiewe bepaling van hierdie aktiewe bestanddele in vastedosiskombinasies vir tuberkulose gebruik word.

Die dissolusiestudie is uitgevoer in ooreenstemming met die kondisies soos voorgeskryf in 'n konsepdokument van die WGO met riglyne vir produkte wat BCS-gedefinieerde hoogs oplosbare aktiewe bestanddele bevat (BCS = biofarmaseutiese klassifikasiesisteem). Die studie en die aanvaarbaarheidsgrens vir die dissolusietoets is deur die WGO aanvaar en in die monografie vir isoniasied en etambutolhidrochloriedtablette ingesluit. Disintegrasie is ingesluit as 'n alternatief vir dissolusie; die aanvaarbaarheidsgrens is gebaseer op die ooreenstemming tussen die dissolusieprofiele en disintegrasietye soos bepaal vir produkte wat deur die WGO verskaf is.

Gevolgtrekking: Die monografieë in ooreenstemming met die vereiste formaat is opgestel en aan die WGO voorgelê. Die monografieë is vir hersiening binne die WGO se adviserende kanale versprei en is by vergaderings van die WGO se konsultasie- en deskundige komitees bespreek. Terugvoer is oorweeg en die monografieë is dienooreenkomstig aangepas. Die monografieë is tydens 'n vergadering van die 40ste WGO-vergadering van die "Expert Committee on Specifications for Pharmaceutical Preparations" vir opname in Die Internasionale Farmakopie goedgekeur. Die monografieë is op die WGO se webbladsy bekendgemaak en sal in die vierde uitgawe van Die Internasionale Farmakopie ingesluit word.

BACKGROUND

The World Health Organization (WHO) is the United Nations' specialised agency for health and was established on 7 April, 1948 (WHO, 2005a).

The WHO Expert Committee on Specifications for Pharmaceutical Preparations was originally created to prepare The International Pharmacopoeia. Contrary to other pharmacopoeias, priority is given to active pharmaceutical ingredients (APIs) and products included in the WHO Model List of Essential Drugs. The quality control specifications published in The International Pharmacopoeia are developed in an independent manner *via* an international consultative procedure (WHO, 2003a:22).

The ultimate goals are the promotion of good quality pharmaceutical products, and the establishment of quality control methods to detect counterfeit drugs as to assure the safety and efficacy of medical treatments worldwide. This is also crucial for promoting successful programmes for eradication or control of diseases and to avoid the emergence of drug resistance. The development of pharmacopoeial monographs for therapeutic agents, including combination products, to treat tuberculosis, malaria and AIDS, currently receives highest priority (WHO, 2003a:23).

WHO requested the Research Institute for Industrial Pharmacy, at the North-West University, Potchefstroom, South Africa, to develop monographs for antituberculosis products, including FDCs, for The International Pharmacopoeia. The required monographs were for:

* Rifampicin capsules;
* Rifampicin tablets;
* 2FDC tablets containing isoniazid and ethambutol hydrochloride;
* 2FDC tablets containing rifampicin and isoniazid;
* 3FDC tablets containing rifampicin, isoniazid and pyrazinamide; and
* 4FDC tablets containing rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride.

A specific format is required in the development of a monograph for tablets or capsules for The International Pharmacopoeia. Typically a monograph for tablets will include the following (IntPh, 2003:170):

* Category;
* Storage;
* Additional information; and
* Requirements;
    - General
    - Definition
    - Identity tests
    - Assay
    - Related substances
    - Dissolution test.

For the products listed above, methods were developed for:

* Identity tests (the term used by the IntPh for identification tests);
* Assay; and
* Related substances.

Identification testing should establish the identity of the API(s) in the new drug product and should be able to discriminate between compounds of closely related structure which are likely to be present. Identity tests should be specific for the API, e.g. infrared spectroscopy (ICH,1999:7).

General requirements that were established for HPLC methods for assay and the determination of related substances were:

* If possible one method should be applicable for the assay of more than one API;
* The column(s) should be easily available;
* The use of an isocratic profile instead of a gradient profile, so that the test could be readily carried out by any test laboratory;

* The method(s) should be stability-indicating; and
* It would be advantageous if the related substances could be determined with the same method(s) used for assay.

WHO did not request the development of dissolution tests for any of these products. However, it was decided to attempt developing a dissolution test for isoniazid and ethambutol hydrochloride tablets, for which WHO had supplied a number of different brands of products. The aims were to compare the dissolution profiles being generated during this study with those presented in the relevant WHO draft guideline (Chapter 4), and to establish whether the recommended dissolution conditions and specifications were appropriate for control of these FDC products.

STUDY OBJECTIVES

The main purpose of the study was the development of pharmacopoeial monographs for antituberculosis therapeutic agents for The International Pharmacopoeia. These include monographs for the following antituberculosis products and fixed dose combinations:

* Rifampicin capsules (150 mg);
* Rifampicin tablets (150 / 300 mg);
* Isoniazid and ethambutol hydrochloride tablets (150 / 400 mg);
* Rifampicin and isoniazid tablets (300 / 150 mg);
* Rifampicin, isoniazid and pyrazinamide tablets (150 / 75 / 400 mg); and
* Rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride tablets (150 / 75 / 400 / 275 mg).

The following study objectives hence included:

* The identification of fixed dose combination products, and their dosages, currently being used in the treatment of tuberculosis;
* Establishing the required format of product monographs as prescribed by the IntPh;
* Developing identity tests for the different product types;
* Developing and validating HPLC methods for assay and related substances for the different product types;

* Developing a dissolution test for isoniazid and ethambutol hydrochloride tablets; and
* Constructing pharmacopoeial monographs for antituberculosis products for The International Pharmacopoeia.

CHAPTER 1

Tuberculosis

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. The World Health Organization declared tuberculosis (TB) a global health emergency in 1993, the first time it had ever made such a declaration for any disease. According to Reichman and Hopkins Tanne (2001), however, every TB death is unnecessary, since the disease is preventable and curable. When treated with appropriate antibiotics, more than 90 percent of TB patients can be cured.

1.1. THE HISTORY OF TUBERCULOSIS

Unless otherwise stated, the information in this paragraph has been obtained from Sarrel (2006).

According to the Global Tuberculosis Institute (1996), *Mycobacterium tuberculosis* has been present in the human population since ancient times. Fragments of the spinal columns from Egyptian mummies from 2 400 BC show definite pathological signs of tubercular decay.

The earliest references to the infectious nature of the disease appear in seventeenth century Italian medical literature. An announcement issued by the Republic of Lucca in 1699 states: "Human health should no longer be endangered by objects remaining after the death of a consumptive. The names of the deceased should be reported to the authorities, and measures undertaken for disinfection".

The introduction of the sanatorium cure provided the first real step against TB. Hermann Brehmer, a Silesian botany student suffering from TB, was instructed by his doctor to seek out a healthier climate. He travelled to the Himalayan Mountains where he could continue his botanical studies while trying to free himself of the disease. He returned home cured and began to study medicine. In 1854, he presented his doctoral thesis "Tuberculosis is a curable disease". In the same year, he built an institution in Gorbersdorf where patients were exposed on their balconies to continuous fresh air. They also received high-quality nutrition. This setup became the blueprint for the subsequent development of sanatoria, a powerful weapon in the battle against a dangerous opponent.

New advances then followed in fast progression. In 1865, the French military doctor Jean-Antoine Villemin independently demonstrated that consumption could be passed from humans to cattle and from cattle to rabbits. On the basis of this ground-breaking evidence, he claimed a specific micro organism as the cause of the disease, finally ending the centuries-old belief that consumption occurred spontaneously.

In 1882, Robert Koch discovered a staining technique that enabled him to see *Mycobacterium tuberculosis*. This excited the world, as it was certain that now the fight against humanity's deadliest enemy could really begin.

The measures available to doctors were still modest. Improving social and sanitary conditions and ensuring adequate nutrition were all that could be done to strengthen the body's defences against the TB bacillus. Sanatoria, now to be found throughout Europe and the United States, provided a twofold function: they isolated the sick, the source of infection, from the general population, while the obligatory rest, together with a suitable diet assisted the healing processes.

These efforts were reinforced by the observation of the Italian, Forlanini, that lung collapse tended to have a positive impact on the outcome of the disease. With the introduction of artificial pneumothorax and surgical methods to reduce the lung volume, active therapy had begun.

A further noteworthy development came in 1895 when Wilhelm Konrad von Rontgen discovered radiation. Now the progress and severity of a patient's disease could be accurately followed and reconsidered.

The French bacteriologist, Calmette, who, together with Guerin, used specific culture media to lower the virulence of the bovine TB bacterium, created the basis for the BCG vaccine still in widespread use today. Then, in the middle of World War II, came the final breakthrough, chemotherapy.

The chemotherapy of infectious diseases, using sulfonamides and penicillins, had been underway for several years, but these molecules were ineffective against *Mycobacterium tuberculosis*. Since 1914, Selman A. Waksman had been methodically screening soil bacteria and fungi and discovered the noticeable inhibitory effect of certain fungi, especially actinomycetes, on bacterial growth. In 1940, he and his team were able to isolate an effective anti-TB antibiotic, actinomycin; but unfortunately, this proved to be too toxic for use in humans or animals.

On November 20, 1944, streptomycin was administered for the first time to a critically ill TB patient. The effect was noticed almost straight away. The bacteria disappeared from his sputum, and he made a speedy recovery. The new drug had side effects, especially on the inner ear, but *Mycobacterium tuberculosis* was no longer a bacteriological exception.

A fast succession of antituberculosis drugs appeared in the following years. These were important because with streptomycin monotherapy, resistant mutants began to appear within a few months. It was soon demonstrated that this problem could be overcome with the combination of two or three drugs.

Following streptomycin, para-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampicin (1963) were introduced as antituberculosis agents. Aminoglycosides such as capreomycin, viomycin, kanamycin and amikacin, and the newer quinolones (for example ofloxacin and ciprofloxacin) are only used in drug resistance situations. Combinations of a *B*-lactam antibiotic with a *B*-lactamase inhibitor improve treatment effectiveness, but the newer drugs, including the macrolides, have not received much clinical testing.

1.2. TUBERCULOSIS INFECTION AND TRANSMISSION

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis.* It is spread through the air when a person with untreated pulmonary TB coughs or sneezes. Prolonged exposure to a person with untreated TB usually is necessary for infection to occur. TB mainly affects the lungs (pulmonary TB), but other parts of the body can also be affected, for example lymph nodes, kidneys, bones and joints (European Bioinformatics Institute, 2006).

Unlike most bacteria, *Mycobacterium tuberculosis* escapes detection by the immune system for a long time. Its unique cell wall, a waxy coating primarily composed of mycolic acids, masks it from the host's immune system. Once inside a human lung, *M. tuberculosis* hides inside macrophages (the cells designed to eliminate it) and reproduces slowly but steadily. Surviving dormant for up to many years, the mycobacterium makes its presence known only once it has launched its infectious attack. While the lungs are the most commonly affected organ, TB can also affect other organs (Global Alliance for Tuberculosis Drug Development, 2005b).

Mycobacterium tuberculosis is one of the most successful pathogens, infecting a third of the global population and claiming over two million lives every year. The bacteria's ability to persist in the form of a long-term asymptomatic infection, known as latent tuberculosis, is

pivotal to the biology of the disease and can subsequently reactivate to cause the active disease. The latent state of infection poses a major obstacle in eradicating tuberculosis. In latent tuberculosis, the host immune response is capable of controlling the infection, but yet unable to eradicate the pathogen (Amdekar, 2005:334).

1.3. ACTIVE AND LATENT TUBERCULOSIS

According to the Centre for Disease Control (CDC, 2005), TB bacteria become active if the immune system cannot stop it from growing. The active bacteria begin to multiply in the body and cause active TB disease. The bacteria attack the body and destroy its tissue. If this occurs in the lungs, the bacteria can actually create a hole in the lung. Some people develop active TB disease soon after becoming infected, before their immune system can fight the TB bacteria. Other people may get sick later, when their immune system becomes weak for another reason.

Infants, young children and people infected with HIV have weak immune systems. People with the following conditions, are particularly susceptible to diseases (CDC, 2005):

* Substance abuse;
* Diabetes mellitus;
* Cancer of the head or neck;
* Leukaemia or Hodgkin's disease;
* Severe kidney disease;
* Low body weight;
* Certain medical treatments (such as corticosteroid treatment or organ transplants); and
* Specialised treatment for rheumatoid arthritis or Crohn's disease.

Symptoms of TB depend on where in the body the TB bacteria are growing. TB bacteria usually grow in the lungs. TB in the lungs may cause symptoms as listed in Table 1.1, where the difference between latent TB infection and active TB disease is indicated.

Table 1.1: The difference between latent TB infection and active TB disease (CDC, 2005)

A person with latent TB infection:	A person with active TB disease:
Has no symptoms.	Has symptoms that may include:
Does not feel sick.	• a bad cough that lasts 3 weeks or longer;
Cannot spread TB to others.	• pain in the chest;
Usually has a positive skin test.	• coughing up sputum with blood;
Has a normal chest x-ray and sputum test.	• weakness or fatigue;
	• weight loss;
	• no appetite;
	• chills;
	• fever; and
	• sweating at night.
	May spread TB to others.
	Usually has a positive skin test.
	May have an abnormal chest x-ray, or positive sputum smear or culture.

1.4. TUBERCULOSIS AS A GLOBAL THREAT

Someone in the world is newly infected with the TB bacillus every second. Overall, one-third of the world's population is presently infected with the bacillus, and more than two thirds of infected people do not receive full and proper treatment. Over the centuries, TB has taken over 1 billion lives (Global Alliance for Tuberculosis Drug Development, 2006). Regions with the highest rate of TB infection can be seen in Figure 1.1.

Between 2000 and 2020, it is estimated that nearly one billion people will be newly infected with TB, 200 million people will become sick from TB, and at least 35 million people will die from the disease (The Global Fund, 2005:24).

Along with the increase of global conflicts, so are refugees and TB. As many as 50 % of the world's refugees could be infected with TB; and since they are on the move, it is difficult to treat them fully because of the lengthy regimen required by existing drugs (Global Alliance for Tuberculosis Drug Development, 2006).

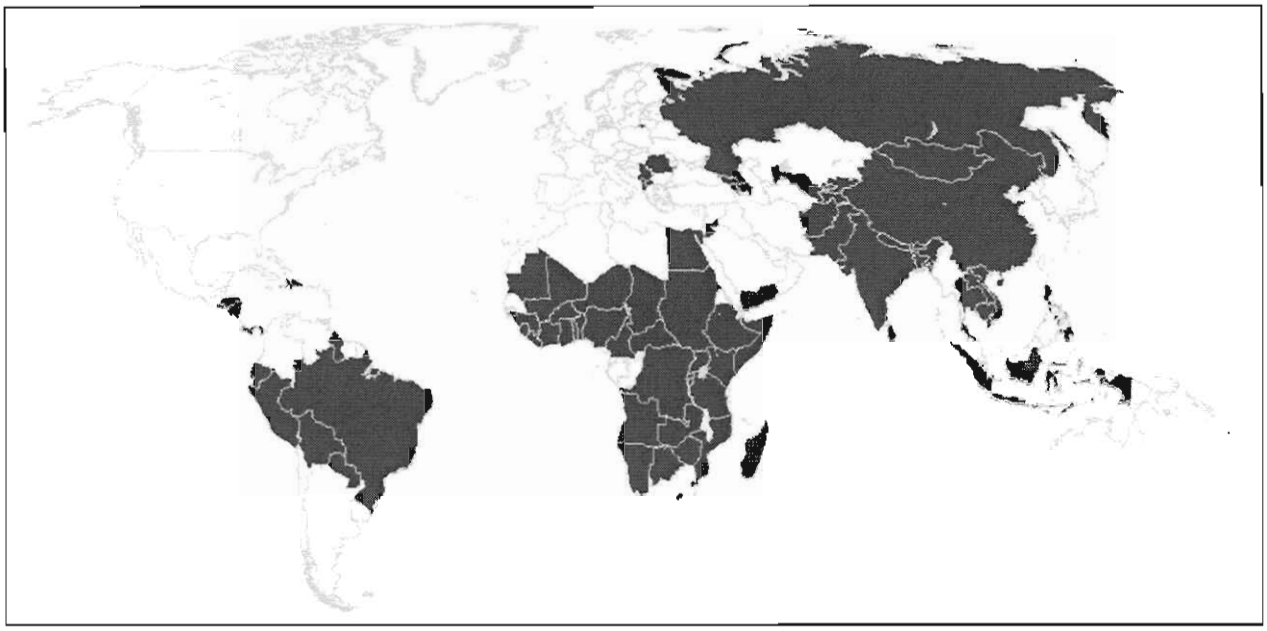


Figure 1.1: Regions of the world with high TB infection rates (The Global Fund, 2005:25)

The World Health Organization estimates that the largest number of new TB cases in 2004 occurred in WHO's South-East Asia Region, which accounted for 33 % of incident cases globally. However, the estimated incidence per capita in sub-Saharan Africa is nearly twice that of the South-East Asia Region, at nearly 400 cases per 100 000 population (WHO, 2005d).

It is estimated that 1.7 million deaths resulted from TB in 2004. Both the highest number of deaths and the highest mortality per capita are in the WHO Africa region, where HIV has led to rapid expansion of the TB epidemic, and increases the probability of dying from TB (WHO, 2005d).

In 2004, the estimated per capita TB incidence was stable or falling in five out of six WHO regions, but growing at 0.6 % per year globally. The exception is the African region, where TB incidence was still increasing, in line with the spread of HIV. However, the number of cases notified from the African region is increasing more slowly each year, probably because the HIV epidemics in African countries are also slowing. In Eastern Europe (mostly countries of the former Soviet Union), incidence per capita increased during the 1990s, but peaked around 2001, and has since dropped (WHO, 2005d). The estimated incidence, prevalence and TB mortality for 2004, are supplied in Table 1.2.

Table 1.2: Estimated incidence, prevalence and TB mortality for 2004 (WHO, 2005d)

WHO region	Incidence[a]				Prevalence[a]		TB Mortality	
	All forms		Smear-positive[b]					
	Number (thousands) (% of global total)	Per 100 000 population	Number (thousands)	Per 100 000 population	Number (thousands)	Per 100 000 population	Number (thousands)	Per 100 000 population
Africa	2 573 (29)	356	1 098	152	3 741	518	587	81
The Americas	363 (4)	41	161	18	466	53	52	5.9
Eastern Mediterranean	645 (7)	122	289	55	1 090	206	142	27
Europe	445 (5)	50	199	23	575	65	69	7.8
South-East Asia	2 967 (33)	182	1 327	81	4 965	304	535	33
Western Pacific	1 925 (22)	111	865	50	3 765	216	307	18
Global	**8 918 (100)**	**140**	**3 939**	**62**	**14 602**	**229**	**1 693**	**27**

[a] Incidence - new cases arising in given period; prevalence - the number of cases which exist in the population at a given point in time.

[b] Smear-positive cases are those confirmed by smear microscopy, and are the most infectious cases.

Because of neglect, this preventable problem has become the largest cause of death of any single infectious disease, killing more than 2 million people every year (Reichman & Hopkins Tanne, 2001).

1.5. THE CAUSE AND EFFECT OF POVERTY

The mutual relationship between disease and poverty is a vicious cycle: poverty increases the disease risk and disease causes or increases poverty. TB is recognised as playing a significant role in slowing the economic growth for entire countries and regions. Low-income households have less access to resources, information and products to protect them from disease. The poor have limited access to adequate health services and may lose badly needed income because

they are too sick to work. The direct costs of treatment and care to households in the poorest countries and the indirect costs of lost earnings escalate according to the severity and duration of the disease. The long and expensive treatment for TB can cost from 5 to 21 % of the annual household income. As TB exhausts the incomes of the world's poorest communities by 12 billion US dollar per year, the burden of this disease at the individual and community level leads to a significant macro-economic impact. TB imposes heavy burdens on already-weak health systems by straining limited health budgets, laboratories and clinics; overcrowding hospitals; and hindering existing efforts to increase access to treatment (The Global Fund, 2005:7).

Studies in India have shown that the incidence of TB is between two and four times higher among groups with low income and no education. Overcrowded conditions, poor nutrition and insufficient sanitation increase the likelihood of being infected and developing active TB. Once they are ill, those who have limited access to health services are less likely to be diagnosed and treated for TB. The greater the numbers of people with active TB in a community, the more likely others are to become infected. This becomes a vicious circle in poor communities where TB flourishes. Over 90 % of TB cases and 90 % of deaths from TB occur in developing countries. A major challenge for TB control today is finding those people who have limited access to effective TB treatment and curing them. Expanding ground-breaking approaches such as linking the public and private sectors in the treatment and referral of such cases will be critical in reducing TB deaths among the poor (The Global Fund, 2005:26).

1.6. THE SOCIO-ECONOMIC IMPACT OF TUBERCULOSIS

TB is a foremost cause of death among women of reproductive age, because they are less likely than men to be tested and treated for TB. In addition, as greater numbers of women become infected with HIV, more are also becoming sick with TB. Over 250 000 children die every year of TB. Children are particularly vulnerable to TB infection because of frequent household contact and weaker immune systems. Children also suffer when their parents are infected. Every year in India alone, more than 300 000 children leave school because their parents are infected with TB (The Global Fund, 2005:26).

Because TB typically affects the most productive and economically active sections of the population, the impact of illness on communities and households is very high. The socio-economic burden of TB is often discussed in terms of the direct and indirect costs to

households. These costs are higher for TB than most other diseases due to the length of the treatment period (six to eight months). Direct costs include paying for visits to clinics, tests and medication. In addition, TB imposes high non-medical costs, such as the cost of additional nourishing foods and transport to and from clinics. The indirect costs of a long illness are also devastating for poor households (The Global Fund, 2005:26).

1.7. TUBERCULOSIS AND HIV

According to Mkele (1998:166), HIV infection increases the risk that a patient infected with *Mycobacterium tuberculosis* will develop the active disease. This happens either from the reactivation of latent infection or from rapid progression of a newly acquired infection. The overlap of the HIV-infected population (mostly in the 15 to 45 year age groups) with the population infected with *Mycobacterium tuberculosis* has resulted in a dramatic rise in TB in this age group. HIV-infected individuals with tuberculosis, who are not treated, progress to AIDS more rapidly than those without infection or those who receive prophylactic antituberculosis treatment. HIV-infected patients with tuberculosis require aggressive therapy to account for issues such as non-compliance, more rapid disease progression, and drug resistance.

TB is among the principal killers of people with HIV as up to 50 % of people with HIV or AIDS develop TB. In some regions of Africa, up to 77 % of TB patients also have HIV. Worldwide, more than 21 million people are co-infected with TB and HIV, and 70 % are concentrated in Africa. A large number of these people will develop active TB if they do not receive treatment (The Global Fund, 2005:24).

1.8. TREATMENT OF TUBERCULOSIS

First line antituberculosis medicines include: rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin. Second line treatment include: amikacin, kanamycin, capreomycin, cycloserine, ethionamide, ofloxacin, prothionamide and para-aminosalicylic acid (Davidson & Le, 1992:661). For the purpose of this study, first line antituberculosis medicines refer to: rifampicin, isoniazid, pyrazinamide and ethambutol.

A long but effective treatment regimen, endorsed by the World Health Organization, is used in 128 countries, but only 23 % of TB patients actually receive this treatment (Reichman & Hopkins Tanne, 2001). This regimen includes monotherapy and combinations.

Since the introduction of an effective chemotherapy fifty years ago, the required treatment period has been halved and treatment simplified through fixed dose combinations (FDCs). Yet the standard dosage must still be taken for a period of six to nine months (Global Alliance for Tuberculosis Drug Development, 2005a). FDCs commonly used today and included in WHO's Essential Medicines List are listed in Table 1.4.

In human disease, the *Mycobacterium tuberculosis* organism exists in several sub-populations, each of which has a characteristic metabolic status and therefore varying vulnerability to antituberculosis drugs. The sub-population that favours high oxygen content and a neutral pH is vulnerable to isoniazid and to a lesser degree to rifampicin, streptomycin, and ethambutol. Pyrazinamide is active in an acidic medium and is very effective in killing a sub-population that exists in an acidic environment. A third sub-population is located mainly in material with neutral pH and poor oxygenation and is killed most effectively by rifampicin, because its bactericidal action starts very quickly. A fourth sub-population is completely dormant and antituberculosis drugs have no action against it (Davidson & Le, 1992:653).

There are three main properties of antituberculosis drugs: bactericidal activity, sterilising activity and the ability to prevent resistance. Isoniazid and rifampicin are the most powerful bactericidal drugs, active against all populations of TB bacilli. Rifampicin is the most influential sterilising drug available. Pyrazinamide is also bactericidal against certain TB bacilli populations, but is only active in an acid environment. Ethambutol is used in association with more powerful drugs to prevent the emergence of resistant bacilli (WHO, 2003b).

Therapy with two drugs, each possessing a different mechanism of action, is effective because the probability that a bacillus is resistant to both, is 1 in 10^{12}, which is at least three orders of magnitude less than the number of infecting organisms (Chambers, 1997:1268).

Table 1.3 shows that the three separate properties of TB are addressed for effective treatment.

Table 1.3: The properties of first line antituberculosis drugs commonly used in FDCs
(Global Alliance for Tuberculosis Drug Development, 2005b)

TB drug	Introduction date	Antibacterial activity	Inhibits resistance	Effective against persisting organisms
Ethambutol	1962		*	
Isoniazid	1952	*	*	
Pyrazinamide	1954			*
Rifampicin	1963	*	*	

Three effective actions will have a sustained impact on the TB and HIV epidemics and will save the lives of millions: Halting the spread of multi-drug resistant TB, implementation of Directly Observed Therapy Short Course (DOTS), and implementation of collaborative TB / HIV activities (The Global Fund, 2005:27). DOTS is further discussed in Paragraph 1.11.

Treatment of tuberculosis takes six to nine months and sometimes longer. Typical side effects of tuberculosis medication include (Victoria State Government, 2005):

* Continual itchiness (caused by isoniazid);
* Upset stomach, nausea or loss of appetite (caused by isoniazid and pyrazinamide);
* Numbness or tingling in the hands or feet (caused by isoniazid);
* A rash, bruising or yellowing of the skin (caused by pyrazinamide and rifampicin);
* Dark coloured urine (caused by rifampicin);
* Visual problems (caused by ethambutol); and
* Yellowing of the eyes (caused by rifampicin).

1.9. DRUG RESISTANT TB

Until 50 years ago, there were no medicines to cure TB. Not only have strains that are resistant to a single drug been documented in every country surveyed; but strains of TB resistant to all major antituberculosis drugs have also emerged. Drug-resistant TB is caused by inconsistent or partial treatment, when patients do not take all their medicines regularly for the required period because they start to feel better, because doctors and health workers prescribe the wrong treatment regimens, or because the drug supply is unreliable. A particularly dangerous form of drug-resistant TB is multi-drug resistant TB (MDR-TB), which is defined as the disease caused by TB bacilli resistant to at least isoniazid and rifampicin, the two most powerful antituberculosis drugs. While drug-resistant TB is generally treatable, it requires up to two years of treatment that is often 100 times more expensive than treatment of drug-susceptible TB, and is also more toxic to patients (WHO, 2005d).

Epidemics of MDR-TB can spread quickly from city to city, country to country and even spanning continents. A survey in 72 countries suggested that the MDR-TB problem is more widespread than previously thought and likely is worsening. In some TB hot spots, at least 20 % of all registered TB cases were due to MDR-TB. Some have estimated that between

185 000 and 415 000 new cases of MDR-TB will develop every year. The enormity of the global MDR-TB problem is unclear since only half of the countries with highest levels of TB cases have relevant data, and many national TB programs do not have the resources to screen all TB cases for resistance. While MDR-TB afflicts countries with poor health infrastructure, MDR-TB is just as likely to break out in industrialised economies. During the late 1980s and early 1990s, outbreaks of MDR-TB in North America and Europe killed over 80 % of those who contracted it. The major TB outbreak in New York in the early 1990s was primarily a MDR-TB epidemic, with one in ten cases being drug-resistant. Another hot spot is the Russian Federation, where MDR-TB has spread like wildfire, particularly in prisons. One-third of Russia's TB population is in prison, and 24 % of those have MDR-TB. In parts of Eastern Europe, nearly half of all TB cases are resistant to at least one first line drug. Other areas where MDR-TB is a serious problem include: Estonia, Latvia, and China. MDR-TB strains are also appearing in significant numbers in Mexico and cases appear to be increasing in Denmark and Germany (Global Alliance for Tuberculosis Drug Development, 2006).

The worldwide occurrence of MDR-TB is shown in Figure 1.2.



Figure 1.2: The worldwide spreading of multi-drug resistant tuberculosis (Global Alliance for Tuberculosis Drug Development, 2006)

Multi-drug resistant tuberculosis commonly occurs when patients do not finish their full course of medication. According to Mkele (1998:165) patients may also stop treatment for the following reasons:

* They feel better after the first few months of treatment;
* They may move to a different area because of work;
* Their health worker may fail to supervise them appropriately; and / or
* Drug misuse.

1.10. TREATMENT OF MULTI-DRUG RESISTANT TB

The WHO recommends the use of fixed dose combination products for the treatment of TB and multi-drug resistant TB. The formulations and dosages currently recommended by the WHO, are shown in Table 1.4.

Table 1.4: WHO list of essential first line antituberculosis FDC products
(WHO, 2005b)

Active ingredient	Dosage form and strength
Isoniazid + ethambutol	Tablet, 150 mg + 400 mg
Rifampicin + isoniazid	Tablet, 60 mg + 30 mg; 150 mg + 75 mg; 300 mg + 150 mg; 60 mg + 60 mg; 150 mg + 150 mg (for intermittent use three times weekly)
Rifampicin + isoniazid + pyrazinamide	Tablet, 60 mg + 30 mg + 150 mg; 150 mg + 75 mg +400 mg; 150 mg + 150 mg + 500 mg (for intermittent use three times weekly)
Rifampicin + isoniazid + pyrazinamide + ethambutol	Tablet, 150 mg + 75 mg + 400 mg + 275 mg

According to Davidson & Le (1992:654) multi-drug resistant TB is treated in the following manner: the patient should receive at least three drugs that he / she has not received before and to which the tubercle bacilli are likely to be susceptible. A single drug should never be added to a failing regimen. In the case of disease resistance to both isoniazid and rifampicin, a 3-drug regimen containing pyrazinamide or ethambutol or ethionamide (if they have not been previously used) and one injectable drug (usually streptomycin or capreomycin) should be used. Some clinicians prefer adding a fourth of fifth drug (cycloserine, ciprofloxacin or

p-aminosalicylic acid). Tuberculosis, which is resistant only to isoniazid, responds well to a regimen containing rifampicin, pyrazinamide and ethambutol or streptomycin.

During the final stages of completing this thesis, the media reported an outbreak of a new TB strain in South Africa (in the KwaZulu Natal area). This new strain of TB could not be effectively treated by available medicines being used in the treatment of multi-drug resistant TB at the time. The inclusion of *p*-aminosalicylic and capreomycin to the regimen were highly recommended.

1.11. DIRECTLY OBSERVED THERAPY SHORT COURSE (DOTS)

In 2006, WHO launched its most recent Stop TB Strategy. The core of this strategy is DOTS, the TB control approach introduced by WHO in 1995 (WHO, 2005d).

Directly Observed Therapy Short Course (DOTS) emerged from the realisation that compliance was essential to successful chemotherapy and that the only way to ensure compliance, was through direct observation of the ingestion of every dose by someone other than the patient. It comprises that the health worker, in consultation with the patient, selects a supervisor, not necessarily a health worker, but for example a member of the family, a neighbour, or even the local shopkeeper. The supervisor safeguards the supply of medicines, the patient's treatment chart and should on a daily basis observe the patient as (s)he takes the medication, and record this in an appropriate manner. Health workers visit supervisors regularly to provide the necessary support and encouragement, and to check on the patients' treatment charts (Mkele, 1998:164).

The DOTS strategy produces cure rates of up to 95 %, prevents the development of drug resistance by ensuring that the full course of treatment is followed, and costs as little as 10 US dollar for a six- to eight-month treatment period (The Global Fund, 2005:28).

By the end of 2003, DOTS had been implemented in 181 countries; 69 % of the world's population was living in areas covered by DOTS and 121 countries had made DOTS available to over 90 % of their TB population. Data from 2003 showed that few countries were approaching the targets for case detection and treatment success (70 % and 85 %, respectively). Vietnam was the only high burden country to have reached targets for both detection and treatment (The Global Fund, 2005:29).

1.12. GLOBAL EFFORTS TO STOP TUBERCULOSIS

The Global Fund is part of concerted efforts to address the total global resource needs in the fight against AIDS, TB and malaria, alongside important partners such as WHO, UNAIDS and its co-sponsors. The Global Fund has already enabled the tuberculosis treatment of 385 000 people under DOTS (the WHO-recommended TB control strategy) (The Global Fund, 2005:28).

In the early 1990s, the world health spotlight fell on infectious diseases, and TB was recognized as both a serious public health problem and as one with potential for cost-effective solutions. New tools and strategies for TB control were adopted, and in 1998 the international Stop TB Partnership was launched. Hosted by the World Health Organization, the Stop TB Partnership aims to mobilize health, economic and social sectors for TB control. Health and Finance Ministers of high TB burden countries endorsed the Stop TB Partnership and committed to global goals for controlling the disease. In 2001, the Stop TB Partnership initiated a 9.1 billion US dollar, five-year Global Plan to Stop TB. Its strategic objectives were to (The Global Fund, 2005:28):

* Increase global coverage of the DOTS TB control strategy;
* Face up to the emerging challenges of TB / HIV co-infection and drug-resistant TB;
* Improve treatment by developing new drugs, vaccines and diagnostics; and
* Reinforce the Global Partnership to Stop TB so that proven TB control strategies were effectively applied around the world.

The focus of the plan was to control TB in the 22 high-burden countries which account for 80 % of the world's TB cases. By early 2004, all high-burden countries had made national plans for TB control, and membership of the Stop TB Partnership had grown to 280 organizations. This growing commitment has led to more coordination and support for national strategies to eliminate TB, and several countries have achieved outstanding successes. With two million people dying every year, however, there is still a long way to go (The Global Fund, 2005:28).

1.13. WHO's INVITATION FOR EXPRESSION OF INTEREST

Following is an extract from the WHO's sixth invitation for expression of interest in antituberculosis products as posted during May 2005 on the WHO's Prequalification website (WHO, 2005c:1). The list of products fairly represents the TB medicines currently being used worldwide.

> *In the context of facilitating access to anti-tuberculosis drugs for use in countries with a high burden of tuberculosis (TB), WHO, together with other UN agencies participating in the Stop TB Partnership, with its Global Drug Facility (GDF), i.e. UNICEF, UNAIDS, UNFPA and the Green Light Committee, are inviting* ***Expressions of Interest****, from manufacturers of such pharmaceutical products. For more information please refer to the web-site: www.stoptb.org / GDF and the web-sites of the above mentioned organizations.*
>
> *Interested* ***manufacturers or their authorised agents*** *are invited to submit their expression of interest to UNICEF Supply Division for the following products*
>
> *1) Ethambutol Hydrochloride 400 mg film coated tablets*
>
> *2) Pyrazinamide 400 mg tablets*
>
> *3) Isoniazid 300 mg tablets*
>
> *4) 2FDC: Rifampicin 150 mg / Isoniazid 75 mg film coated tablets*
>
> *5) 2FDC: Rifampicin 150 mg / Isoniazid 150 mg film coated tablets*
>
> *6) 2FDC: Ethambutol Hydrochloride 400 mg / Isoniazid 150 mg film coated tablets*
>
> *7) 3FDC: Rifampicin 150 mg / Isoniazid 75 mg / Ethambutol Hydrochloride 275 mg film coated tablets*
>
> *8) 4FDC: Rifampicin 150 mg / Isoniazid 75 mg / Pyrazinamide 400 mg / Ethambutol Hydrochloride 275 mg film coated tablets*
>
> *9) Streptomycin Sulfate 1 g vial (injection)*
>
> *10) Water for injection 5 ml vial (injection)*
>
> *11) Amikacin 500 mg / 2 ml vial (injection)*
>
> *12) Kanamycin 1 g powder for injection, vial*

13) Capreomycin 1 g powder for injection, vial

14) Cycloserine 250 mg capsules

15) Ethionamide 125 mg or 250 mg tablets

16) Ofloxacin 200 mg tablets

17) Prothionamide 125 mg or 250 mg tablets

18) Para-aminosalicylic acid 4 g granules and para-aminosalicylic sodium 100 g granules

Formulations for children.

Dosage forms should be soluble tablets, tablets with break line (film coated), and / or sachets.

1) 3FDC: Rifampicin 60 mg / Isoniazid 30 mg / Pyrazinamide 150 mg

2) 2FDC: Rifampicin 60 mg / Isoniazid 30 mg (R60/H30)

3) 2FDC: Rifampicin 60 mg / Isoniazid 60 mg (R60/H60)

4) Isoniazid 100 mg tablets.

The medicines listed in this Invitation for Expression of Interest are those for which a need has been identified by the TB department, WHO.

WHO, together with other UN agencies participating in the Stop TB Initiative, with its Global Drug Facility (GDF), i.e. UNICEF, UNAIDS, UNFPA and the Green Light Committee are especially interested in receiving Expressions of Interest for the possible procurement and supply of these products. Products should be made available in different pack sizes: in blister packs in carton boxes and loose tablets in suitable containers e.g. HDPE.

1.14. CONCLUSION

In this chapter it was suggested how TB poses a worldwide health threat and how this disease has a huge socio-economic impact.

TB is also among the major killers of people infected with HIV. Because of the side-effects caused by currently available medicines and the fact that patients start to feel better before the

course of treatment is completed, patient compliance is generally poor. This led to the development of DOTS, which is highly effective in producing higher cure rates.

The WHO recommends the use of fixed dose combination products for the treatment of TB and multi-drug resistant TB. To date no monographs for FDC products of first line antituberculosis APIs appear in The International Pharmacopoeia. The WHO hence requested the Research Institute for Industrial Pharmacy to develop monographs for antituberculosis FDC products for inclusion in The International Pharmacopoeia.

CHAPTER 2

Fixed Dose Combination Products: Guidelines and Requirements

The development of fixed dose combinations (FDCs) is becoming ever more important from a public health perspective. It is being used in the treatment of a wide range of conditions such as in the management of HIV / AIDS, malaria and tuberculosis, which are considered the foremost infectious disease threats in the world (WHO, 2005e:95).

Fixed dose combination products provide a simple approach to delivering the correct number of medicines at the correct dosage as all the necessary medicines are combined in a single dosage form. Complete treatment is given by changing the number of tablets or capsules according to the patient's weight (WHO, 1999:5).

FDCs have advantages when there is a specific patient population for whom treatment with a particular combination of actives in a fixed ratio of doses has been shown to be safe and effective, and when all of the actives add to the overall therapeutic effect. There can also be real clinical benefits in the form of increased efficacy and / or a reduced occurrence of adverse effects, but such claims should be verified (WHO, 2005e:96).

Kaplan (2004:4) defines fixed dose combination products as two or more APIs in a single formulation, each API having independent modes of action, or the combination of which are synergistic or additive or complementary in their effect.

The World Health Organization distinguishes between the concepts fixed dose combination (FDC), co-packaged product (such as co-blistered tablets) and fixed dose combination finished pharmaceutical product (FDC-FPP) as follows (WHO, 2005e:107):

Fixed dose combination (FDC)

> A combination of two or more actives in a fixed ratio of dose. This term is used generically to mean a particular combination of actives irrespective of the formulation or brand. It may be administered as single entity products given concurrent or as a finished pharmaceutical product.

Co-packaged product

A product consisting of two or more separate pharmaceutical products in their final dosage forms that are packaged together for distribution to patients in co-packaging.

Fixed dose combination finished pharmaceutical product (FDC-FPP)

A finished pharmaceutical product that contains two or more actives.

Finished pharmaceutical product (FPP)

A product that has undergone all stages of production, including packaging in its final container and labelling. An FPP may contain one or more actives.

This study comprises the development of monographs for rifampicin tablets / capsules and TB FDC products containing combinations of two or more of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride. In the development of monographs, it is important to consider the safety, efficacy, and in particular the quality requirements for FDC products, which include stability, assay and identification testing, as well as the determination of degradation products / related substances.

Examples of FDC products used in the treatment of TB are listed in Table 1.4. Note that the term *product* is used in a broad sense, which may include *finished pharmaceutical product.*

2.1. FDC GUIDELINES

Combination therapy is not a new concept, as it is also used in the treatment of infections other than HIV / AIDS, malaria and tuberculosis, as well as in non-infectious diseases such as hypertension, diabetes and epilepsy.

Before 2004, there were no comprehensive guidelines on FDCs, hence no detailed uniform principles, guidelines or international standards that addressed the development of FDCs, and that described their potential benefits, or possible disadvantages in treating diseases, existed (Global Health, 2004b). However, since the beginning of 2004 the development of guidelines for FDCs received considerable attention, of which this study is an essential part.

The first movement towards setting guidelines was through a panel meeting consisting of regulators and other representatives of the co-sponsors, as listed below, in February 2004 in Cape Town (South Africa) with the aim to draft scientific and technical principles for FDCs. The drafted document titled "Scientific and technical principles for fixed dose combination

drug products" was posted on the Global Health website for comments. The panel meeting was followed by a conference on FDC products held during 29 to 31 March 2004 in Gaborone (Botswana). The co-sponsors were: Joint United Nations Programme on HIV / AIDS (UNAIDS), World Health Organization (WHO), Southern African Development Community (SADC) and United States Department of Health and Human Services (HHS). This conference was attended by government officials and representatives of drug regulatory agencies from 23 nations, the research-based and generic pharmaceutical industries, public health leaders, health care providers, advocacy groups (including persons living with HIV / AIDS), academia and members of non-governmental organisations (Global Health, 2004a; HHS, 2004).

The outcome of the Botswana conference was the so called "Scientific and technical principles for fixed dose combination drug products for the treatment of HIV / AIDS, malaria and tuberculosis". The purpose of these principles was to increase the general awareness of scientific and technical issues related to the development of FDCs (Global Health, 2004b). (This document and other information on the conference can be found on the webpage: http://www.globalhealth.gov/Final_FDC_index.html).

Shortly after the Botswana conference, the US FDA released in May 2004 a draft guidance titled "Guidance for industry: Fixed dose combination and co-packaged drug products for treatment of HIV" (US FDA, 2004). Of importance for the development of HIV / AIDS FDCs, this guideline has three significant attachments (US FDA, 2004), namely:

Attachment A: "Scenarios for approval of FDC / co-packaged combinations for treatment of HIV";

Attachment B: "Examples of combinations for treatment of HIV supported by current clinical data for FDC / co-packaging"; and

Attachment C: "Combinations for treatment of HIV not acceptable for FDC / co-packaging".

Soon after this, in June 2005, WHO published the first comprehensive guidelines on FDCs titled: "Guidelines for registration of fixed dose combination medicinal products" (WHO, 2005e:94). Note that this guideline covers all prescription medicines versus the draft guidance of the US FDA that is only relevant to HIV medication. For the purpose of this study, the WHO's guidelines are applicable.

Paragraph 5 of the WHO's guidelines on specifications for pharmaceutical preparations, argues on balancing the advantages and disadvantages of FDCs. From a scientific or medical perspective, FDCs are more likely to be useful when several of the following factors apply (WHO, 2005e:111):

* There is a medical rationale for combining the active ingredients;
* There is an identifiable patient group for which this combination of actives and doses is suitable therapy (the larger the patient group, the more significant this factor is);
* The combination has a greater efficacy than any of the component actives alone at the same dose;
* The occurrence of adverse reactions in response to treatment with the combination is lower than the response to any of the component actives given alone;
* For antimicrobials, the combination results in a reduced incidence of resistance;
* One active ingredient acts as a booster for another;
* The component actives have compatible pharmacokinetics and / or pharmacodynamics;
* Therapy is simplified, particularly when the existing therapy is complex;
* One of the ingredients is intended to lessen abuse of the other ingredient; and
* The active ingredients are chemically and physicochemically compatible, or special formulation techniques have been used that adequately address any incompatibility.

From a scientific or medical perspective, FDCs are less likely to be useful when one or more of the following factors apply (WHO, 2005e:112):

* The component actives are usually separately titrated to meet the patient's needs;
* There is a higher occurrence or greater severity of adverse reactions to the combination than with any of the ingredients given alone, or there are adverse reactions not seen in response to treatment with any of the individual ingredients;
* There are adverse pharmacokinetic interactions between the ingredients;
* Dose alteration is needed in special populations, for example people with renal or hepatic impairment; and
* The product (tablets or capsules) is so large that patients find it difficult to swallow.

According to the WHO (2005e:96), FDCs must be proven safe and effective with regards to the claimed indications. It should not be assumed that benefits outweigh the risks. Accordingly, a large portion of this guideline is devoted to the safety and efficacy of FDC products and how these should be addressed in product design and development.

Kaplan (2004:10) listed the advantages and disadvantages of FDCs as follows:

Advantages:

* Patient compliance and treatment outcomes are improved by a simpler dosage schedule;
* Medication errors are reduced;
* Attainment of antimicrobial resistance is prevented by eliminating monotherapy;
* Drug shortages are reduced by simplifying drug storage and handling, and thus lowers risk of being "out of stock";
* Only one expiry date simplifies dosing as single products may have different expiry dates;
* Procurement, management and handling of drugs are simplified; and
* Production, packing and shipping costs are lowered.

Disadvantages:

* FDCs are sometimes more expensive than the sum of the costs of the separate single-drug tablets;
* Potential quality problems when drugs are combined, especially with rifampicin in FDCs for TB. This necessitates bio-availability testing; and
* Drug interactions may lead to alteration of the therapeutic effect.

There will be cases where individual drugs must be utilised for certain patients because of the challenges of dose titration, allergies to one or more of the components, different pharmacokinetic and / or pharmacodynamic profiles, and pharmaceutical development (Global Health, 2004a).

2.2. GUIDELINE REQUIREMENTS FOR QUALITY

The WHO's FDC guidelines pay specific attention to the quality aspects of FDC products. Quite similar principles apply to FDC FPPs than to single entity products, although there are added complexities arising from the need to consider two or more actives instead of one. Quality requirements for FDC products are discussed below (WHO, 2005e:116).

Aspects that are specifically important when considering FDCs and their specifications include:

* Chemical and physicochemical compatibility of the APIs in an FDC with one another;
* The degradability of each API under stress conditions in the presence of each other in addition to API excipient incompatibility. This aspect is discussed in the next chapter with regards to the products investigated in this study;
* Analytical procedures should be validated for each active in the presence of the others during development of analytical methods for quality control of the finished product, stability testing and dissolution testing;
* Different assay procedures may be required for the different actives in the finished product, and for different purposes; and
* The dissolution rate of the APIs may differ in different media, and dissolution conditions and acceptance criteria should be developed for each API.

Acceptance criteria for impurities in FDC FPPs should be expressed with reference to the parent API. This requirement is unique to FDC products. If an impurity results from a reaction between two APIs, its acceptance limits should be expressed in terms of the API that represents the worst case (WHO, 2005e:117). The implication is that unknown impurities should also be related to the API that represents the worst case.

In addition to WHO guideline requirements, identification tests should be specific in order to identify each of the components, and when possible give an indication of the relative strength, as for example thin-layer chromatography.

According to the "Botswana principles" (Global Health, 2004b), the following three factors need to be taken into account in the development or evaluation of FDCs:

* The safety and efficacy of the individual active components;
* The safety and efficacy of a multi drug regimen; and
* The possible interaction between active components when they are formulated into an FDC.

The following factors need to be considered when developing a practical FDC dosage regimen (Global Health, 2004b):

* Combining active components with different pharmacokinetic characteristics may present problems; for example, combining antimicrobials with different half-lives may lead to drug resistance;
* Conflicting effects of food on the bioavailability of the active ingredients might complicate a dosing strategy; and
* The active ingredients in FDCs should be chemically and physically compatible, unless formulation techniques have been shown to overcome any incompatibility.

2.3. CONCLUSION

Development studies and quality control considerations of FDC products can be much more complex than those of monotherapeutic products, because of the possible chemical and physical incompatibility of the APIs with each other. These issues are further discussed in Chapter 3.

It is important to consider the specific requirements relating to FDC products during the development of their monographs. In 2004, the development of guidelines for FDCs received considerable attention for the first time, of which this study is evidence. The "Botswana principles" and draft guidance on HIV / AIDS FDCs by the US FDA, eventually led to the development of comprehensive guidelines on FDC products. Of particular importance was the assay of the individual APIs in an FDC product, as well as the determination of related substances.

CHAPTER 3

Physical and Chemical Properties of APIs in 4FDC TB Products

A medicinal product is designed to possess certain desirable properties of which the following are of major importance (Lund, 1994:277):

* When the product is administered by the specified route, the active constituent should achieve the required rate and extent of bioavailability;
* The product itself should be efficacious, safe and acceptable to the patient; it should be convenient for use and stable; and
* The stability of a product relates to its resistance to the various chemical, physical and microbiological reactions that may change the original properties of the preparation during transport, storage and use.

In this chapter, the major physical properties of ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin are discussed, as well as stability aspects, degradation pathways, and especially the reaction between isoniazid and rifampicin. The stability of rifampicin in solution, and the effect of environmental conditions and packaging type on the stability of the drugs are also investigated.

3.1. PHYSICOCHEMICAL PROPERTIES OF APIs

The chemical structures of ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin are shown in Figure 3.1. Ethambutol hydrochloride and rifampicin are both intrinsically chiral, and specific stereo isomers are used medicinally. Conversely, Isoniazid and pyrazinamide are non-chiral compounds.

The physicochemical properties of these four antituberculosis APIs are summarised in Table 3.1.

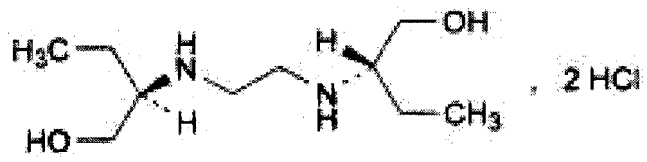
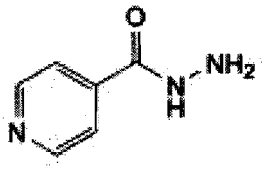
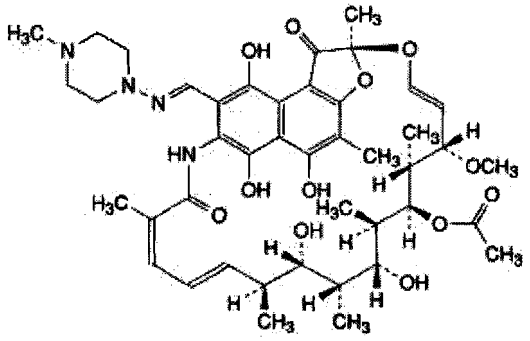
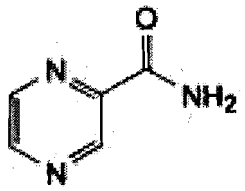
Ethambutol hydrochloride ($C_{10}H_{24}N_2O_2$, 2HCl)	Isoniazid ($C_6H_7N_3O$)
	
Rifampicin ($C_{43}H_{58}N_4O_{12}$)	**Pyrazinamide ($C_5H_5N_3O$)**
	

Figure 3.1: Structures of antituberculosis APIs (BP, 2005)

Rifampicin solubility is higher at low pH values. At 25 °C, rifampicin has the following solubility values in water at different pH values (Lund, 1994:1040):

* 0.3 % m/v at pH 7.5;
* 0.4 % m/v at pH 5.3; and
* 10 % m/v at pH 2.0.

However, as will be seen later, the stability of rifampicin is pH dependent and rifampicin decomposes fast at low pH values.

The hygroscopicity of ethambutol can cause problems in pharmaceutical products, and this may be the reason why the USP (2006:1922) sets a loss on drying limit of 3.0 % for the 4FDC tablets.

According to the biopharmaceutics classification system (BCS) an API is defined as highly soluble if the therapeutic dose is soluble in 250 ml water or less over the pH range 1.2 to 6.8 (WHO, 2006g:395). APIs with high solubility are classified according to the BCS as Class I or Class III APIs, whereas APIs with low solubility will belong to Class II or Class IV. See Table 3.1 for the BCS classification of ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin.

Table 3.1: The physicochemical properties and BCS classification of antituberculosis APIs

Properties	Ethambutol hydrochloride	Isoniazid	Pyrazinamide	Rifampicin
Molecular weight[1]	277.24	137.14	123.11	822.94
Description	A white, crystalline powder, Hygroscopic when exposed to high relative humidity[3]	A white, crystalline powder or colourless crystals[2]	A white, crystalline powder[2]	A reddish-brown or brownish-red, crystalline powder[2]
pH (in water)	2 % w/v solution: 3.7 to 4.0[3]	5 % solution: 6.0 to 8.0; 1 % solution: 5.5 to 6.5[2,3]	1.5 % solution: 5.0 to 7.0[4]	1 % w/v suspension: pH of 4.5 to 6.5 [3]
Dissociation constant	6.3; 9.5 (20 °C)[3]	1.8 (hydrazine nitrogen); 3.5 (pyridine nitrogen); 10.8 (acidic group)[3]	0.5[5]	1.7 (naphthalene hydroxy groups); 7.9 (piperazine nitrogen)[3]
Melting point	199 – 204 °C[3]	170 – 174 °C[3]	188 – 191 °C[2,5]	185 °C (decompose)[3]
Solubility in water (m/v)	50 % Soluble in water[3]	Soluble 1 in 8 of water[3]	1.5 % Soluble in water[2]	Slightly water soluble[2,3]
Solubility in organic solvents (m/v)	20 % Soluble in ethanol; 0.1 % Soluble in chloroform[3]	Ethanol: 1 in about 45; Chloroform: 1 in 1 000 Very slightly soluble in ether and in benzene[3]	0.7 % Soluble in chloroform; 0.6 % Soluble in ethanol[2,3]	Slightly soluble in ethanol; Freely soluble in chloroform[2,3]
BCS classification[6]	3	3 or 1	3 or 1	2

[1] (O' Neill, 2001:663; 928; 1424; 1474)
[2] (BP, 2005)
[3] (Lund, 1994:863; 928; 1039)
[4] (IntPh, 1988:268)
[5] (Moffat, 1986:947)
[6] (WHO, 2006g:407; 410; 415-416)

3.2. STABILITY OF ANTITUBERCULOSIS APIs

Gallo & Radaelli (1976:493) reported that rifampicin undergoes degradation to form 3-formyl rifamycin, rifampicin-quinone, rifampicin N-oxide and 25-desacetyl rifampicin. Jindal *et al.* (1995:422) found rifampicin to be unstable in highly acidic solutions, with maximum stability at pH 4.0.

Isoniazid is incompatible with aldehydes and ketones as it reacts with reducing sugars to produce isonicotinyl hydrazones. Reducing sugars include lactose, and maltose, which should not be used as excipients in isoniazid formulations. Degradation reactions for isoniazid include both hydrolysis and oxidation under stress conditions, and the route and the resultant degradation products depend on the conditions under which the reaction occurs (Lund, 1994:930).

Pyrazinamide exhibits good stability in the solid state. There is no apparent degradation of bulk sample, either in wet or in dry atmosphere (Felder & Pitrè, 1983:450).

Solutions of ethambutol hydrochloride are reported to be stable when heated at 121 °C for 10 minutes (Lund, 1994:863).

3.3. DEGRADATION OF ANTITUBERCULOSIS APIs

3.3.1 In formulations

The main concern with regards to the stability of antituberculosis FDCs is the interaction between isoniazid and rifampicin. Bhutani *et al.* (2004:1073) attributed the greater instability in three- or four-drug combinations to the fact that pyrazinamide and ethambutol hydrochloride may be catalytic towards the reaction between isoniazid and rifampicin. They undertook a further study to confirm this catalytic reaction by combining various organic bases and amides, related in structure to pyrazinamide and ethambutol hydrochloride, individually with rifampicin and isoniazid. The mixtures were exposed to accelerated stability test conditions of 40 °C at 75% RH for 15 days. The nature of the degradation products that were formed and the changes in relative concentrations of the APIs and degradation products were established by means of HPLC. The APIs showed different extents of degradation, producing the isonicotinyl hydrazone, and in some cases degradation products relating to rifampicin only,

such as 25-desacetyl rifampicin, rifampicin quinone and rifampicin N-oxide (Bhutani *et al.*, 2005b:892).

It was found that ethambutol hydrochloride influences the reaction between rifampicin and isoniazid to a greater extent than pyrazinamide. This can be attributed to the hygroscopicity and acidic nature of ethambutol hydrochloride (Bhutani *et al.*, 2005b:895).

The direct interaction between isoniazid and rifampicin can be best explained through a transhydrazone formation, via nucleophilic attack on the imine group of rifampicin by the amino group of isoniazid, following a tetrahedral mechanism as shown in Figure 3.2.

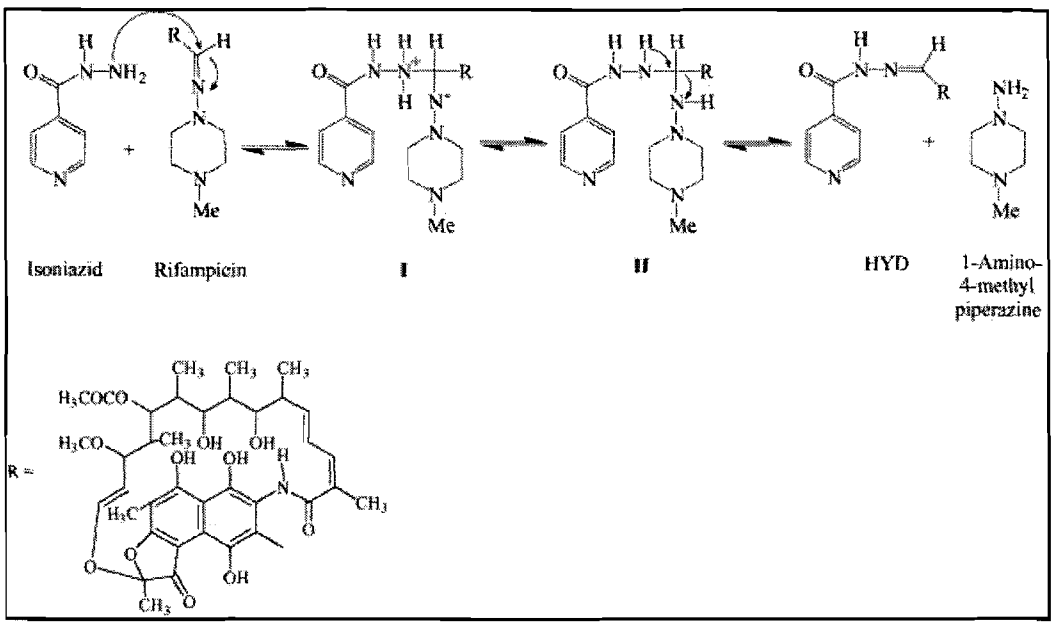


Figure 3.2: Postulated mechanism of formation of the isonicotinyl hydrazone on direct interaction between isoniazid and rifampicin (Bhutani *et al.*, 2005b:897)

Bhutani *et al.* (2005b:897) felt that pyrazinamide also plays a role in catalysing the decomposition of rifampicin and isoniazid in a fixed dose combination of rifampicin, isoniazid and pyrazinamide, and not merely because of a direct transhydrazone formation involving isoniazid and rifampicin. They proposed that the increased decomposition rate in the presence of pyrazinamide is due to base catalysed transhydrazone formation (see Figure 3.3), in which pyrazinamide acts as a proton scavenger via a five membered ring transition state involving *intra*-molecular hydrogen bond formation between one of the hydrogens of $CONH_2$ and the nitrogen of the adjacent ring.

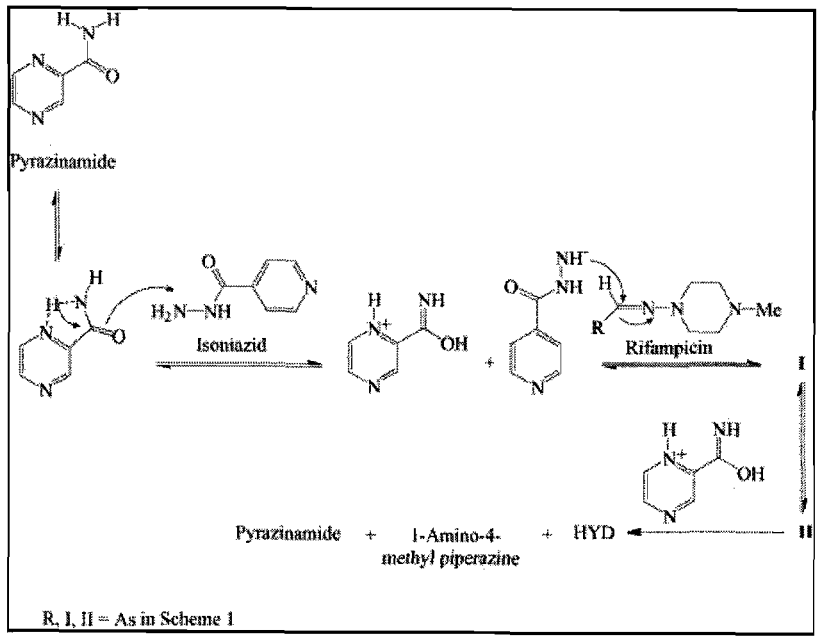


Figure 3.3: Mechanistic explanation to the catalytic effect of pyrazinamide on the direct interaction between isoniazid and rifampicin (Bhutani *et al.*, 2005b:898)

Figure 3.4 demonstrates the probable mechanism for the catalysis of the interaction between isoniazid and rifampicin in the presence of ethambutol, either as a free base, or hydrochloride salt. It is suggested that this reaction follows a similar path than that of pyrazinamide as described and illustrated above.

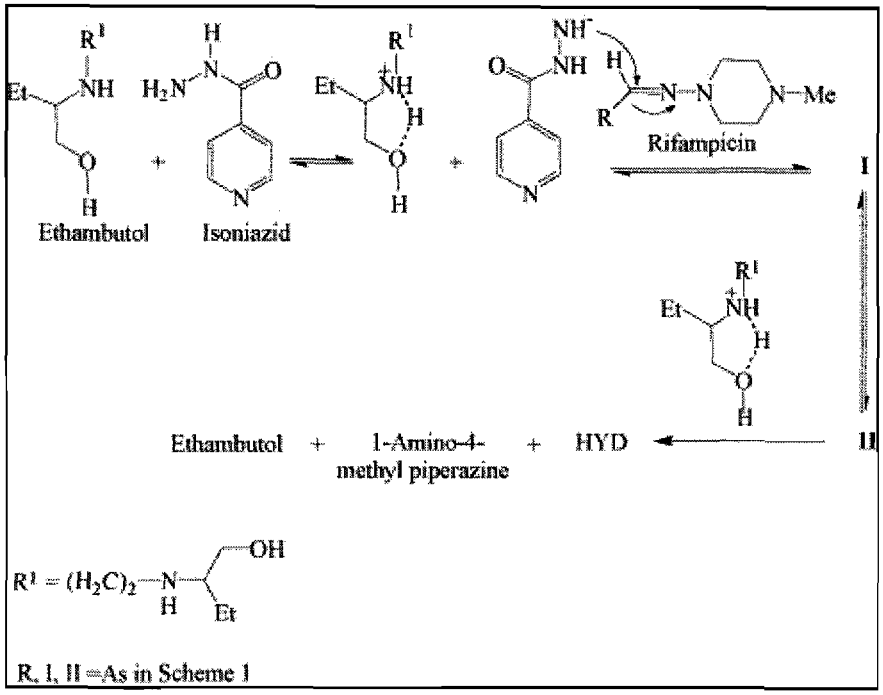


Figure 3.4: Mechanistic explanation to the catalytic effect of ethambutol on the direct interaction between isoniazid and rifampicin (Bhutani *et al.*, 2005b:899)

The study of the interaction of rifampicin with isoniazid, in the presence of various organic bases and amides, showed that the reaction leading to the formation of the isonicotinyl hydrazone even occurs in a non-acidic environment. It therefore lends support to the formation of the isonicotinyl hydrazone on storage of solid antituberculosis FDC formulations under accelerated stability testing conditions. The fact that some bases were able to convert isoniazid and rifampicin quantitatively into the isonicotinyl hydrazone, confirmed the possibility of catalysis of the reaction by pyrazinamide and ethambutol hydrochloride. It was thus postulated that pyrazinamide and ethambutol hydrochloride play a catalytic role through participation of *intra*-molecular proton transfer during the reaction between rifampicin and isoniazid (Bhutani *et al.*, 2005b:899).

Mariappan *et al.* (2005:646) reported that any amount of the isonicotinyl hydrazone that may be present initially in antituberculosis FDC formulations containing rifampicin and isoniazid or that is formed *in situ* in the stomach upon administration of these products, would result in a loss of therapeutic activity of rifampicin. This is because the isonicotinyl hydrazone lacks *in vivo* activity. Even its conversion to 3-formyl rifamycin does not help because it is also inactive *in vivo*.

3.3.1.1 The Influence of Temperature, Humidity, Light and Packaging Type on the Stability of Antituberculosis Products

Insufficient storage and distribution of pharmaceutical products can lead to their physical decline and chemical decomposition. This would result in reduced activity and, sometimes, in the formation of toxic degradation products. Degradation is particularly likely to occur under tropical conditions with high ambient temperature and humidity (WHO, 1997:4).

Singh & Mohan (2003:298) carried out a stability study to determine possible reasons for the poor bioavailability of rifampicin in certain FDC antituberculosis products. Earlier data indicated that rapid decomposition of rifampicin in the presence of isoniazid in FDC antituberculosis products under acidic stomach conditions was the likely reason behind the problem (Singh *et al.*, 2000a:405; 2000b:491; 2001:5). It was suggested by Pillai *et al.* (1999:S309) that there were perhaps, in addition to *in situ* stomach decomposition, some formulation-specific problems in those FDC preparations being collected from the market and subjected to bioequivalence studies.

The products studied by Singh & Mohan (2003:299) were stored at 40 °C / 75 % RH in the presence of light. The control samples showed no visual or chemical change from the original

state on storage at room temperature for the duration of the study. The strip-packed products kept in humidity and photo stability chambers were also not affected when compared with the control samples. The blister-packed products were stable in the humidity chamber, but colour changes occurred in the light chamber. The changes were extensive in the unpacked tablets under both dark and light conditions (Singh & Mohan, 2003:299). The physical changes after 5 days are shown in Figure 3.5.

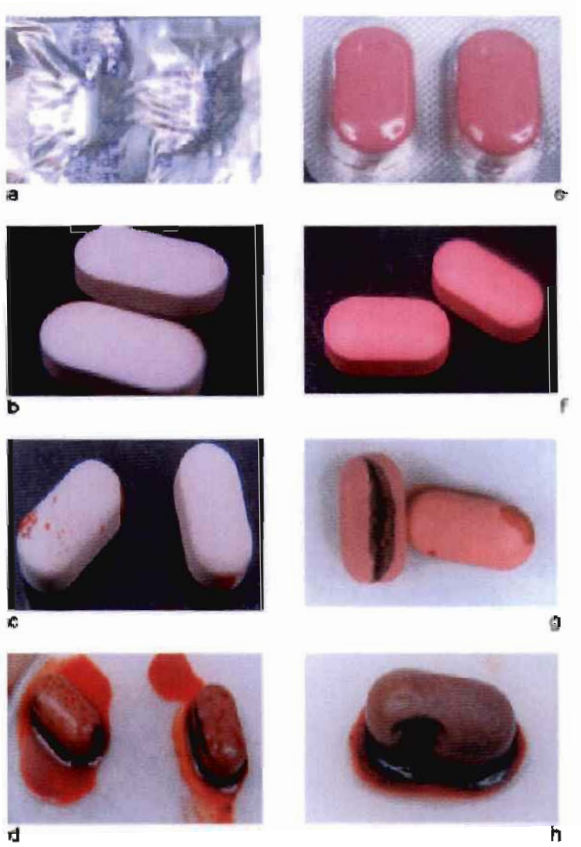


Figure 3.5: Packed and unpacked marketed products after exposure to humidity and light for 5 days. (a) Strip pack, packed control; (b) unpacked control; (c) exposed to 40 °C / 75 % RH; (d) exposed to 40 °C / 75 % RH / light; (e) blister pack, packed control; (f) unpacked control; (g) exposed to 40 °C / 75 % RH; (h) exposed to 40 °C / 75 % RH / light (Singh & Mohan, 2003:300)

Red specks, as shown in Figure 3.5(c), appeared on the surfaces of two brands of unpacked tablets after storage in a dark humidity chamber. In two other brands, bursting of the tablets

was apparent (see Figure 3.5(g)). In the photo stability chamber the colour initially faded, followed by the appearance of red specks and then "bleeding" (see Figures 3.5(d) and 3.5(h)).

The "bleeding" can be attributed to ethambutol hydrochloride's hygroscopicity. The hygroscopic ethambutol hydrochloride dissolves in the absorbed moisture. This solution may then serve as a solvent for the other APIs in the tablet, resulting in the so-called "bleeding". Figure 3.6 shows one such example.



Figure 3.6: "Bleeding" of a 4FDC tablet in the pocket of a marketed strip pack that was within one year of the expiry date (Singh & Mohan, 2003:299)

The products in unpacked conditions showed rifampicin decomposition of about 60 % and extensive physical changes. The main decomposition product in the solid state was the isonicotinyl hydrazone resulting from 3-formyl rifamycin (the hydrolysis product of rifampicin and isoniazid). It was suggested that there is a possibility of instability of rifampicin within the formulation environment, which might be responsible, in addition to *in situ* decomposition of rifampicin, for the poor bio-availability.

In light of the abovementioned significant study performed by Singh & Mohan, commercially available 4FDC antituberculosis tablets supplied by the WHO, were exposed to 40 °C / 75 %RH conditions for a period of one week as part of this study. In Figure 3.7, the control samples are on the left. The samples on the right were pricked with a pin before exposure, but the samples in the middle were exposed intact.

It can be seen that "bleeding" might start after a longer period of exposure, indicating that these products are of better quality as those used in the abovementioned study.

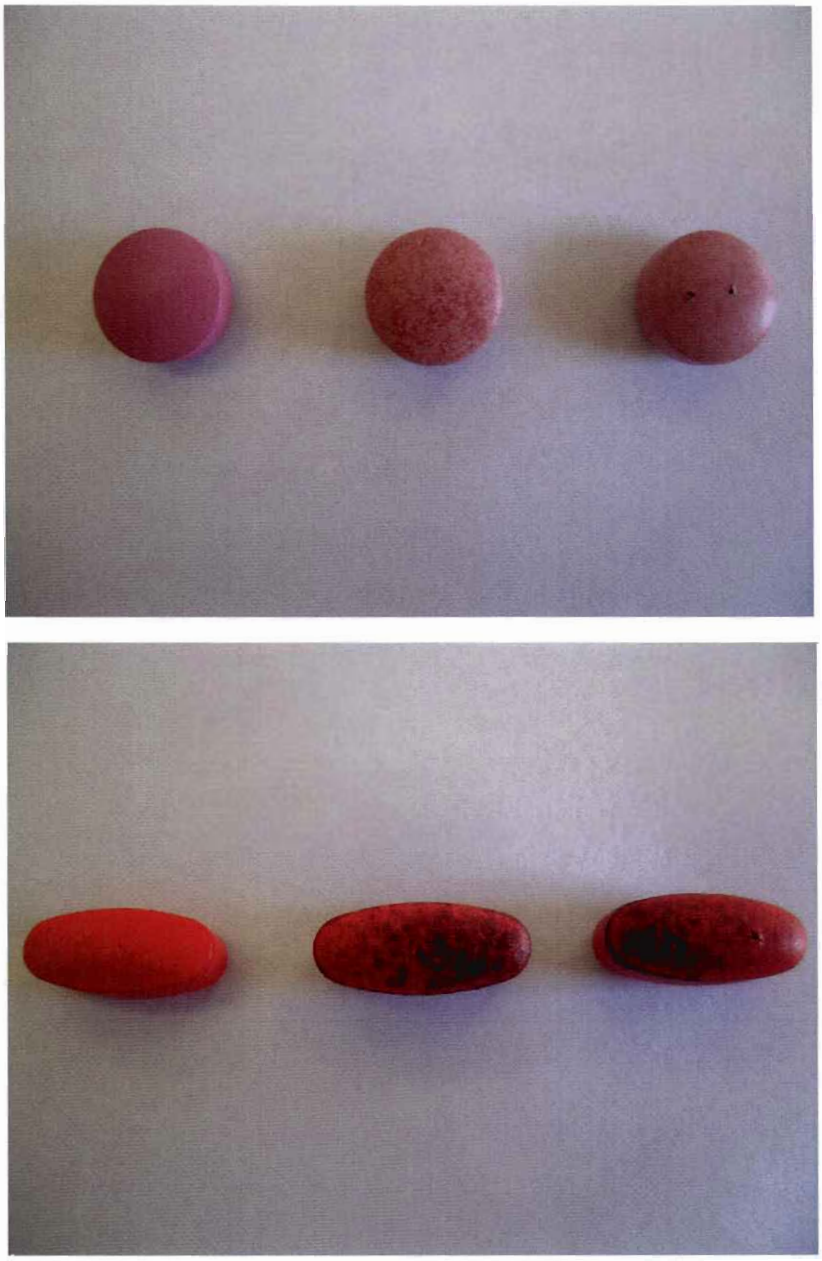


Figure 3.7: 4FDC antituberculosis products exposed to 40 °C / 75 %RH for one week. Left: control; middle: exposed; right: pricked with pin and exposed

3.3.2 *In situ* under stomach acid conditions

Among the diverse physical and chemical factors that can possibly be held accountable for the variable bio-availability of rifampicin from FDC antituberculosis products, one that explains most of the issues related to this typical problem is the rapid decomposition of rifampicin in the presence of isoniazid *in situ* in stomach acid conditions (Sing *et al.*, 2001:16).

Singh *et al.* (2000b:491) conducted a study under stomach conditions to determine the extent of degradation of rifampicin, isoniazid and pyrazinamide in marketed preparations containing single, two, three and four APIs.

In all pure API mixtures, rifampicin was decomposed between 17.8 to 24.4 %, while isoniazid was decomposed between 3.2 and 4.7 % only. Pyrazinamide was found to be stable and like ethambutol, did not influence the decomposition of rifampicin under these conditions.

In comparison with the pure drugs, wide variations in the decomposition of rifampicin (7.5 to 33.3 %) and isoniazid (1.4 to 5.3 %) were found when marketed FDCs were exposed to the same acidic conditions. This signified that the formulation and packaging / storage conditions may strongly affect the degradation, or not, of rifampicin and isoniazid. The results further suggested that the poor bioavailability of rifampicin and isoniazid in FDC products may also, to some extent, be due to the decomposition of the drug in the stomach.

In another publication, Singh *et al.* (2000a:405) proposed a reason for the increase in decomposition of rifampicin in the presence of isoniazid under acidic conditions. Degradation studies were performed in 0.1 M HCl at 37 °C, in the absence of and in combination with isoniazid. Both rifampicin and isoniazid were analysed. They proposed that once 3-formyl rifamycin is formed under acidic conditions, it interacts with isoniazid to form the hydrazone (through a second-order reaction). The hydrazone regenerates isoniazid and 3-formyl rifamycin by a pseudo first-order reaction. Because the second-order forward reaction is faster than the former reaction (rifampicin to 3-formyl rifamycin) and the following (hydrazone to 3-formylrifamycin and isoniazid) first-order reactions, the formation of the hydrazone is largely favoured. The decomposition of rifampicin to 3-formyl rifamycin is pushed forward and an increase of the degradation of rifampicin is observed.

The pathway for the decomposition of rifampicin under acidic conditions is shown in Figure 3.8. The reaction is reversible.

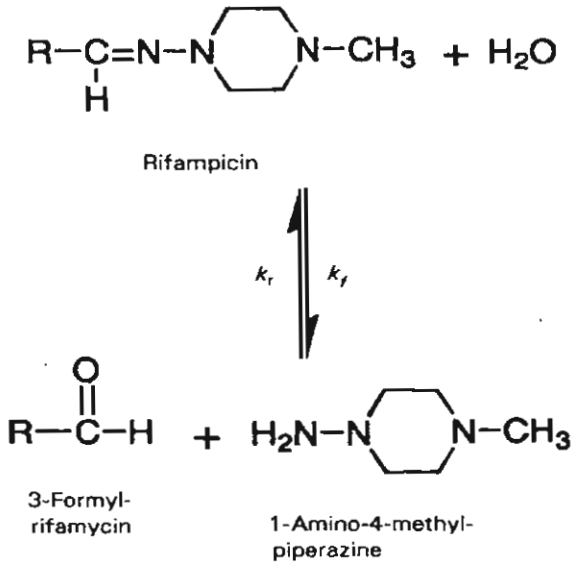


Figure 3.8: Pathway for the decomposition of rifampicin under acidic conditions (Singh *et al.*, 2000a:409)

The pathways for the formation of the isonicotinyl hydrazone, and for the decomposition of rifampicin in the presence of isoniazid are shown in Figures 3.9 and 3.10, respectively.

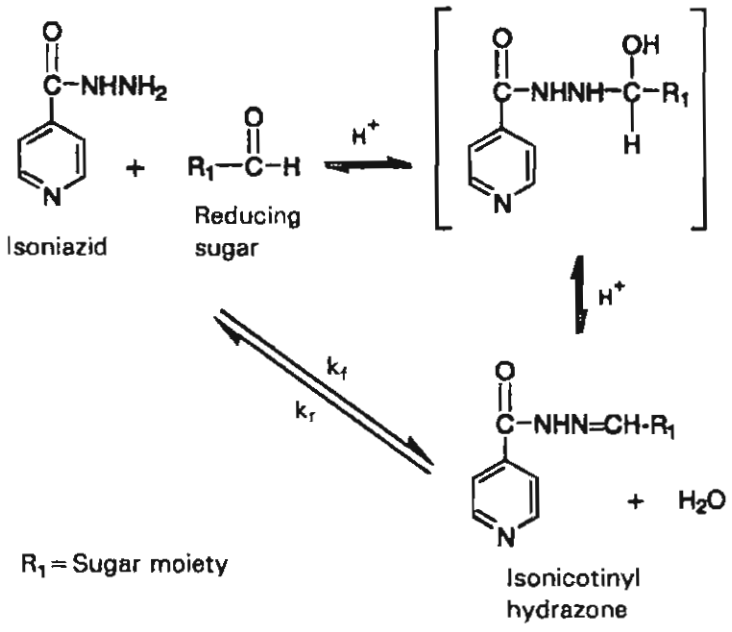


Figure 3.9: Pathway for the hydrazone formation (Singh *et al.*, 2000a:409)

The formation of the isonicotinyl hydrazone of 3-formyl rifamycin in solution needs not be through the hydrolysis product (3-formyl rifamycin) of rifampicin. A reactive group on rifampicin, namely the hydrazone or imino group, can react with the hydrazine group of isoniazid. This reaction will be pH-dependent, since the isoniazid hydrazine group will only be reactive in the unprotonated form (Dekker & Lötter, 2003:205).

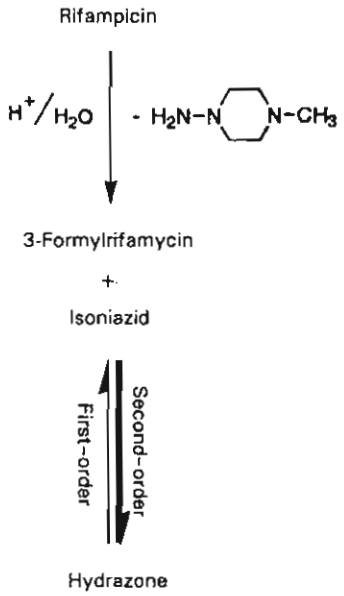


Figure 3.10: Proposed pathway for the decomposition of rifampicin in the presence of isoniazid (Singh *et al.*, 2000a:409)

3.4. CONCLUSION

Solubility properties are important considerations in the development of monographs, for instance regarding the preparation of standard and sample solutions. According to the BCS classification, ethambutol, isoniazid and pyrazinamide are each highly soluble over a wide pH range. Rifampicin, however, is highly soluble at a lower pH only. Such lower pH values could thus lead to a decrease of rifampicin stability, especially when in combination with isoniazid, due to the formation of the isonicotinyl hydrazone.

The low pH of the stomach fluids hence causes an increase in the degradation of rifampicin that is augmented when rifampicin and isoniazid are in combination . Low pH values should clearly be avoided during analysis.

Unsuitable storage conditions of 4FDC products, containing rifampicin, may lead to decomposition and the formation of degradation products. If packaging is inappropriate, moisture may be absorbed by the product and cause the hygroscopic ethambutol hydrochloride to dissolve. This could cause the other APIs in the tablet to dissolve also, i.e. so-called "bleeding". Pyrazinamide and ethambutol hydrochloride may be catalytic towards the reaction between isoniazid and rifampicin (Bhutani *et al.*, 2004:1073), with ethambutol hydrochloride affecting this reaction to a larger extent than pyrazinamide. This can be ascribed to the hygroscopicity and acidic nature of ethambutol hydrochloride (Bhutani *et al.*, 2005b:895).

The specifications of FDC products containing rifampicin and isoniazid should not only provide for the known degradants, namely rifampicin-quinone, rifampicin N-oxide and 3-formyl rifamycin (BP, 2005; USP, 2006:1917), but also for another degradation product, namely isonicotinyl hydrazone.

Methods to quantify these degradation products individually were to be developed for products containing rifampicin and isoniazid in combination, as discussed in Chapter 5.

Factors that require consideration in the quality control of FDCs, include formulation, and suitable packaging and storage, as these may affect API degradation, bioavailability, patient compliance and subsequently the successful curing of TB.

CHAPTER 4

Monograph Development

A pharmacopoeia (from the Greek word, *pharmakopoiia*, meaning: preparation of drugs) is a book describing drugs and drug preparations used in medicine. The text is issued by an official authority and considered as the authoritative standard. Originally pharmacopoeias were collections of recipes to guarantee unfailing communication between physician and pharmacist, but have changed with time into a book of standards describing the quality specifications of raw materials and pharmaceutical products (Globepharm, 2005).

According to Globepharm (2005), the major pharmacopoeias in use worldwide today are:

* The International Pharmacopoeia (IntPh) – primarily used in the developing nations;
* British Pharmacopoeia (BP) – primarily used in the 54 member countries of the British Commonwealth;
* United States Pharmacopoeia (USP) – primarily used in North America and in 27 other countries; and
* European Pharmacopoeia (PhEur) – primarily used in the 26 countries which are member states of the Council of Europe.

The first edition of the United States Pharmacopoeia (USP) was issued in 1820, and the first edition of the British Pharmacopoeia (BP) in 1864. The need for an International Pharmacopoeia (IntPh) was perceived early and work on the IntPh dates back to 1874 (Globepharm, 2005). The first volume of the first edition of The International Pharmacopoeia was published in 1951 with the aim of harmonizing quality requirements for pharmaceutical substances worldwide (WHO, 2003a:23).

The WHO requested the Research Institute for Industrial Pharmacy to develop the following monographs for antituberculosis products for The International Pharmacopoeia: rifampicin capsules; rifampicin tablets; 2FDC tablets containing isoniazid and ethambutol hydrochloride; 2FDC tablets containing rifampicin and isoniazid; 3FDC tablets containing rifampicin, isoniazid and pyrazinamide; and 4FDC tablets containing rifampicin, isoniazid, pyrazinamide

and ethambutol hydrochloride. The strengths of these products are given in the WHO model list of essential medicines (available at: http://whqlibdoc.who.int/hq/2005/ a87017_eng.pdf). These monographs should conform in structure and specifications to the standard format of the IntPh.

4.1. PURPOSE OF PHARMACOPOEIAL MONOGRAPHS

The International Pharmacopoeia provides international standards for the content, purity and quality of APIs, pharmaceutical products and excipients affecting international trade. A product is not of pharmacopoeial quality, unless it complies with all the stated requirements. The goals are the promotion of good quality pharmaceutical products, and the existence of quality control methods to detect counterfeit drugs as to assure the safety and efficacy of medical treatments worldwide. This is also crucial for promoting successful programmes for eradication or control of diseases and to avoid the emergence of drug resistance. The development of screening tests and pharmacopoeial monographs for therapeutic agents, including combination products, to treat tuberculosis, malaria and HIV / AIDS, currently receive highest priority (WHO, 2003a:23,24).

According to the British Pharmacopoeia Announcement website (2000) (available at: http://www.pharmacopoeia.org.uk/monographdev.cfm), each monograph taken as a whole for formulated preparations should present a reliable basis for making an independent judgement as to the quality of the preparation in the interest of the safety of the public.

A USP-NF monograph for an official substance or preparation includes the article's definition (packaging, storage and other requirements) and a specification. The specification consists of a series of universal (description, identification, impurities, and assay) and specific tests, one or more analytical procedures for each test, and acceptance criteria (USP, 2006:v).

4.2. FORMAT OF THE INTERNATIONAL PHARMACOPOEIA MONOGRAPHS

The monographs of the IntPh have a specific format required for full control of the medicine. Typically a monograph for tablets will include the following (IntPh, 2003:170):

* Category

  (For example: Antituberculosis);

* Storage

  (For example: Rifampicin tablets should be kept in a tightly closed container, protected from light);

* Additional information

  (For example: Strength in the current WHO Model List of Essential Medicines; and other important information such as polymorphic form);

* Requirements

  - General

    (For example: Complies with the monograph for "Tablets");

  - Definition

    (For example: Isoniazid tablets contain not less than 90.0 % and not more than 110.0 % of the amount of isoniazid ($C_6H_7N_3O$) stated on the label);

  - Identity tests

    (The identification tests should be specific for identification of the API(s) and more than one test may apply for this purpose);

  - Assay

    (The assay method is normally specific; a non-specific method may be used if the related substances method is specific) (ICH Q6A, 1999:7);

  - Related substances

    (This tests is important for the safety of the product); and

  - Dissolution test

    (Currently, references only state the general chapter).

4.3. METHOD DEVELOPMENT

4.3.1 Identity tests

The ICH guideline Q6A (1999:7) states the following requirements for the identification of APIs in a pharmaceutical product:

Identification testing should establish the identity of the new drug substance(s) in the new drug product and should be able to discriminate between compounds of closely related structure which are likely to be present. Identity tests should be specific for the new drug substance, e.g., infrared spectroscopy. Identification solely by a single chromatographic retention time, for example, is not regarded as being specific. However, the use of two chromatographic procedures, where the separation is based on different principles, or combination of tests into a single procedure, such as HPLC / UV diode array, HPLC / MS, or GC / MS, is generally acceptable.

The requirements are equally applicable for the identification of well-known APIs in generic products.

An infrared spectroscopic method was developed as a stand-alone test (Test A) for rifampicin tablets and capsules. This was achieved through extraction of the tablets with dichloromethane, followed by evaporation of the solvent and comparing the IR spectrum of the residue with that of rifampicin reference standard.

As an alternative way of identifying rifampicin in the rifampicin tablets and capsules, a set of tests (B, C and D) were developed.

* Test B is based on a purple colour that is produced when treating a hot solution of an aqueous extract of the tablet / capsule powder with copper(II) sulfate.
* Test C is a TLC test, based on the TLC test for the related substances test in the monograph of rifampicin in The International Pharmacopoeia (1988:280). The mobile phase in the latter monograph consists of chloroform:methanol (85:15). Because of safety issues, chloroform has been substituted for dichloromethane with good effect. The chromatogram could be examined immediately in daylight, considering the fact that rifampicin has a reddish-brown colour.
* Test D is based on the retention time similarity of the sample and standard solutions in the HPLC assay method.

Infrared spectroscopy was not considered for identification of the individual APIs in FDC products. This would have required separation of the APIs, which was considered impractical. Consequently two identification tests (HPLC and TLC) were developed. The HPLC test was completely based on the assay method (see below), involving a comparison of the retention times

of the relevant API peaks in the test and reference solutions. The HPLC method being used is completely described in Chapter 5.

The TLC method has the option of observing the spots by either UV or daylight detection.

For the UV detection of TLC spots, a method that was supplied by the WHO (2002b) was used. The mobile phase consisted of methanol:strong ammonia (100:1.5), with commercial TLC plates covered with silica gel F254 as stationary phase. The spots were best observed by examining the plate under UV light (254 nm) after its exposure to iodine vapours. The plate could be inspected in daylight after iodine exposure, although pyrazinamide was only vaguely visible. The spot-intensity of isoniazid, pyrazinamide and ethambutol hydrochloride is concentration dependent and can be used for the semi-quantitative determination of these APIs in antituberculosis FDCs. This TLC method is completely described in Chapter 6.

4.3.2 The assay

One or more specific HPLC methods were to be developed for determining the API content in FDCs. General requirements set for HPLC methods were:

* If possible one method should be applicable for the assay of more than one API;
* The column(s) should be easily available;
* The use of an isocratic profile instead of a gradient profile, so that the test could be readily carried out by any test laboratory;
* The method(s) should be stability-indicating; and
* It would be advantageous if the related substances could be determined with the same method(s) used for assay.

It was possible to develop two basic methods that would enable:

1. The assay of ethambutol hydrochloride, isoniazid and pyrazinamide simultaneously in FDC products, containing any one or more of these APIs; and
2. The assay of rifampicin in FDC products and in rifampicin tablets / capsules. This method was also suitable for the determination of all known rifampicin related substances in these products.

HPLC methods were thus developed and validated for the analysis of:

* Ethambutol hydrochloride and isoniazid content in a 2FDC product containing ethambutol hydrochloride and isoniazid:
* Isoniazid and pyrazinamide content in a 3FDC product containing isoniazid, pyrazinamide and rifampicin;
* Ethambutol hydrochloride, isoniazid and pyrazinamide content in a 4FDC product containing ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin; and
* Rifampicin content in FDC products containing rifampicin and in rifampicin tablets / capsules.

The HPLC methods are fully described in Chapter 5.

4.3.3 Related substances

Related substances may already be present in the API raw material, prior to its use in manufacture, or they may form during the manufacturing process, or in the final product during storage. Upon consultation with WHO, it was decided to limit the related substances to the most unstable API present in the proposed FDC formulations. In this study it was necessary to develop a method for rifampicin degradation products (rifampicin-quinone, rifampicin N-oxide and 3-formylrifamycin) and for the measurement of the degradation product being formed due to the interaction between isoniazid and rifampicin, i.e. the isonicotinyl hydrazone (refer to discussion in Chapter 3). The HPLC method used for determining the rifampicin content in FDCs, proved to be useful also for analysing these related substances (refer to Chapter 5).

4.3.4 Dissolution

Although not requested by the WHO, it was decided to develop a dissolution test for isoniazid and ethambutol hydrochloride tablets, for which the WHO had supplied a number of different brands of products.

APIs belonging to BCS Class I and Class III are highly soluble, thus isoniazid, ethambutol and pyrazinamide can be considered as highly soluble (WHO, 2006g:397).

The purpose of this study was:

* To generate the dissolution profiles of the products being tested in this study, according to the recommended WHO conditions, and to compare the resulting outcomes with those as outlined in the WHO draft guideline "*In vitro* dissolution testing methods for oral immediate release drug products containing Biopharmaceutics Classification System (BCS) Class I drugs" (WHO, 2005f:16); and
* To establish if the dissolution conditions and specifications being recommended in the said guideline are appropriate for controlling this type of FDC product.

A dissolution method was successfully developed. The assay method for ethambutol hydrochloride and isoniazid tablets was used to analyse the dissolution samples.

This method, together with the results, were submitted to the WHO. These were accepted for inclusion in the monograph for ethambutol hydrochloride and isoniazid tablets. For the first time ever a comprehensive dissolution method was included in a monograph in The International Pharmacopoeia.

This method is completely described in Chapter 7.

4.3.5 Peer review and approval of monographs for The International Pharmacopoeia

The developed monographs were compiled and submitted to WHO by June 2004. They were distributed for review within the WHO advisory channels and were discussed at two WHO consultation meetings during 2004 and 2005. During this process the feed-back of WHO was considered and the monographs updated accordingly.

The monographs served at the following two committee meetings:

- WHO 39th Expert committee on specifications for pharmaceutical preparations, Geneva, 25-29 October 2004; and
- WHO 40th Expert committee on specifications for pharmaceutical preparations, Geneva, 24-28 October 2005.

The monographs were accepted at the latter meeting.

The monographs were officially approved by the resolution in WHO's Technical report series 937 (WHO, 2006h:7) which reads:

> *It was noted that preliminary drafts of monographs for various new antituberculosis drugs had been discussed at previous meetings. The Committee discussed and adopted the monographs for the following finished dosage forms:*
>
> - *rifampicin tablets*
> - *rifampicin capsules*
> - *rifampicin + isoniazid tablets*
> - *rifampicin + isoniazid + pyrazinamide + ethambutol HCl tablets*
> - *isoniazid + ethambutol HCl tablets*
> - *rifampicin + isoniazid + pyrazinamide tablets.*

These will be included in the fourth edition of the IntPh. In the meanwhile they are posted on the WHO website at:

http://www.who.int/medicines/publications/pharmacopoeia/mon_tb/en/index.html

4.4. CONCLUSION

Pharmacopoeial monographs for the following antituberculosis products were developed according to the requirements of the IntPh:

* Rifampicin capsules;
* Rifampicin tablets;
* Isoniazid and ethambutol hydrochloride tablets;
* Rifampicin and isoniazid tablets;
* Rifampicin, isoniazid and pyrazinamide tablets; and
* Rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride tablets.

For these monographs, methods were developed for identification, assay and related substances. The development of the TLC identification method, the HPLC methods for assay of all the APIs, and the determination of the rifampicin related substances are described comprehensively in the chapters to follow.

A dissolution test was also successfully developed for isoniazid and ethambutol hydrochloride tablets.

Through a series of peer reviewing and WHO meetings, the proposed monographs were approved for The International Pharmacopoeia, during a meeting of the 40th WHO Expert Committee on Specifications for Pharmaceutical Preparations (WHO, 2006h:7). These approved monographs are available on the WHO website, of which copies are included in this thesis in Appendices H to M.

CHAPTER 5

Development of HPLC Methods for Analysis of Antituberculosis Products: Assay and Rifampicin Related Substances

5.1. OBJECTIVE

To develop and validate HPLC methods for the analysis of the active pharmaceutical ingredients (APIs) and related substances of rifampicin in antituberculosis FDC products.

The FDC products for which monographs were developed for the IntPh included:

* Rifampicin capsules;
* Rifampicin tablets;
* Isoniazid and ethambutol hydrochloride tablets;
* Rifampicin and isoniazid tablets;
* Rifampicin, isoniazid and pyrazinamide tablets; and
* Rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride tablets.

All APIs in each of these products required analysis, using a method(s) that is specific for each API. It would be advantageous to limit the number of methods so that each API would not need a separate HPLC assay.

For this study existing HPLC methods were used as point of departure, due to the general specificity of the HPLC technique, as well as information from available literature. The chosen methods needed full validation.

According to the WHO guidelines for FDCs (WHO, 2005e:94), careful consideration of the compatibility of APIs in FDC products is essential (see Chapter 2). Of the four APIs being

examined, rifampicin is generally known for its instability (see Chapter 3), while the other three APIs intrinsically are relatively stable.

In Chapter 3, the reaction between rifampicin and isoniazid to form the isonicotinyl hydrazone, was discussed. Since this reaction may occur in solid oral dosage formulations, a method for identifying and determining the presence of the hydrazone had to be developed.

In consultation with WHO, it was decided that only those degradation products relating to rifampicin would be included in the monographs.

The steps that were followed in developing the monographs included:

* Identifying existing HPLC methods from available literature and from methods used by manufacturers;
* Screening those methods for their potential;
* Selecting the best alternatives for application and / or further development; and
* To start with the analysis of the more complex 4FDC tablets and to adjust those methods for the lower combinations and eventually for rifampicin tablets and capsules. It was believed that the methods suitable for the 4FDC product analysis would apply to the 3FDCs, etc.

The purposes of screening the HPLC methods were to establish:

* Their validation performance;
* Their suitability to analyse the different APIs; and
* Their stability indicating ability.

5.2. USEFUL EXISTING HPLC METHODS

Possible methods that were identified for the analysis of 4FDC tablets from literature and from those used by manufacturers are summarised in Table 5.1.

Table 5.1: Useful existing HPLC methods for the development of new HPLC methods (Continue on next two pages)

Active Ingredients	Source of Information	Mobile phase	Column	Flow rate (ml/min.)	Injection volume (µl)	Detection (UV) (nm)
Isoniazid, pyrazinamide and rifampicin	Calleri *et al.* (2002:1091)	Acetonitrile (A) and 50 mM phosphate buffer (pH 3.5) (B). Gradient profile (A:B) 3:97 v/v for 5 min., linear gradient to 50:50 v/v at 30 min., 3:97 v/v in 10 min.	Lichrospher 100 RP18, 5 µm, 250 x 4.0 mm; and Lichrospher 100 RP18, 5 µm (guard column)	1.0	Not available	Rifampicin:254 Isoniazid:261 Pyrazinamide:265
Isoniazid, pyrazinamide and rifampicin	Gaitonde & Pathak (1991:1203)	20 % 0.005 N Tetrabutyl ammonium hydroxide (pH 3.0) and 80 % methanol	Cyano-bonded phase ODS	1.5	Not available	265
Ethambutol hydrochloride	Manufacturer A (2004)	940 ml Buffer (pH 5.0, consisting of 0.2 g copper(II) acetate and 50 g ammonium acetate) and 60 ml methanol	Prodigy ODS (2), 5 µm; or Lichrospher 60 RP Select B, 5 µm, 125 x 4.0 mm; or Luna C_{18} (2), 5 µm, 150 x 4.0 mm	2.0	20	270
Isoniazid	Manufacturer A (2004)	600 ml Docusate sodium buffer and 600 ml water, pH adjusted to 2.5	µBondapak C_{18}, 10 µm, 250 x 4.6 mm	1.5	10	254
Pyrazinamide	Manufacturer A (2004)	1 000 ml Phosphate buffer solution (pH 8.0) and 10 ml acetonitrile	µBondapak C_{18}, 10 µm, 150 x 3.9 mm	1.0	20	270
Rifampicin	Manufacturer A (2004)	Water:acetonitrile:phosphate buffer:1.0 M citric acid:0.5 M sodium perchlorate (510:350:100:20:20)	Lichrospher 60 RP Select B, 5 µm, 125 x 4.0 mm	1.0	50	254

Table 5.1: Useful existing HPLC methods for the development of new HPLC methods (Continued from previous page)

Active Ingredients	Source of Information	Mobile phase	Column	Flow rate (ml/min.)	Injection volume (µl)	Detection (UV) (nm)
Ethambutol hydrochloride	Manufacturer B (2004)	980 ml Buffer (pH 4.3, consisting of 11.5 g ammonium dihydrogen orthophosphate, 2.0 ml triethyl-amine and 0.3 g copper(II) acetate) and 20 ml methanol	Cosmosil C8 MS, 5 µm, 250 x 4.6 mm; or YMC Pack C8, 5 µm, 250 x 4.6 mm	1.0	50	260
Isoniazid and pyrazinamide	Manufacturer B (2004)	970 ml Ammonium dihydrogen phosphate buffer (pH 5.0) and 30 ml acetonitrile	Cosmosil C8 MS, 5 µm, 250 x 4.6 mm; or YMC Pack C8, 5 µm, 250 x 4.6 mm	1.0	20	254
Rifampicin and its related substances	Manufacturer B (2004)	550 ml Phosphate buffer and 450 ml acetonitrile	Cosmosil C8 MS, 5 µm, 250 x 4.6 mm; or YMC Pack C8, 5 µm, 250 x 4.6 mm	2.0	20	254
Ethambutol hydrochloride, Isoniazid and pyrazinamide	Manufacturer C (2004)	1920 ml Ammonium acetate buffer (with added 2 % cupric acetate solution))(pH 4.2) and 80 ml methanol	Reversed phase µBondapak C_{18}, 10 um, 300 x 3.9 mm	1.5	20	290
Rifampicin	Manufacturer C (2004)	Acetonitrile:phosphate buffer:1.0 M citric acid:0.5 M sodium perchlorate (510:350:100:20). Adjust pH to 7.0	Reversed phase stainless steel column NOVA-PAK, C_{18}, 4 µm, 150 x 3.9 mm	0.8	15	254

Table 5.1: Useful existing HPLC methods for the development of new HPLC methods (Continued from previous page)

Active Ingredients	Source of Information	Mobile phase	Column	Flow rate (ml/min.)	Injection volume (µl)	Detection (UV) (nm)
Rifampicin and its related substances	Mohan *et al.* (2003:608)	Buffer pH 6.8:acetonitrile (96:4) (A) and buffer pH 6.8:acetonitrile (45:55) (B). Gradient profile (A:B) 100:0 for 5 min., linear gradient to 0:100 in 1 min., 0:100 to 25 min.	C_{18} column , 5 µm, 250 x 4.6 mm	1.0	20	238
Rifampicin and its related substances	Sankar *et al.* (2003:734)	65 % Methanol and 35 % 0.01 M phosphate buffer at pH 7.0	Supelcosil LC-18-DB, 5 µm, 250 x 4.6 mm	1.0	20	254

5.3. THE SCREENING OF EXISTING HPLC METHODS

The methods were subjected to a screening process and general observations that were made are summarised below.

5.3.1 Manufacturer A (2004)

This method was found suitable for the analysis of ethambutol hydrochloride, as it required a C_{18} column (currently having the broadest range of HPLC applications and a wide pH stability). The detection wavelength of 270 nm is also ideal for detecting isoniazid and pyrazinamide. Resolution between the isoniazid, ethambutol hydrochloride and pyrazinamide peaks was good and this method appeared suitable for the assay of all three APIs.

For the analysis of rifampicin, Manufacturer A uses a mobile phase comprising of sodium perchlorate, an oxidant that can cause degradation of rifampicin. This method is similar to the USP method for the assay of rifampicin capsules.

5.3.2 Calleri *et al.* (2002:1091)

A gradient profile method is used by Calleri *et al.* (2002:1091) for the analysis of isoniazid, pyrazinamide and rifampicin. Since the WHO preferred that a gradient profile should not be used, the method was not considered any further.

5.3.3 Gaitonde and Pathak (1991:1203)

When this method was tested during the screening process, it appeared that the ODS column used in the analysis of isoniazid, pyrazinamide and rifampicin, caused reproducibility problems. The method was not considered any further.

5.3.4 Mohan *et al.* (2003:608)

This altered USP gradient method for the analysis of rifampicin and its related substances was was found unsuitable during screening. Different C_{18} columns were tried, for example a Hypersil ODS column, a μBondapak column and also a Luna C_{18} column. The retention times were shorter on the Hypersil ODS and μBondapak columns than on the Luna C_{18} column, but the resolution between peaks was poor. Unacceptable peak tailing occurred with the Luna C_{18} column.

5.3.5 Manufacturer B (2004)

The methods for the analysis of ethambutol hydrochloride, and of isoniazid and pyrazinamide were tested, but resulted in unsatisfactory peak tailing for all three APIs.

The pH of the mobile phase used in the analysis of rifampicin and its related substances was found to be 2.56, which is too low for maximum rifampicin stability.

5.3.6 Sankar *et al.* (2003:734-735)

The method used by Sankar *et al.* (2003:734) for determining the decomposition of rifampicin when in combination with isoniazid over a low pH range, gave satisfactory separation of the isoniazid, rifampicin and rifampicin related substances peaks. The peak for the isonicotinyl hydrazone (the degradation product resulting from the interaction of rifampicin with isoniazid) was also well separated from the other peaks. A chromatogram showing separation of rifampicin, isoniazid, isonicotinyl hydrazone, rifampicin quinone and 3-formyl rifamycin, is presented in Figure 5.1.

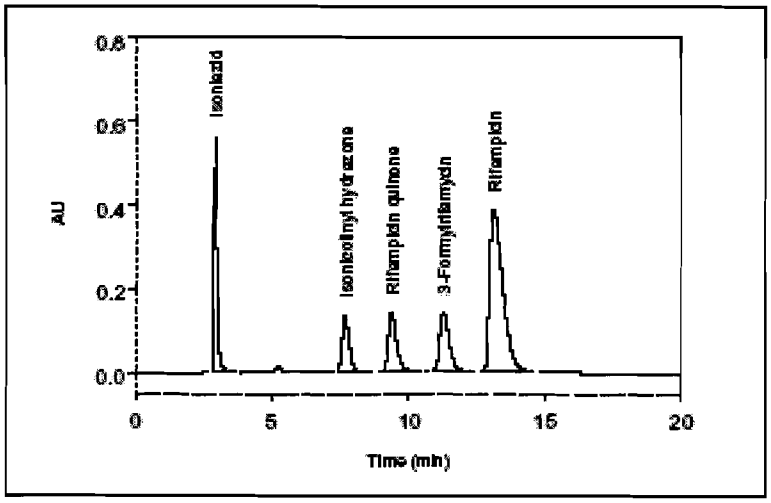


Figure 5.1: Chromatogram showing separation of rifampicin, isoniazid, the isonicotinyl hydrazone, rifampicin-quinone and 3-formyl rifamycin
(Sankar *et al.*, 2003:735)

5.3.7 Conclusion

Two of the methods being screened showed the best potential, namely:

* The method supplied by Manufacturer A (2004) for the analysis of ethambutol hydrochloride, appeared useful for the simultaneous analysis of ethambutol hydrochloride, isoniazid and pyrazinamide; and
* The method which Sankar *et al.* (2003:735) used for studying the decomposition of rifampicin when in combination with isoniazid, over a low pH range, appeared promising as a method for the analysis for rifampicin and its related substances.

It was decided to further investigate both methods, and if successful, only two methods would be needed for the analysis of 4FDC products.

5.4. DEVELOPMENT OF HPLC METHODS

5.4.1. HPLC methods for analysing ethambutol hydrochloride, isoniazid and pyrazinamide

Experimental conditions and results

It was decided to first develop a method for analysis of ethambutol hydrochloride, isoniazid and pyrazinamide combinations, and then to apply this method on products containing any one or more of the three APIs.

The method of Manufacturer A (2004) was used as point of departure. Essentially this method is similar to those of Manufacturers B and C, the difference being in the mobile phase buffer pH. The method of Manufacturer B for the analysis of ethambutol hydrochloride is at pH 4.3. The effect of changes in the buffer over the pH range of 4.2 to 5.0 was studied. Results obtained indicated that a pH of 5.0 was best for analysing an ethambutol hydrochloride, isoniazid and pyrazinamide combination (see figure 5.2). Lower pH values resulted in poor peak resolution, as indicated in Figures 5.3 and 5.4.

Ethambutol hydrochloride lacks absorption in the visible and UV region (Mariappan *et al.*, 2000:345). Copper(II) acetate should therefore be included in the mobile phase buffer, since a complex, with measurable UV absorption, forms between ethambutol and the copper(II) ions.

The addition of copper to the mobile phase did not influence the analysis of isoniazid and pyrazinamide. However, with copper(II) acetate in the mobile phase, the column required longer rinsing times, in order to remove all traces of copper.

Separate solutions of each of the APIs were prepared by using the two variations of the mobile phase, i.e. the one containing copper(II) acetate, and the other without it. These samples were analysed by means of a UV spectrophotometer to determine the wavelengths of maximum absorption for each API. The UV spectra are shown in Figures 5.5 and 5.6.

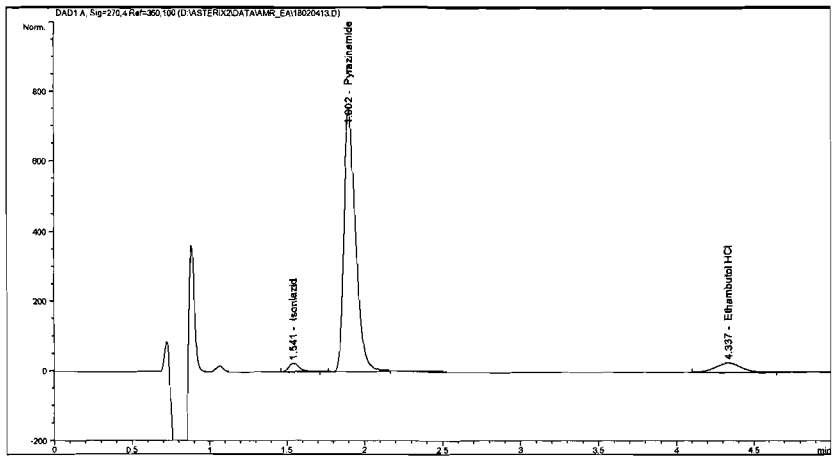


Figure 5.2: Chromatogram of a non-representative mixture containing ethambutol hydrochloride, isoniazid and pyrazinamide (buffer pH 5.0)

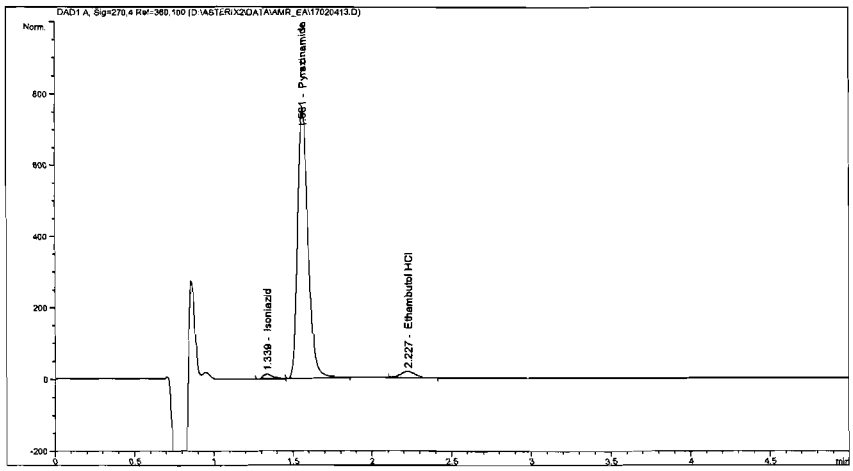


Figure 5.3: Chromatogram of a non-representative mixture containing ethambutol hydrochloride, isoniazid and pyrazinamide (buffer pH 4.5)

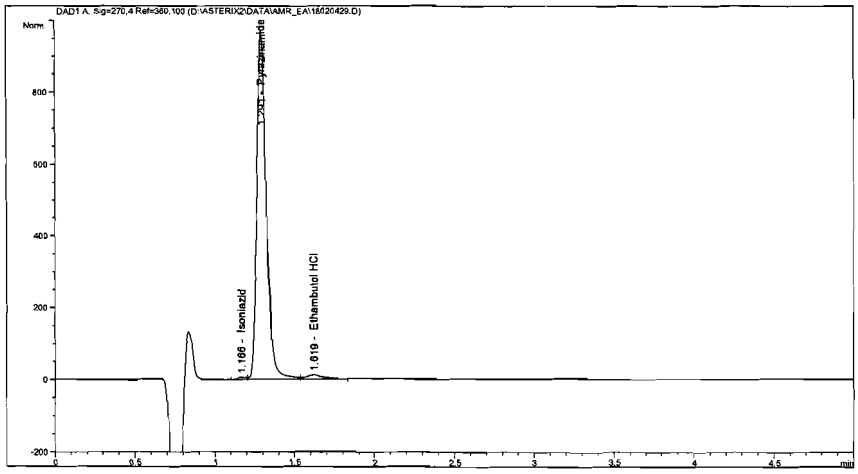


Figure 5.4: Chromatogram of a non-representative mixture containing ethambutol hydrochloride, isoniazid and pyrazinamide (buffer pH 4.2)

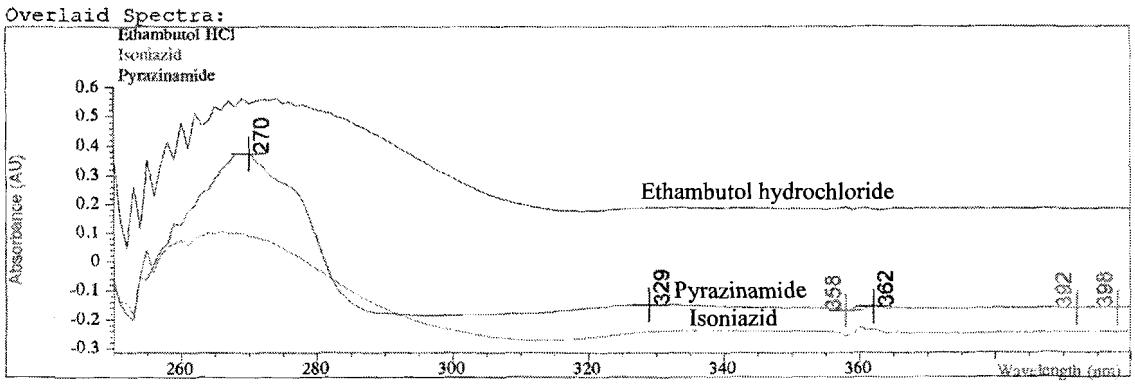


Figure 5.5: UV spectra of the mobile phase solutions (with added copper(II) acetate) containing ethambutol hydrochloride, isoniazid and pyrazinamide

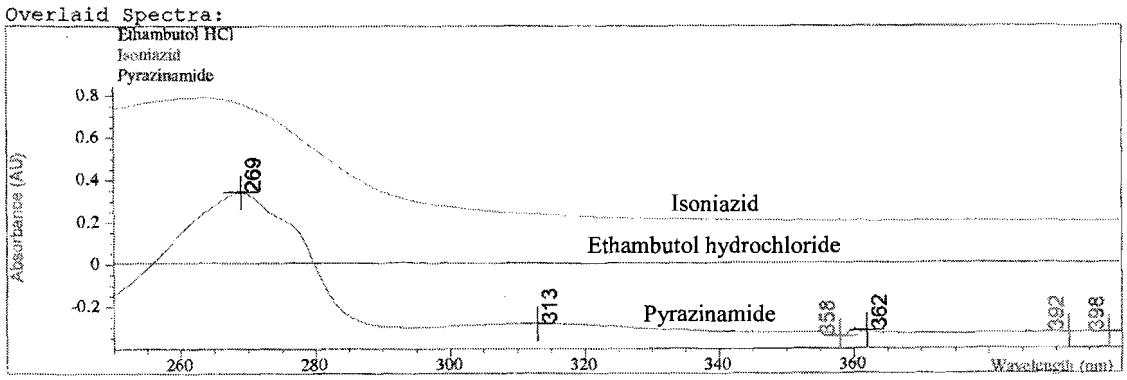


Figure 5.6: UV spectra of the mobile phase solutions (in absence of copper(II) acetate) containing ethambutol hydrochloride, isoniazid and pyrazinamide

Conclusion

Ethambutol hydrochloride did not exhibit UV absorption in the absence of copper. It showed maximum absorption at 270 to 280 nm where copper(II) acetate was present, confirming the necessity of copper in the mobile phase. Isoniazid displayed maximum absorption at about 240 to 265 nm in the absence of copper. Pyrazinamide showed maximum absorption at 270 nm in both the absence and presence of copper.

It was concluded that this method was suitable for analysing ethambutol hydrochloride, isoniazid and pyrazinamide in FDC products, containing any one or more of the three APIs.

5.4.1.1 ANALYSIS OF ETHAMBUTOL HYDROCHLORIDE, ISONIAZID AND PYRAZINAMIDE IN 4FDC PRODUCTS

A 4FDC mixture containing ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin was analysed at a UV wavelength of 270 nm, with copper(II) acetate in the mobile phase. Isoniazid showed acceptable absorption at this wavelength. Satisfactory separation of the ethambutol hydrochloride, pyrazinamide and isoniazid peaks, eluting in this order, was obtained (see Figure 5.7). This assay would be valid if the resolution between the isoniazid and pyrazinamide peaks was at least 2.0. During validation (see Appendix D) the required resolution was achieved, hence confirming the suitability of this method.

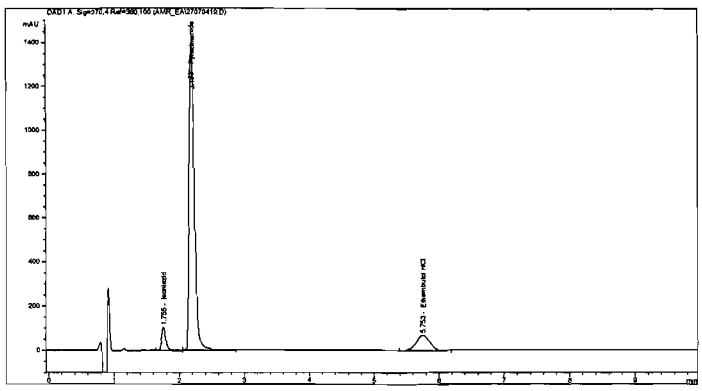


Figure 5.7: Chromatogram of a 4FDC mixture containing ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin

5.4.1.2 ANALYSIS OF ISONIAZID AND PYRAZINAMIDE IN 3FDC PRODUCTS

A 3FDC mixture containing isoniazid, pyrazinamide and rifampicin was analysed at a UV wavelength of 240 nm and without copper(II) acetate in the mobile phase. Isoniazid and pyrazinamide both showed acceptable absorption at this wavelength. This wavelength was chosen to favour the response factor of isoniazid in a typical 3FDC product. Satisfactory separation of the isoniazid and pyrazinamide peaks was obtained, with the resolution being above the required 2.0 (see Figure 5.8). The suitability of this method was also confirmed by the validation outcomes (see Appendix C).

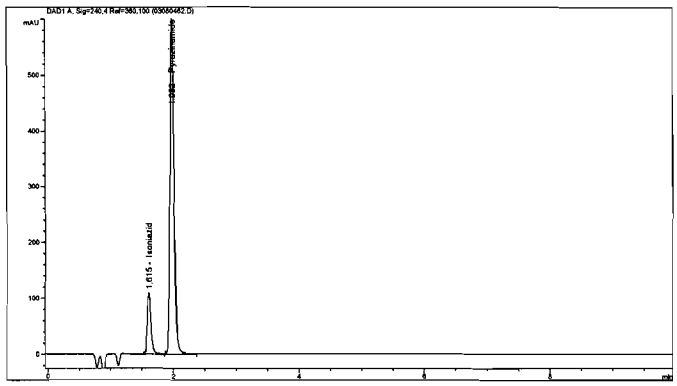


Figure 5.8: Chromatogram of a 3FDC mixture containing isoniazid, pyrazinamide and rifampicin

5.4.1.3 ANALYSIS OF ETHAMBUTOL HYDROCHLORIDE AND ISONIAZID IN 2FDC PRODUCTS

A 2FDC mixture containing ethambutol hydrochloride and isoniazid was analysed at a UV wavelength of 270 nm, with copper(II) acetate in the mobile phase. Both APIs displayed acceptable absorption at this wavelength. Satisfactory separation of the ethambutol hydrochloride and isoniazid peaks was obtained, with the resolution being above the minimum requirement of 10.0 (see Figure 5.2). The validation (see Appendix B) confirmed the suitability of the method.

5.4.2 HPLC method for analysing rifampicin and its related substances

Experimental conditions and results

The method for the analysis of rifampicin and its related substances was partly based on the article by Sankar *et al.* (2003:734), in which the study outcomes of the decomposition of rifampicin in the presence of isoniazid, in the pH range 1 – 3, was reported. In Figure 5.1, it can

be seen that Sankar *et al.* focused on rifampicin, the isonicotinyl hydrazone, rifampicin-quinone and 3-formyl rifamycin, ignoring the degradation product, rifampicin N-oxide.

In this study, however, all of the above related substances, including rifampicin N-oxide, were analysed. The isonicotinyl hydrazone was kindly supplied by Doctor Saranjit Singh, from the National Institute of Pharmaceutical Education and Research, India. A chromatogram of a solution containing rifampicin and its degradation products is shown in Figure 5.9.

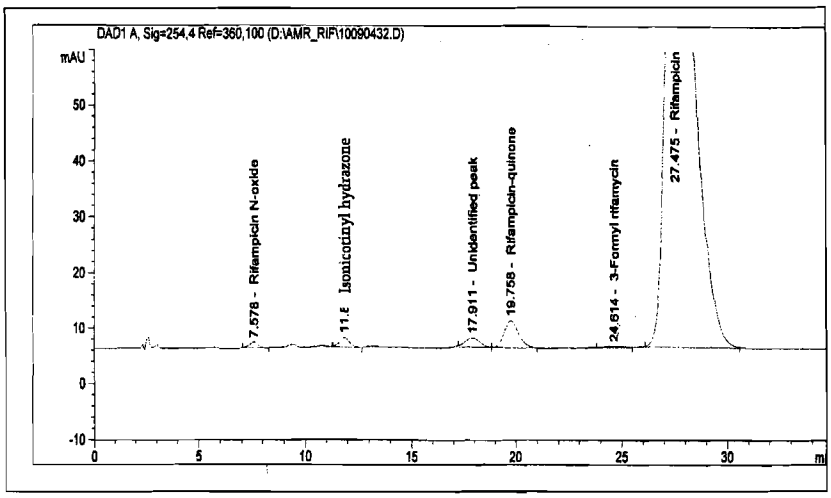


Figure 5.9: Chromatogram of a solution containing rifampicin, the isonicotinyl hydrazone, rifampicin N-oxide, rifampicin-quinone and 3-formyl rifamycin

In Figure 5.10 the chromatogram of a 4FDC mixture that was analysed, using this method, is shown. Due to the absence of copper in the mobile phase, no ethambutol hydrochloride peak was detected. Isoniazid and pyrazinamide, with very short retention times, did not interfere with the analysis of rifampicin and its related substances.

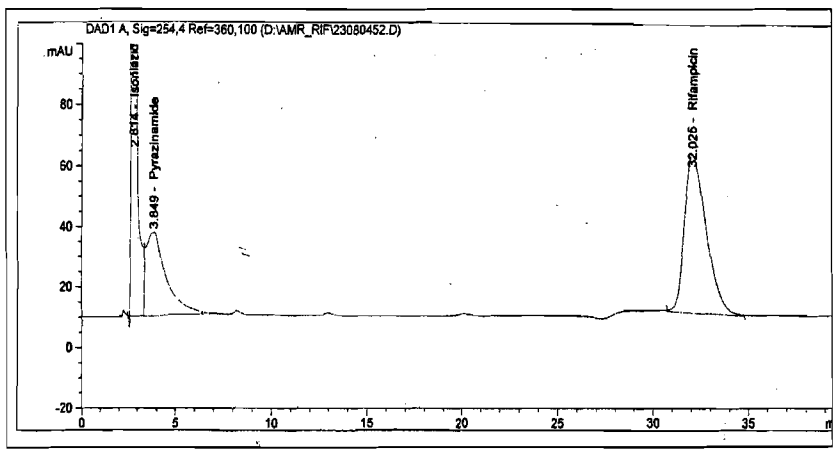


Figure 5.10: Chromatogram of a 4FDC solution analysed by the HPLC method for rifampicin and its related substances

Retention times were approximately 0.7; 0.9, 0.3 and 0.5 for rifampicin-quinone, 3-formyl rifamycin, rifamycin N-oxide and the isonicotinyl hydrazone, respectively. Robustness of this method was indicated by its ability to tolerate small changes in the chromatographic conditions. The assay was valid due to the resolution between the rifampicin and 3-formyl rifamycin peaks being at least 1.5. The validations (see Appendix E to G) confirmed the suitability of the method.

Limits for related substances were based on the British Pharmacopoeia monograph for rifampicin capsules. The isonicotinyl hydrazone limits were based on stability specifications that were obtained from several manufacturers.

Conclusion

This method is ideal for the analysis of rifampicin in combination products, as well as in rifampicin tablets and capsules. Sample solutions should be protected from light, as light increase the degradation rate. Samples should also be analysed promptly after preparation.

5.5. FORCED DEGRADATION

ICH guideline Q1A(R2) (ICH, 2003:2) states the importance of stress testing (forced degradation) as follows:

> *Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.*

Experimental conditions and results

An amount of placebo (see Appendix B), equivalent to a specified amount of sample powder was used to prepare a solution. A sample solution was prepared and diluted 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively. The sample solutions were then stored overnight in closed test tubes at 40 °C / 75 %RH to entice degradation. The placebo and sample solutions were subsequently analysed by means of HPLC.

The resulting chromatograms of all solutions were examined in order to determine whether any additional peaks were formed.

Remaining analyte peaks in the water sample, after exposure to the said conditions of stress, were analysed by means of diode array peak testing in order to determine whether these peaks remained pure. Figures 5.11 and 5.12 show the chromatograms being generated from these samples. Detailed results of the forced degradation studies are reported in Appendices B to G.

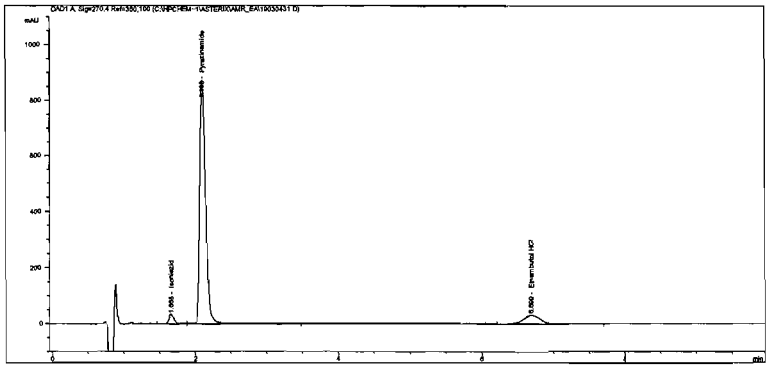


Figure 5.11: Chromatogram of a 4FDC sample diluted with water that has been stressed overnight at 40 °C / 75 %RH and analysed by the HPLC method for analysing ethambutol hydrochloride, isoniazid and pyrazinamide

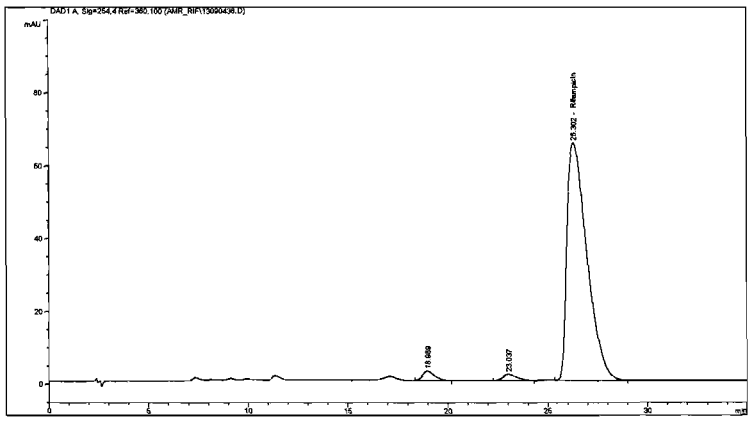


Figure 5.12: Chromatogram of a tablet sample containing rifampicin mixed with water that has been stressed overnight at 40 °C / 75 %RH and analysed by the HPLC method for analysing rifampicin and its related substances

None of the ingredients in the placebo interfered with the analyte peaks (chromatograms are shown in the validation study included in Appendices B to G). Those extra peaks that formed during forced degradation did not interfere with any of the API peaks and their related

substances. Peak purity testing of all the peaks remaining after forced degradation in the water sample, resulted in purity values of more than 999 out of 1 000.

Conclusion

Peak purity testing showed that the peaks remained pure despite forced degradation, thus proving that the method is stability indicating.

5.6. CONCLUSION

The analysis of 4FDC products was successfully achieved by employing only two HPLC methods: one for analysing ethambutol hydrochloride, isoniazid and pyrazinamide, and a second for analysing rifampicin and its related substances.

Since ethambutol hydrochloride lacks absorption in the UV region, cupric ions were added to the mobile phase in order for ethambutol hydrochloride to form a complex with measurable UV absorption.

The conditions of the HPLC method for analysing ethambutol hydrochloride, isoniazid and pyrazinamide in a 4FDC product differed from the HPLC method used when ethambutol hydrochloride was absent from the FDC, for example a 3FDC containing isoniazid, pyrazinamide and rifampicin. When analysing a 4FDC combination containing ethambutol hydrochloride, copper(II) acetate was added to the mobile phase, with detection set at 270 nm. However, copper(II) acetate was not required when ethambutol hydrochloride was absent.

The HPLC method that was reported in literature for the analysis of rifampicin and its related substances had originally not been used for testing rifampicin N-oxide. However, it was found that the method was suitable for the analysis of rifampicin and all of its related substances, i.e. rifampicin-quinone, 3-formyl rifamycin, rifamycin N-oxide and the isonicotinyl hydrazone.

During the analysis of a 4FDC with this method, isoniazid and pyrazinamide did not interfere with the analysis of rifampicin and its related substances. No peak was detected for ethambutol hydrochloride, as it was not UV absorbing.

The separation of the API and related substances peaks was satisfactory and validations (see Appendices B to G) confirmed the suitability of the methods being developed. Peak purity testing after forced degradation confirmed that the methods were stability indicating and thus suitable for stability testing, quality control and batch release of antituberculosis products.

CHAPTER 6

Development of a TLC Method for Identification of Antituberculosis APIs

6.1. OBJECTIVE

To develop a thin layer chromatographic (TLC) method for the identification of the APIs in antituberculosis FDC products.

Existing TLC methods were identified and screened for their applicability. The purpose of the screening was to establish:

* The effectiveness of the observation techniques;
* The possible use of this test for semi-quantitative purposes; and
* The application volume and plate size.

Initially TLC was considered as a limit test for related substances of rifampicin. This was, however, achieved through HPLC analysis (refer to Chapter 5).

6.2. USEFUL EXISTING TLC METHODS

6.2.1 **Kenyon** (1993:3)

Kenyon (1999:3) developed a TLC method, suitable for the identification and quantification of APIs in TB FDC products. Two reference concentrations, representing the upper and lower concentrations for the dosage limits (85 % and 115 or 120 %), are spotted on a TLC plate alongside the sample solution, representing 100 %. The sample solution is spotted between the two reference solutions. The spots are examined visually either by ultraviolet light, or by iodine staining. The drug is considered within specification if the intensity of the sample spots is between the intensity of the high and low reference solution spots.

The conditions for the analysis of isoniazid, pyrazinamide and rifampicin tablets are as follows:

Developing chamber: Polyethylene bag.

Plate: 5 x 10 cm Silica 60 F254 plate.

Mobile phase: Methanol:acetone:ammonium hydroxide (13:17:1).

Application volume: 3 µl.

Detection: The spots of rifampicin can be visually detected by observing the spots in daylight. Spots of isoniazid and pyrazinamide are detected under UV light at 254 nm.

6.2.2 WHO (2002b)

The following TLC conditions have been recommended for the analysis of TB 4FDC products:

Conditioning: Room temperature, TLC-tank lined with filter paper saturated with the mobile phase.

Plate: 10 x 10 cm Silica gel 60 plate (or silica gel 60 F254).

Mobile phase: Methanol:strong ammonia solution (100:1.5).

Application volume: 5 µl.

Detection: Iodine vapour for 15 minutes (sensitivity can be improved by using UV at 254 nm).

6.2.3 The International Pharmacopoeia (1988:280)

The following TLC conditions are recommended for the testing of rifampicin related substances in the IntPh monograph for rifampicin:

Plate: Silica gel.

Mobile phase: Chloroform:methanol (85:15).

Application volume: 20 µl.

Detection: Examine in daylight.

6.3. THE SCREENING OF EXISTING TLC METHODS

The identified methods were subjected to a screening process, by using them for testing antituberculosis products, supplied by the WHO. Observations made regarding each method are discussed below.

6.3.1 **Kenyon** (1999:3)

The mobile phase (methanol:acetone:ammonia (13:17:1) used by Kenyon for the identification and semi-quantitative analysis of rifampicin, isoniazid and pyrazinamide tablets was tested. It gave good separation of the isoniazid and pyrazinamide spots, as well as a well defined rifampicin spot.

6.3.2 **WHO** (2002b)

The TLC method, as described by the WHO, produced well separated spots with the use of the mobile phase consisting of methanol:strong ammonia solution (100:1.5). Unfortunately the rifampicin spot was not well defined, whilst the pyrazinamide spot was only vague after subjecting the plate to iodine vapours. However, visibility was improved when these plates were examined under UV light, after being subjected to iodine vapours. After consultation with WHO, it was decided that in principle this method should be used.

6.3.3 **The International Pharmacopoeia** (1988:280)

The mobile phase chloroform:methanol (85:15), used for related substances in rifampicin (IntPh, 1988:280), produced well separated spots. This method was used for the identification of rifampicin tablets and capsules, by replacing chloroform with dichloromethane.

6.4. TLC METHOD (WITH DAYLIGHT AND UV DETECTION) FOR THE IDENTIFICATION OF FDCs

The following TLC method from the 4FDC guideline was used as a basis for the identification of tablets containing 150 mg rifampicin, 75 mg isoniazid, 400 mg pyrazinamide and 275 mg ethambutol hydrochloride (WHO, 2002b).

Carry out the test as described under "Thin-layer chromatography" (Vol. 1, p. 83), using silica gel R1 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 2 mg rifampicin RS, 1 mg isoniazid RS, 5.3 mg pyrazinamide RS and 3.7 mg ethambutol hydrochloride RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in daylight. The sensitivity can be improved by using silica gel R2 as the coating substance and examining the chromatogram in ultraviolet light (254 nm).

The principal spots obtained with solution A correspond in position, appearance and intensity with those obtained with solution B. Rifampicin may exhibit a second spot with slightly lower Rf value, due to oxidation on the plate before development.

6.4.1. Experimental procedure

Standard solutions were prepared, containing 80 %, 100 % and 120 % of the quantities specified for solution (B) in the above method.

Solution (A) was prepared for one 4FDC tablet, according to the prescribed procedure above.

5 µl each of the standard solutions (80 %, 100 % and 120 %) was applied on 20 x 20 cm plates and on 10 x 20 cm plates. 2.5 µl of each of the 100% standard solutions was also applied on 20 x 20 cm plates.

6.4.2 Results and discussion

Observation techniques

After development of the TLC plate (20 x 20 cm size, and 5 µl application volume), spots were observed in the order as described in Table 6.1. In Figures 6.1 to 6.4 three concentrations (80 %, 100 % and 120 %) of each standard solution were been applied as reference.

Table 6.1: Observation of spots in the TLC chromatogram for FDC tablets

Observation technique	Observation
In daylight, prior to iodine exposure (See Figure 6.1)	Rifampicin spots were visible, although slightly deformed.
Under UV light (254 nm), prior to iodine exposure (See Figure 6.2)	Rifampicin, isoniazid and pyrazinamide were clearly visible. (The intensity of the spots for isoniazid and pyrazinamide was concentration dependent.) Ethambutol hydrochloride was not visible.
In daylight, after iodine exposure (See Figure 6.3)	Rifampicin, isoniazid and ethambutol hydrochloride were clearly visible. (The intensity of the spots for isoniazid and ethambutol was concentration dependent.) Pyrazinamide was vaguely visible (it was not clearly visible on the scanned image).
Under UV light (254 nm), after iodine exposure (See Figure 6.4)	The spots of all APIs were clearly visible. (The intensity of the spots for isoniazid, pyrazinamide and ethambutol hydrochloride was concentration dependent.)

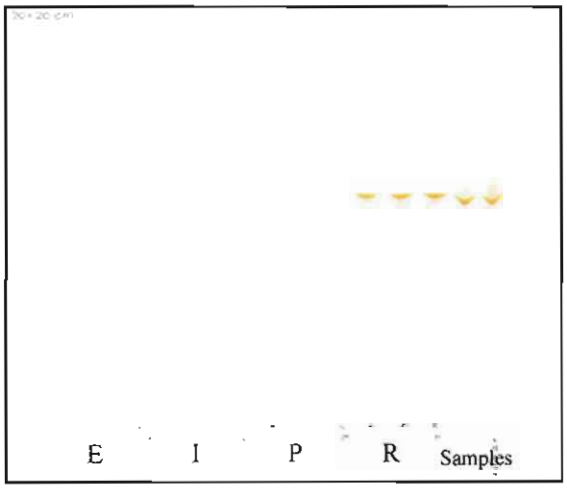


Figure 6.1: Observation of the TLC plate in daylight prior to iodine exposure[1]

[1] E=Ethambutol HCl, I=Isoniazid, P=Pyrazinamide, R=Rifampicin

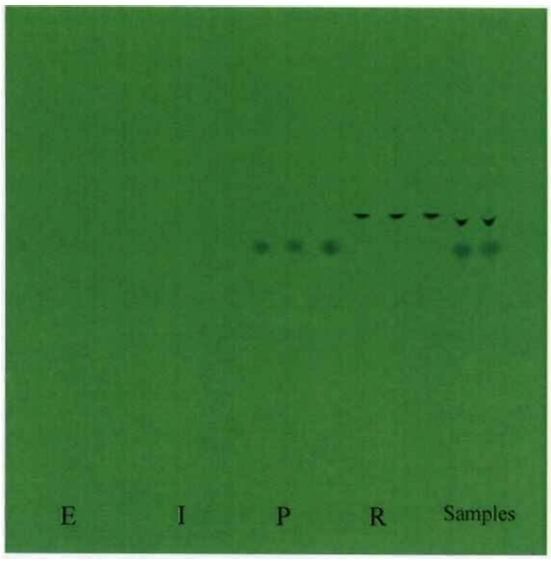


Figure 6.2: Observation of the TLC plate under UV light (254 nm) prior to iodine exposure

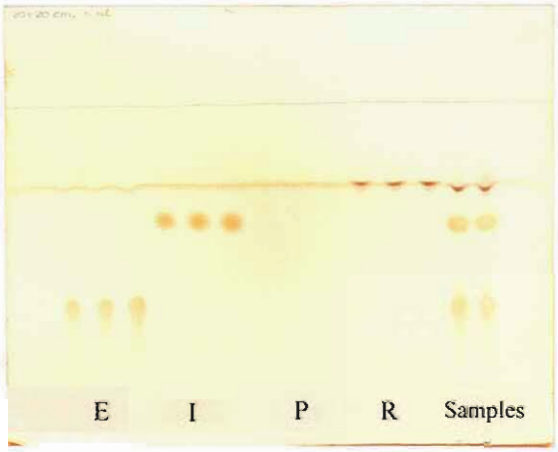


Figure 6.3: Observation of the TLC plate in daylight after iodine exposure

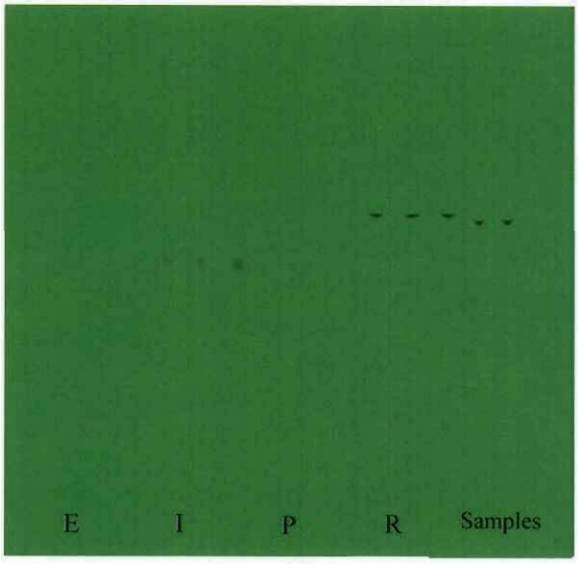


Figure 6.4: Observation of the TLC plate under UV light (254 nm) after iodine exposure

Conclusion

The spots were best observed by examining the plate under UV light (254 nm), following exposure to iodine vapours. In the absence of a UV 254 nm light source, the plate could be inspected in daylight, after iodine exposure, although pyrazinamide would only be vaguely visible.

Semi-quantitative application

The spot-intensity of isoniazid, pyrazinamide and ethambutol hydrochloride was concentration dependent. Thus, TLC can be used for semi-quantitative determination of these APIs in antituberculosis FDCs.

The difference in intensity of the 80 %, 100 % and 120 % spots are better observed when inspecting the scanned image, or digital photo on a computer monitor. The brightness of the photo can be increased, thereby making the spots even vaguer. At some point the 80 % spot will become invisible, while the 100 % and 120 % spots are still visible, thereby increasing the contrast.

Conclusion

The TLC method can be used for semi-quantitative estimation of the content of isoniazid, ethambutol hydrochloride and pyrazinamide, but only when inspected under UV light. The level of accuracy of the semi-quantitative determination is largely improved by altering the brightness of digital photos or scanned images.

Application volume

The same sample preparations that were used in the above studies were spotted in smaller volumes (2.5 μl) on 20 x 20 cm plates. It was found that it was easier to apply 2.5 μl spots evenly than 5 μl spots, and that 2.5 μl spots were better visible when examining plates under UV light (254 nm) than in daylight.

Conclusion

An application volume of 5 μl is recommended when pyrazinamide is present in the sample, as well as when examining samples in daylight. Conversely, when examined under UV at 254 nm, as well as in the absence of pyrazinamide from the sample, the application volume can be

reduced to 2.5 µl. Furthermore, exposure of the plate to iodine vapours is required prior to examination in daylight.

Plate size

A 10 x 20 cm plate was used in another study. Spots were well separated for the purpose of identification of the APIs in 4FDC antituberculosis tablets.

Conclusion

10 x 20 cm TLC plates are also suitable for the identification of the APIs in FDC antituberculosis products, as long as acceptable separation of spots occur.

6.5. CONCLUSION

A TLC method was developed for the identification of the APIs in TB FDC products using a method supplied by WHO (2002b) as point of departure.

The spots were best observed by examining TLC plates under UV light (254 nm), after their exposure to iodine vapours. In the absence of a UV 254 nm light source, the plate could be inspected in daylight, after iodine exposure, although pyrazinamide would only be vaguely visible.

The spot-intensity of isoniazid, pyrazinamide and ethambutol hydrochloride was concentration dependent. Thus, TLC can be used for the semi-quantitative determination of these APIs in antituberculosis FDCs.

In the presence of pyrazinamide, an application volume of 5 µl is recommended, as well as when plates are examined in daylight. Conversely, in the absence of pyrazinamide from the sample, as well as when examined under UV light, the application volume can be reduced to 2.5 µl.

Exposure of the plate to iodine vapours is required prior to examination in daylight.

Initially 20 x 20 cm plates were used, but 10 x 20 cm TLC plates were also found suitable for the identification of APIs in FDC antituberculosis products, as long as satisfactory separation of spots occur.

CHAPTER 7

Development of a Dissolution Method for Isoniazid and Ethambutol HCl Tablets

7.1. OBJECTIVE

Although not requested by the WHO, it was decided to develop a dissolution test for isoniazid and ethambutol hydrochloride tablets, for which the WHO had supplied a number of different brands of products. The aims were to compare the dissolution profiles being generated during this study with those presented in the relevant WHO draft guideline (Chapter 4), and to establish whether the recommended dissolution conditions and specifications were appropriate for control of these FDC products.

A dissolution method was successfully developed. The assay method for isoniazid and ethambutol hydrochloride tablets was used to analyse the dissolution samples.

The resulting method, together with the study outcomes, were submitted to the WHO. These were accepted for the monograph for ethambutol hydrochloride and isoniazid tablets. For the first time ever a comprehensive dissolution method was included in a monograph in The International Pharmacopoeia.

The purpose of this study was:

* To generate the dissolution profiles of the products being tested in this study, according to the recommended WHO conditions, and to compare the resulting outcomes with those as outlined in the WHO draft guideline "*In vitro* dissolution testing methods for oral immediate release drug products containing Biopharmaceutics Classification System (BCS) Class I drugs" (WHO, 2005f:16); and
* To establish if the dissolution conditions and specifications being recommended in the said guideline are appropriate for controlling this type of FDC product.

7.2. BACKGROUND

WHO (2006g:402) divided orally administered drugs being listed on the WHO Model List of Essential Medicines, into different classes, according to their doses, solubility and permeability.

Table 7.1: Solubility classification (according to BCS) of antituberculosis APIs (WHO, 2005f)

API	Solubility
Rifampicin	Low
Isoniazid	High
Pyrazinamide	High
Ethambutol hydrochloride	High

The solubility classification of the four antituberculosis APIs according to BCS is listed in Table 7.1.

> *The Biopharmaceutics Classification System (BCS) was proposed in 1995 by Amidon et al. It is a scientific framework which divides APIs into four groups, according to their solubility and permeability properties. According to the HHS-FDA definitions, the four possible categories for an API according to the BCS are:*
>
> - *BCS class I: "high" solubility – "high" permeability*
> - *BCS class II: "low" solubility – "high" permeability*
> - *BCS class III: "high" solubility – "low" permeability*
> - *BCS class IV: "low" solubility – "low" permeability*
>
> *Depending on the classification, the oral availability of the API may be expected to range from being heavily dependent on the formulation and manufacturing method (e.g. Class II APIs: poorly soluble yet highly permeable) to being mostly dependent on the API permeability properties (e.g. Class III APIs: highly soluble yet poorly permeable)* (WHO, 2006g:392).

With reasonable assurance, isoniazid and ethambutol hydrochloride can be regarded as Class III APIs (WHO, 2006g:407; 410). Thus, isoniazid and ethambutol hydrochloride could also qualify

for the dissolution conditions, as described in Paragraph 7.3, or at least with regards to batch release and stability.

In order to set dissolution acceptance criteria, the available isoniazid / ethambutol hydrochloride tablet formulations (see Table 7.2) were subjected to multipoint dissolution testing, according to the conditions listed in Table 7.3. Multipoint dissolution studies, with their characteristic withdrawal times (see Table 7.3), can be used to determine the similarity of dissolution profiles of two products. The comparison of profiles obtained from multipoint dissolution is explained by the following extract from the recent dissolution guideline for comment, as issued by the South African Medicines Control Council (2006:6):

Two scenarios for comparing the profiles obtained from multipoint dissolution are operative:

1. *If both the test and reference product show more than 85 % dissolution within 15 minutes, the profiles are considered similar (no calculations required). If not, see the next point.*
2. *Calculate the f_2 value. If $f_2 \geq 50$, the profiles are normally regarded similar such that further in vivo studies are not necessary. Note that only one measurement should be considered after 85 % dissolution of both products has occurred and excluding point zero.*

The similarity factor (f_2) is a logarithmic reciprocal square root transformation of the sum of squared errors, and is a measurement of the similarity in the percentage (%) dissolution between the two curves.

$$f_2 = 50.\ log\{[1+ (1/n)\sum_{t=1}^{n} (R_t - T_t)^2]^{-0.5}.100\}$$

Where n is the number of time points, R_t is the dissolution value of the reference batch at time t, and T_t is the dissolution value of the test batch at time t. For curves to be considered similar, f_2 values should be close to 100. Generally, f_2 values greater than 50 (50 to 100) ensure sameness or equivalence of the two curves and, thus, of the performance of the test and reference products.

Table 7.2: Description of products subjected to multipoint dissolution testing during the last quarter of 2004

Product	Label claim (mg/tablet)		Manufacturing country	Manufacturing date (Batch number)
	Isoniazid	Ethambutol 2HCl		
A	300	800	India	02/2004 (X4320)
B	150	400	Pakistan	12/2003 (SHT 18)
C	150	400	India	11/2003 (EI-400/150-007)
D	150	400	Pakistan	06/2002 (P-3)

7.3. EXISTING DISSOLUTION METHOD

The WHO draft guideline, "*In vitro* dissolution testing methods for oral immediate release drug products containing BCS Class I drugs", recommends dissolution test conditions and specifications for inclusion in The International Pharmacopoeia. The recommended medium is 500 ml of buffer at pH 6.8.

Dissolution conditions from the draft guideline (WHO, 2005f) are summarised in Table 7.3.

Table 7.3: *In vitro* dissolution test conditions (WHO, 2005f)

Parameter	Setting
Apparatus	Paddle
Volume dissolution medium	500 ml
Degassing	Degassed or undegassed
Dissolution medium	Phosphate buffer pH 6.8
Agitation	75 rpm
Temperature	37 °C
Withdrawal times	10, 15, 20, 30, 45, 60 minutes
Specification	≥85% of label claim dissolved in 30 minutes

It should also be noted that according to the ICH guideline (1999:11), disintegration may be substituted for dissolution of rapidly dissolving[1] (dissolution greater than 80 % in 15 minutes at pH 1.2, 4.0 and 6.8) products, containing drugs which are highly soluble throughout the physiological range (dose / solubility volume less than 250 ml from pH 1.2 to 6.8).

[1] WHO (2006g:397) defines very rapidly dissolving as a release of ≥85 % of the labelled amount of drug in 15 minutes.

7.4. EXPERIMENTAL PROCEDURE

The products listed in Table 7.2 were tested according to the conditions listed in Table 7.3 (similar to the conditions in the monograph on isoniazid and ethambutol hydrochloride tablets, Appendix K). The dissolution medium was not degassed.

Six units of each product listed in Table 7.2 were subjected to these dissolution studies. Samples were taken manually. Each dissolution sample was filtered through a Type AA 0.8 μm nitrocellulose filter (Millipore) during sampling. The samples were analysed for isoniazid and ethambutol hydrochloride, according to the assay method being developed in this study, as described in the monograph for isoniazid and ethambutol hydrochloride tablets (Appendix K).

7.5. RESULTS AND DISCUSSION

In Table 7.4 f_2 values, if required, are given for comparison of the dissolution profiles of the two APIs present in each product (normally different products are compared, whilst in this study the APIs per product are compared). Disintegration times are also included.

All the products, except for product D, met the dissolution requirement of NLT 85 % within 30 minutes, with respect to both APIs. The APIs of each product have quite similar dissolution profiles as can be judged from the f_2 values being higher than 50 (Table 7.4) and from the product type graphs (Figures 7.1 – 7.6).

The dissolution rates of the products were in the following order:

$$\text{Product A} > \text{Product B} \approx \text{Product C} > \text{Product D.}$$

Product A had a very fast dissolution rate (very rapidly dissolving), with complete dissolution of both APIs after about 15 minutes. Products B and C showed similar dissolution profiles [for isoniazid: $f_2 = 65$ (n=4) and for ethambutol hydrochloride: $f_2 = 79$ (n=3)]. Product D, however dissolved very slowly, with incomplete dissolution for both APIs even after 60 minutes. The difference in dissolution rates cannot be ascribed to product strength, as product A, which had the fastest dissolution rate, had double the strength of the other products. The dissolution rates of these products correlated, relatively speaking, with their disintegration times.

It can be thus concluded that the dissolution rate of isoniazid and ethambutol hydrochloride tablets is formulation / disintegration related, and not API related.

Table 7.4: Dissolution rates at different time points for isoniazid and ethambutol hydrochloride tablets

Time (minutes)	Percentage of label claim dissolved							
	Product A		Product B		Product C		Product D	
	I	E	I	E	I	E	I	E
10	87	94	55	61	57	61	29	34
15	96	99	72	77	78	80	38	41
20	99	99	84	87	91	90	47	50
30	**100**	**99**	**97**	**97**	**100**	**98**	**63**	**64**
45	101	99	104	102	102	99	80	79
60	102	100	105	103	102	99	95	91
f2 (I versus E)	Not applicable (>85%, 15 min)		68 (n=4)		77 (n=3)		74 (n=6)	
Disintegration time in water, without discs (minutes)	7		11		11		21	

I = Isoniazid, E = Ethambutol hydrochloride

The draft guideline of WHO proposed an acceptance criterion of not less than 85 % of the label claim dissolved in 30 minutes (see Table 7.3). The results obtained in this study indicated that this requirement is acceptable. A proposal was submitted to WHO and the conditions and acceptance criteria as set out in Table 7.3 were accepted for inclusion in the monograph of isoniazid and ethambutol hydrochloride tablets.

The data in Table 7.4 show a relationship between the dissolution rate and disintegration time. Based on this data, disintegration was proposed as an alternative to dissolution, with a limit of 15 minutes when conducted in purified water. This proposal was accepted and included as an alternative to the dissolution test in the monograph of isoniazid and ethambutol hydrochloride tablets (see Appendix K).

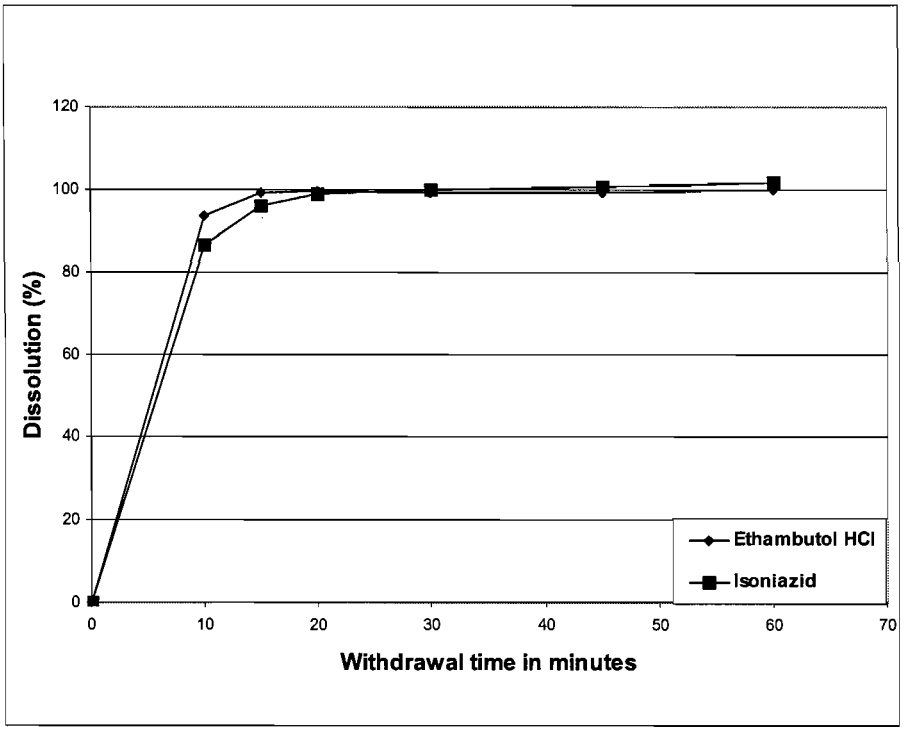


Figure 7.1: Dissolution profiles for Product A depicting both APIs

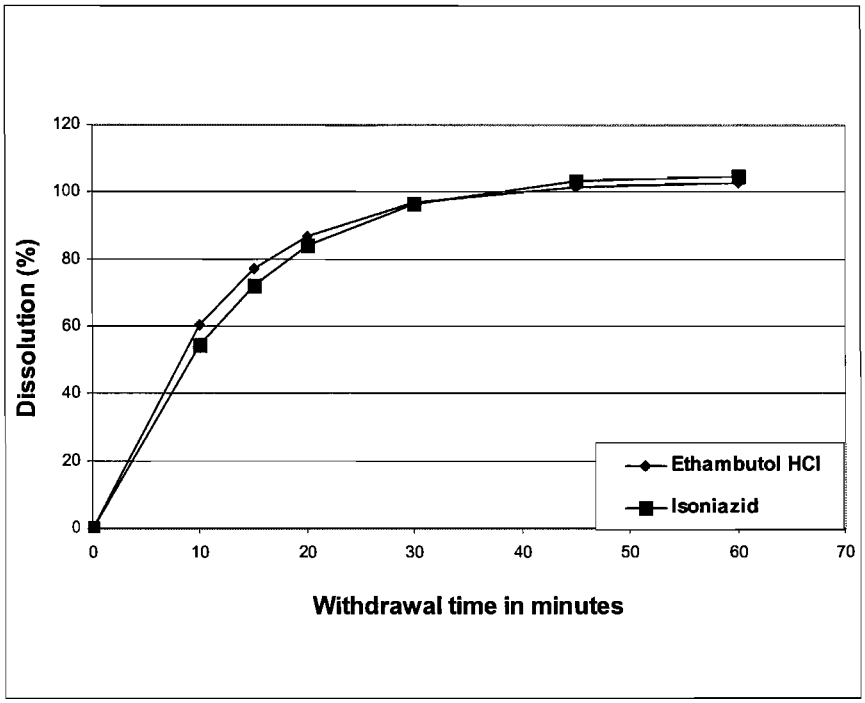


Figure 7.2: Dissolution profiles for Product B depicting both APIs

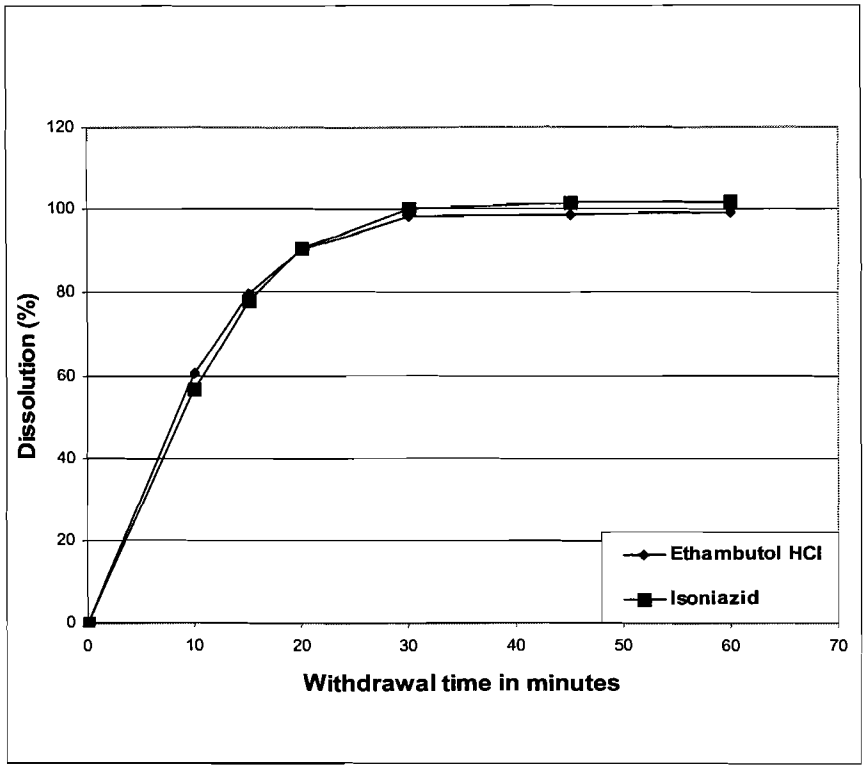


Figure 7.3: Dissolution profiles for Product C depicting both APIs

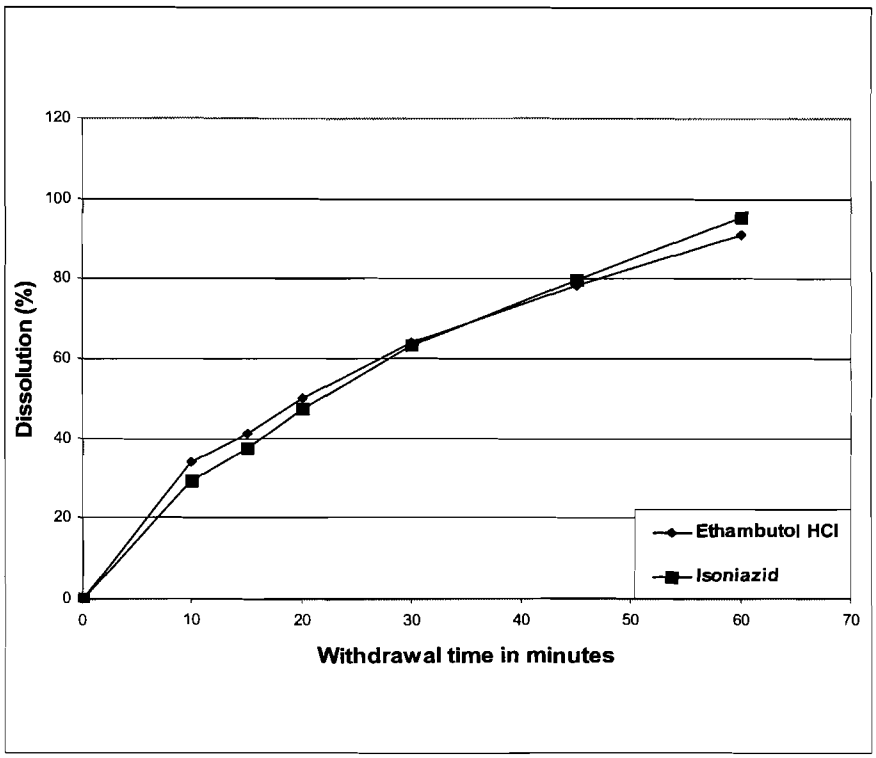


Figure 7.4: Dissolution profiles for Product D depicting both APIs

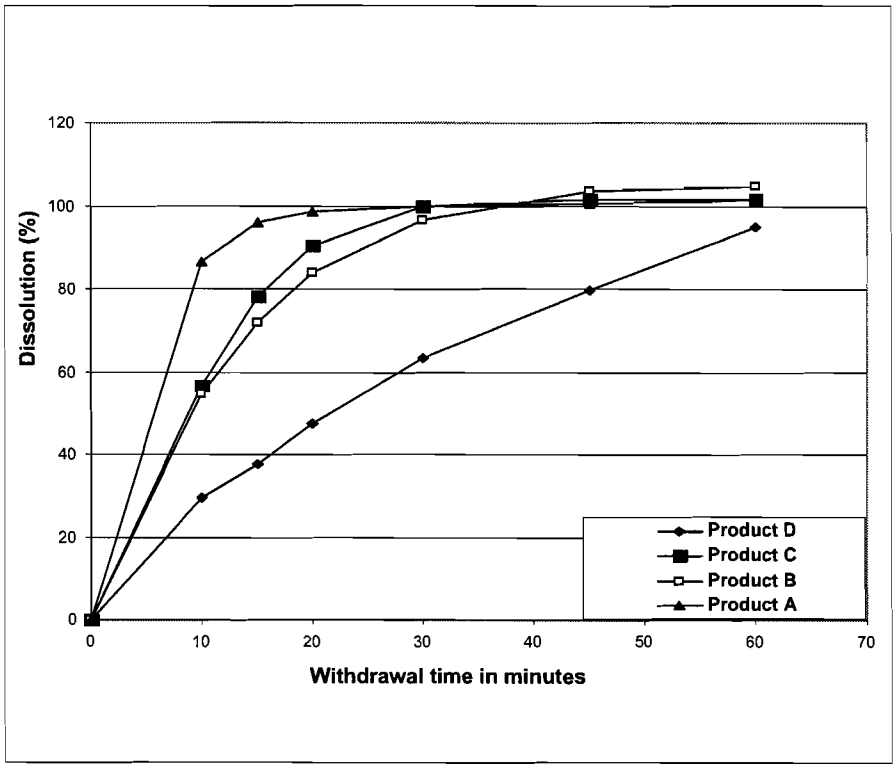


Figure 7.5: Dissolution profiles for isoniazid depicting all four products

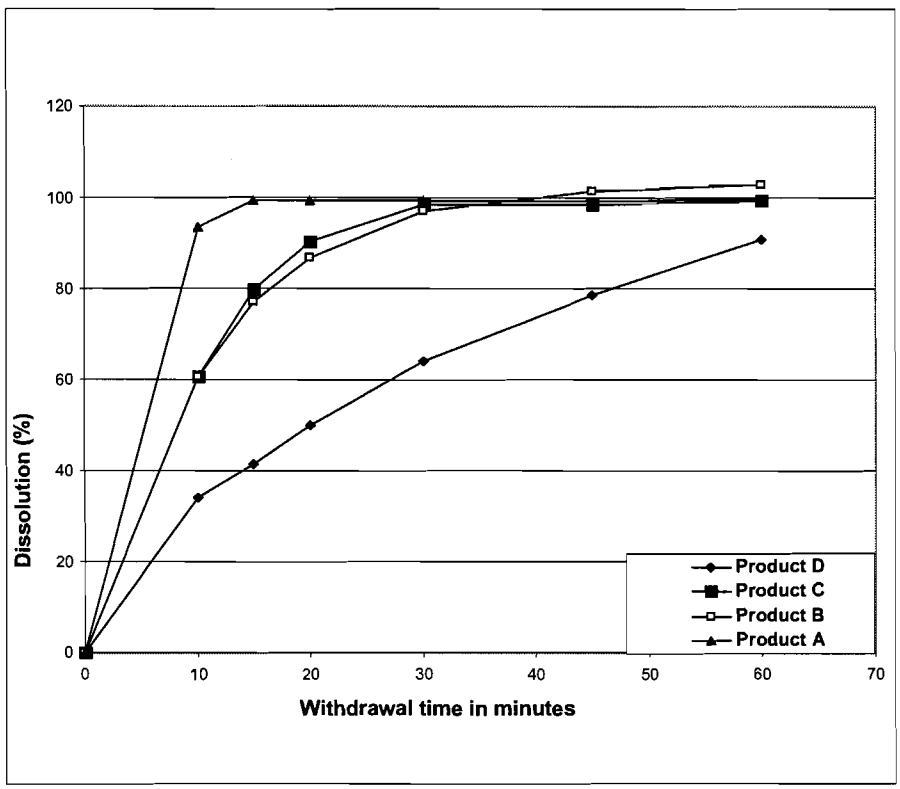


Figure 7.6: Dissolution profiles for ethambutol hydrochloride depicting all four products

7.6. CONCLUSION

Based on the outcomes of this study, it is recommended that the dissolution conditions and specifications as set out in Table 7.3, should be adopted for the isoniazid and ethambutol hydrochloride tablets monograph in The International Pharmacopoeia. Degassing of the dissolution medium is optional.

This study indicates that the APIs (ethambutol hydrochloride and isoniazid) of each product have similar dissolution profiles, which could be expected, since both APIs are highly soluble. In such a case, the use of only one API as a marker in the dissolution test may be considered.

These study outcomes were accepted by WHO and the dissolution method was included in the monograph for isoniazid and ethambutol hydrochloride tablets. The relevant monograph (Appendix K) demonstrates that disintegration has been included as an alternative to dissolution testing. The disintegration limit of 15 minutes was decided upon in consultation with WHO and was based on the disintegration data presented in Table 7.4.

CHAPTER 8

Summary and Conclusion

From the outcomes of this study, valuable contributions were made towards establishing monographs for a number of antituberculosis products for inclusion in The International Pharmacopoeia. This study mainly arose from a request by the WHO to develop monographs for:

* Rifampicin capsules;
* Rifampicin tablets;
* 2FDC tablets containing isoniazid and ethambutol hydrochloride;
* 2FDC tablets containing rifampicin and isoniazid;
* 3FDC tablets containing rifampicin, isoniazid and pyrazinamide; and
* 4FDC tablets containing rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride.

This entire study was motivated by the important role that international health regulatory authorities, such as the WHO, play in promoting good quality pharmaceutical products. Furthermore, the comprehensive guidelines on FDCs, as required by the WHO, were applied in this study.

Among several other pharmacopoeias available, "The International Pharmacopoeia" today comprises one of the major pharmacopoeias in use worldwide, especially among developing nations. The International Pharmacopoeia provides international standards for the content, purity and quality of APIs, for pharmaceutical products, as well as for excipients affecting international trade. Accordingly, a product is not of pharmacopoeial quality, unless it complies with all the stated requirements. Their goal being the promotion of good quality pharmaceutical products and the development of quality control methods, counterfeit drugs are detected, thus assuring the safety and efficacy of medical treatments worldwide. This is also crucial for promoting successful programmes for eradication or controlling diseases, and to avoid the emergence of drug resistance (WHO, 2003a:22-23).

Following is a brief summary of main research activities and findings that formed part of this study.

In the literature study (**Chapter 1**), the impact of tuberculosis (TB) on the global health and socio–economic environments was described. Important facts about TB included the following: TB, which is an airborne disease, is caused by *Mycobacterium tuberculosis* and it mainly affects the lungs. *Mycobacterium tuberculosis* is one of the most successful pathogens, infecting a third of the global population and claiming over two million lives every year (Amdekar, 2005:334). TB is also among the major causes of death among people infected with HIV, since up to 50 % of people with HIV or AIDS develop TB. Similarly, in some regions in Africa, up to 77 % of TB patients also have HIV. Worldwide, more than 21 million people are co-infected with TB and HIV, of which 70 % are concentrated in Africa. A large number of these people will develop active TB if they do not receive treatment (The Global Fund, 2005:24).

In this chapter also, available APIs and recommendations relating to antituberculosis medicines were introduced. Primary antituberculosis medicines include the APIs rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin. The WHO recommends the use of fixed dose combination products (FDCs) for the treatment of TB and multi-drug resistant TB. Currently, those FDCs that are commonly used and included in WHO's Essential Medicines List, are listed in Table 1.4.

Chapter 2 focused on the necessary guidelines and requirements to keep in mind when developing monographs for FDCs.

It was only since 2004 that the development of guidelines for FDCs received considerable attention. The 2004 "Botswana principles" and draft guidance on HIV / AIDS FDCs by the US FDA, eventually led to the development of comprehensive WHO guidelines on FDC products. The possible interaction between APIs had been identified as being a major factor that could lead to quality problems, hence ineffective treatment, when incompatible APIs are formulated into one FDC.

Therefore, in Chapter 2 the importance of considering the safety, efficacy, and in particular the quality requirements for FDC products when developing monographs, was emphasised. It was demonstrated that these are achieved through stability, assay and identification testing, as well as through the determination of degradation products.

In **Chapter 3**, the most important physical properties of ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin were examined, as well as their stability characteristics and degradation pathways. Due to its known volatility, the stability of rifampicin in solution, its interaction with other APIs in combination FDCs, as well as the effect of environmental conditions and packaging on the stability of these APIs, were also investigated.

According to Mariappan *et al.* (2005:646), the reaction between isoniazid and rifampicin to form the isonicotinyl hydrazone could result in a loss of therapeutic activity of rifampicin, because the isonicotinyl hydrazone lacks *in vivo* activity. Failure to take this into account when formulating and storing medicines containing these APIs, could lead to a chain reaction of possible degradation, reduced bioavailability, poor patient compliance and subsequently failure to cure TB.

Chapter 4 dealt with the development of pharmacopoeial monographs. The contents of a monograph for tablets will typically include the following (IntPh, 2003:170):

* Category;
* Storage;
* Additional information; and
* Requirements (including identity tests, assay, related substances and dissolution test).

Methods therefore had to be developed during this study for identity tests, assay and related substances. The development of a dissolution test was not required by WHO.

In **Chapter 5**, the development of HPLC methods for the analysis of the APIs, and for the related substances of rifampicin in the relevant FDCs, was described.

Practical requirements were set for the development of HPLC methods for assay, i.e.:

* If possible one method should be applicable for the assay of more than one API;
* The column(s) should be easily available;
* The use of an isocratic profile instead of a gradient profile, so that the test could be readily carried out by any test laboratory;
* The method(s) should be stability-indicating; and
* It would be advantageous if the related substances could be determined with the same method(s) used for assay.

The strategy followed in developing the HPLC methods included the following steps:

* Identifying existing HPLC methods from available literature and from methods used by manufacturers;
* Screening those methods for their potential suitability through trial testing;
* Selecting the best alternatives for application and / or further development; and
* To start with the analysis of the more complex 4FDC tablets and to adjust those methods for the lower combinations and eventually for rifampicin tablets and capsules. It was believed that the methods suitable for the 4FDC product analysis would apply to the 3FDCs, etc.

The analysis of 4FDC products was successfully achieved by employing only two HPLC methods:

1. One method was developed for analysing any one or more of ethambutol hydrochloride, isoniazid and pyrazinamide in a single HPLC run. Ethambutol hydrochloride that is not UV absorbing, however, forms a complex with cupric ions, with maximum absorption at 270 nm. By including Cu(II) acetate in the mobile phase, ethambutol hydrochloride (as well as isoniazid and pyrazinamide) could be successfully quantified by UV detection (270 nm). For the assay of FDC products not containing ethambutol hydrochloride, Cu(II) acetate was omitted and the detection wavelength changed to 240 nm.
2. A second HPLC method was developed for the analysis of rifampicin and its related substances.

The suitability of these HPLC methods was confirmed through validation (see Appendix B to G). Peak purity testing further confirmed that the methods are stability indicating and that it should be suitable for stability testing, quality control and batch release of antituberculosis products.

The development of a TLC method for the identification of the APIs in TB FDC products was described in **Chapter 6**. An identification test method of WHO (2002b) was used as the point of departure.

Results showed that spots were best observed by examining the TLC plate under UV light (254 nm) after exposing it to iodine vapours. In the absence of an UV 254 nm light source, the plate could also be inspected in daylight after iodine exposure, with pyrazinamide being only vaguely visible.

It was concluded that this TLC method could also be used for semi-quantitative estimation of the content of isoniazid, ethambutol hydrochloride and pyrazinamide, but only when inspected under UV light.

The development of a dissolution method for isoniazid and ethambutol hydrochloride tablets was described in **Chapter 7**. Isoniazid and ethambutol are highly soluble, according to the Biopharmaceutics Classification System (WHO, 2006g:407; 410).

WHO did not request the development of dissolution methods. However, the initiative that was taken in this study to develop a suitable dissolution test for isoniazid and ethambutol hydrochloride tablets, proved very rewarding.

Not only did WHO accept all of the test outcomes for inclusion in the monograph for ethambutol hydrochloride and isoniazid tablets, but for the first time ever was a comprehensive dissolution method included in a monograph in The International Pharmacopoeia.

In developing this method, dissolution conditions were adopted from the draft guideline, "In Vitro Dissolution Testing Methods for Oral Immediate Release Drug Products Containing BCS Class I Drugs" (WHO, 2005f) (summarised in Table 7.3). These study outcomes further confirmed that WHO's recommended dissolution conditions and specifications were appropriate for control of these FDC products.

Disintegration has furthermore been included as an alternative to dissolution, based on data generated for a number of antituberculosis FDC products.

This study can be considered successful, as all study objectives were accomplished. Antituberculosis FDC products, and their dosages, were identified; the required format of IntPh product monographs was determined; identity tests and HPLC methods were developed (the latter were successfully validated); and pharmacopoeial monographs for antituberculosis products were successfully developed for The International Pharmacopoeia. Additionally, a dissolution test was developed for isoniazid and ethambutol hydrochloride tablets.

The developed monographs have since their submission to, and acceptance by WHO, been posted on the WHO website (web address below) and are to be included in the next (fourth edition) of The International Pharmacopoeia.

(http://www.who.int/medicines/publications/pharmacopoeia/mon_tb/en/index.html)

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APPENDIX A

Validation Parameter Definitions

The characteristics for consideration during the validation of analytical procedures are discussed. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A summary of the characteristics applicable to identification, control of impurities and assay procedures is included. Information was taken from the guideline Q2 (R1) on the validation of analytical procedures prepared by the ICH (2005).

A.1 COMMON TYPES OF ANALYTICAL PROCEDURES

The discussion of analytical procedures is directed to the three most common types of analytical procedures, namely:

A.1.1 Identification tests

Identification tests are intended to ensure the identity of an analyte in a sample. This is usually achieved by comparison of a property of the sample, for example spectrum, chromatographic behaviour, chemical reactivity, etcetera, to that of a reference standard.

A.1.2 Testing for impurities

Testing for impurities in a sample can be either a quantitative test or a limit test. The tests are intended to accurately reflect the purity characteristics of the sample.

A.1.3 Assay procedures

Assay procedures are intended to measure the analyte present in a given sample. Validation characteristics exist for a quantitative measurement of the major component(s) in the drug substance, as well as for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures, for example dissolution.

A.2 TYPICAL VALIDATION CHARACTERISTICS

Typical validation characteristics that should be considered are listed below:

* Accuracy;
* Precision (Repeatability and Intermediate precision);
* Specificity;
* Limit of detection;
* Limit of quantitation;
* Linearity; and
* Range.

Table A.1 lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. Robustness is not listed, but should be considered at an appropriate stage in the development of the analytical procedure.

Table A.1: Summary of validation characteristics

Type of analytical procedure	**Identification**	**Testing for impurities**		**Assay** **- Dissolution (measurement only),** **- Content/potency**
		Quantitative	**Limit**	
Accuracy	-	+	-	+
Precision - Repeatability - Intermediate precision	- -	+ + (1)	- -	+ + (1)
Specificity (2)	+	+	+	+
Limit of detection	-	- (3)	+	-
Limit of quantitation	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

-: signifies that this characteristic is not normally evaluated.
+: signifies that this characteristic is normally evaluated.
(1): in cases where reproducibility has been performed, intermediate precision is not needed.
(2): lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).
(3): may be needed in some cases.

A.3 GLOSSARY

A.3.1 Analytical procedure

It refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to the preparation of the sample, the reference standard and the reagents, use of apparatus, generation of the calibration curve, use of the formulae for the calculation, etcetera.

A.3.2 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etcetera.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s) such as:

* Identification to ensure the identity of an analyte;
* Purity tests to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, for example related substances test, heavy metals, residual solvents content, etcetera; and
* Assay (content or potency) to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

A.3.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

A.3.4 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample, it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

A.3.4.1 Repeatability (intra-day)

Repeatability expresses the precision under the same operating conditions over a short interval of time.

A.3.4.2 Intermediate Precision (inter-day)

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etcetera.

A.3.4.3 Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology).

A.3.5 Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

A.3.6 Limit of quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

A.3.7 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

A.3.8 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

A.3.9 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

APPENDIX B

Validation of an HPLC Assay Method:

Determination of Ethambutol HCl and Isoniazid Content in a 2 Fixed Dose Combination product containing Ethambutol HCl and Isoniazid

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B.1. ORIGIN OF METHOD

The method was based in part on a method supplied by Manufacturer A (2004) for the determination of ethambutol hydrochloride in 4FDC tablets.

B.2. SPECIFICATION

The tablet must contain the following:

Ethambutol hydrochloride	400 mg (360 – 440 mg)
Isoniazid	150 mg (135 – 165 mg).

B.3. SUMMARY

In Table B.1 a summary of the validation results is given.

Table B.1: Summary of validation results

Parameter	Acceptance criteria	Results
Specificity	The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients.	Complies
Linearity and Range	The method is linear over the range: 20 – 150 % of the expected sample concentration. R^2 is not less than 0.99.	Complies Ethambutol hydrochloride: Linear from 40 – 300 µg/ml (20 – 150 % of expected sample concentration) R^2: 0.9999 Isoniazid: Linear from 15 – 112.5 µg/ml (20 – 150 % of expected sample concentration) R^2: 0.9994
Accuracy (Recovery)	Recovery must be between 98.0 – 102.0 %.	Complies Ethambutol hydrochloride: 100.0 % Isoniazid: 100.0 %
Precision (Repeatability)	RSD of 2.0 % or less.	Complies Ethambutol hydrochloride: Intra-day = 1.5 %, Inter-day = 0.8 % Isoniazid: Intra-day = 1.2 %, Inter-day = 0.5 %
Ruggedness (Stability)	The sample solution should not be used for a period longer than it takes to degrade by 2.0 %.	Ethambutol hydrochloride: Stable for 6 hours Isoniazid: Stable for 6 hours
Ruggedness (System repeatability)	The peak area and retention times should have an RSD of 2.0 % or less.	Complies Ethambutol hydrochloride: 1.4 % for peak area, 0.1 % for retention time Isoniazid: 0.9 % for peak area, 0.1 % for retention time
Robustness	The method should be able to tolerate about 5.0 % variance in chromatographic conditions.	Complies

B.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: Column L1, USP 29, 2006, p 2649, (Luna C_{18}-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 50 g ammonium acetate and 0.2 g copper(II) acetate monohydrate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 5.0 ± 0.05 with glacial acetic acid. Mix 940 ml of this solution with 60 ml methanol.

Flow rate: 2.0 ml/min.

Injection volume: 20 µl.

Detection: 270 nm (Diode Array Detection).

Retention times: About 1.6 and 5.9 minutes for isoniazid and ethambutol hydrochloride respectively.

Solvent: Water.

B.5. SAMPLE SOLUTION PREPARATION

1. Accurately weigh and powder 20 tablets and mix. Accurately weigh an amount of powder equivalent to 100 mg ethambutol hydrochloride.
2. Transfer the powder into a 500 ml volumetric flask, and dissolve in about 400 ml of water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtrate.
5. Analyse by means of HPLC.

B.6. STANDARD SOLUTION PREPARATION

1. Accurately weigh about 100 mg of ethambutol hydrochloride reference standard[1] and 37.5 mg of isoniazid reference standard[2] into a 500 ml volumetric flask.
2. Dissolve in about 400 ml water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Analyse by means of HPLC (6 injections).

B.7. CALCULATIONS

Ethambutol hydrochloride

The tablet contains 400 mg ethambutol hydrochloride and 150 mg isoniazid. The prepared sample solution will contain 0.2 mg/ml ethambutol hydrochloride.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

Isoniazid

The tablet contains 400 mg ethambutol hydrochloride and 150 mg isoniazid. The prepared sample solution will contain 0.075 mg/ml isoniazid.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

[1] Ethambutol hydrochloride primary standard: USP, B/N 032501195

[2] Isoniazid primary standard: USP, B/N 03452/INH

B.8. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

B.8.1 Specificity

Method

1. Prepare a solution from the placebo powder[3] similar to preparing the sample solution.

   The composition of the placebo used is given Table B.2.

   Table B.2: Standard placebo mixture constitution

Excipient	Quantity (mg)
Aerosil	5
Ascorbic acid	5
Croscarmellose sodium	5
Crospovidone	5.5
Hydroxypropyl methylcellulose	2
Lactose	133
Magnesium stearate	2
Maize starch	134
Polyethylene glycol 4000	2
Polyvinylpyrrolidone	2.5
Silicon oil	2
Sodium lauryl sulphate	2
Talc	2
API(s) where relevant	-

2. Prepare a sample solution and dilute 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.

3. Store the solutions prepared in point 2, overnight in closed test tubes at 40 °C/75 % RH to bring about degradation.

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[3] For the purpose of developing a monograph assay method for the IntPh, the use of a product specific placebo seemed not appropriate. A placebo was constituted from information available from patient information leaflets from a number of TB FDC products.

4. Analyse by means of HPLC.

5. Examine the chromatograms to determine whether any additional peaks were formed.

Acceptance criteria

The placebo should not generate any peaks that will interfere with the determination of the active ingredients.

Extra peaks formed under stress conditions should be discernible from those of the active ingredients.

B.8.2 Linearity and Range

Method

1. Prepare a standard solution as described under Standard Solution Preparation.

2. Inject variable volumes into the chromatograph to obtain standards from 20 – 150 % of the expected sample concentration.

Acceptance criterion

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

B.8.3 Accuracy

Method

1. Weigh amounts of the placebo equivalent to the amount of sample powder that would contain 80 %, 100 % and 120 % of 100 mg of ethambutol hydrochloride. Add 400 ml of solvent in triplicate as described under Sample Solution Preparation.

2. Spike, respectively with known amounts of the active ingredients at concentrations of approximately 80, 100 and 120 % of the expected sample concentration, make up to volume and filter.

3. Analyse in duplicate by means of HPLC.

Acceptance criterion

Recovery must be between 98.0 – 102.0 %.

B.8.4 Precision

B.8.4.1 Intra-day Precision

Method

1. Weigh amounts of tablet powder equivalent to 80 %, 100 % and 120 % of 100 mg of ethambutol hydrochloride. Prepare solutions thereof in triplicate as described under Sample Solution Preparation.
2. Analyse in duplicate by means of HPLC.

B.8.4.2 Inter-day Precision

Method

1. Analyse the same homogenous sample in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more occasions on different days to determine the between-day variability of the method.

Acceptance criteria

Repeatability must have an RSD of 2.0 % or less (n = 9).

Inter-day precision must have an RSD of 2.0 % or less (n = 9).

B.8.5 Ruggedness

B.8.5.1 Stability of sample solution

Method

1. Prepare a sample solution as described under Sample Solution Preparation.
2. Analyse by means of HPLC.
3. Leave the sample solution in the auto sampler tray and re-analyse over a period of 8 hours to determine the stability.

Acceptance criterion

Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %, and in the case of degradation, special precautions should be followed to compensate for the loss.

B.8.5.2 SYSTEM REPEATABILITY

Method

Inject a sample solution six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area and retention times should have an RSD of 2.0 % or less.

B.8.6 Robustness

Method

1. Make deliberate changes to the chromatographic conditions to determine the method's tolerance towards changes.
2. Change the flow rate, wavelength, injection volume, mobile phase pH and use a similar column from a different manufacturer.

Acceptance criterion

The method should be able to tolerate about 5.0 % variance in the chromatographic conditions.

B.8.7 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between peaks and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 2.0 %. The resolution and plate count have to be determined through the validation for the specific system.

B.9. VALIDATION RESULTS

B.9.1 Specificity

A chromatogram of the solution containing the placebo is shown in Figure B.1. Figure B.2 is a chromatogram obtained for a sample solution. Figure B.3 shows a chromatogram of a standard solution. Figures B.4 – B.7 show chromatograms of sample solutions that have been stressed overnight as described under B.8.1. The analyte peaks that remained in the aqueous solution after being stressed were analysed by means of diode array peak purity testing to determine whether the peaks are still pure. The purity test results are given in Figures B.8 – B.9.

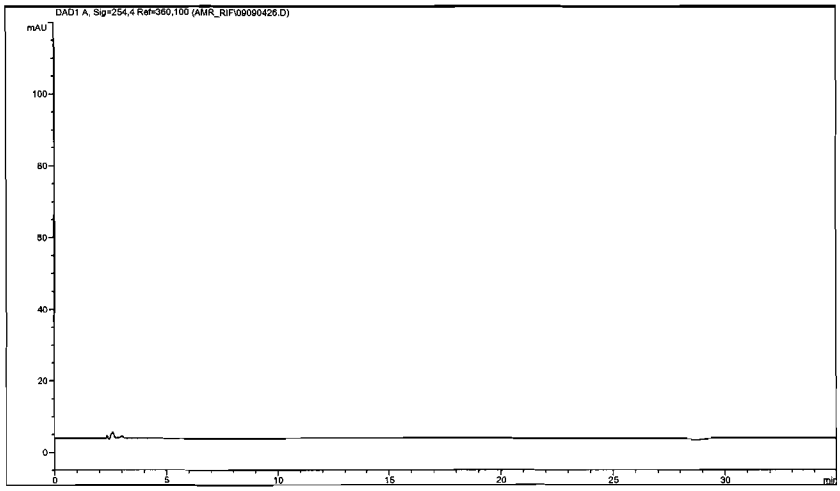


Figure B.1: Chromatogram of a solution containing the placebo

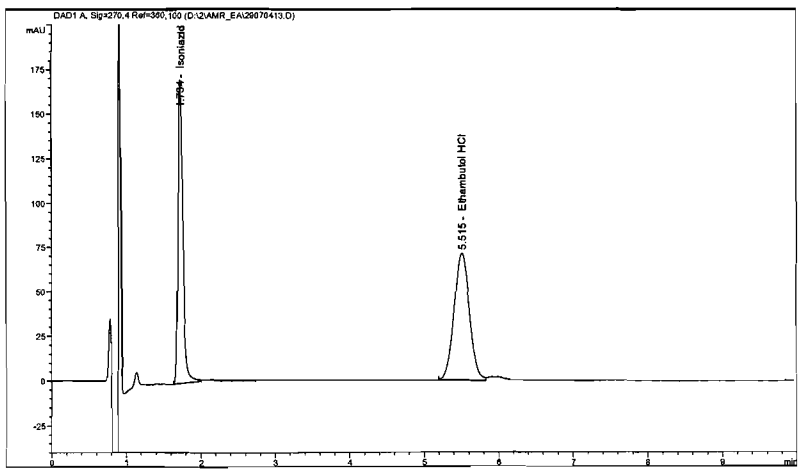


Figure B.2: Chromatogram of a sample solution

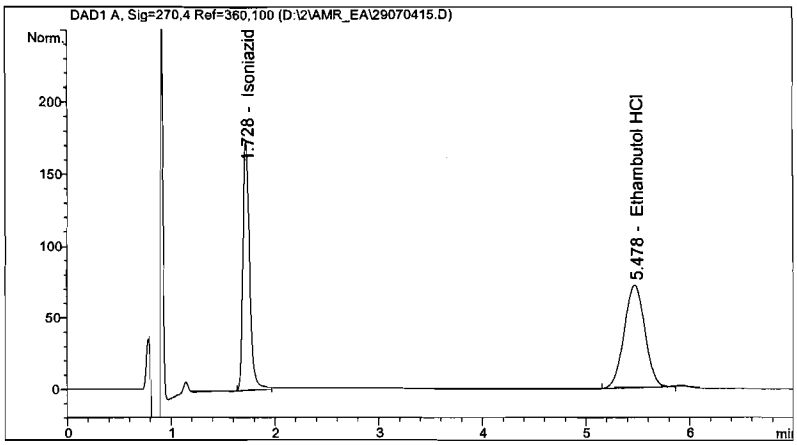


Figure B.3: Chromatogram of a standard solution

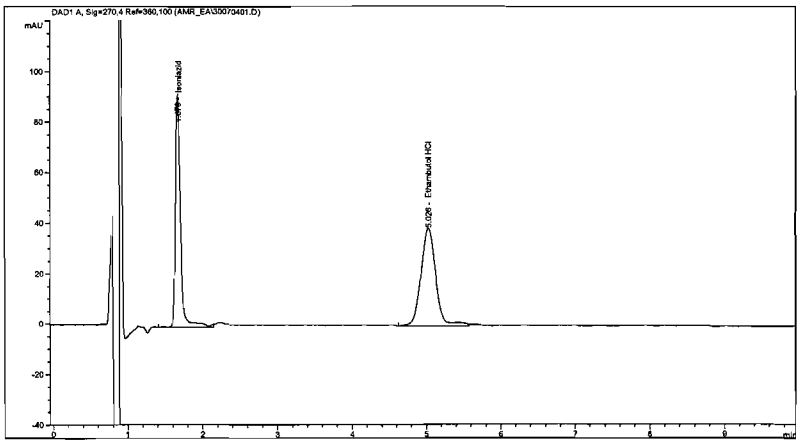


Figure B.4: Sample solution stressed overnight in water at 40 °C/75 % RH

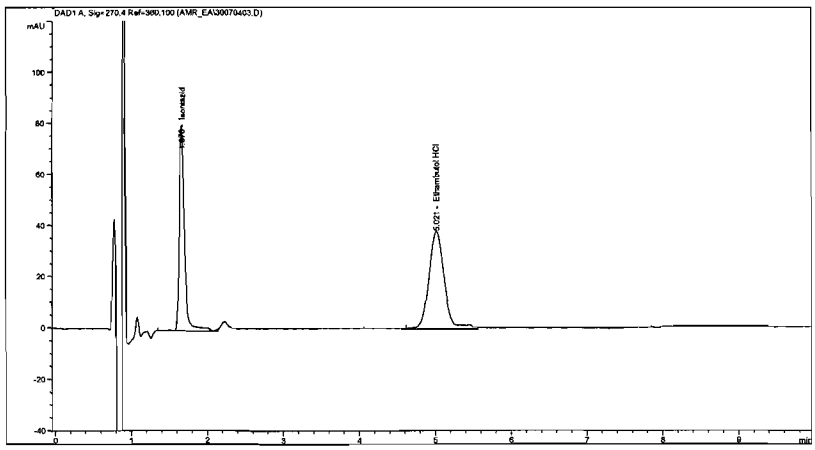


Figure B.5: Sample solution stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

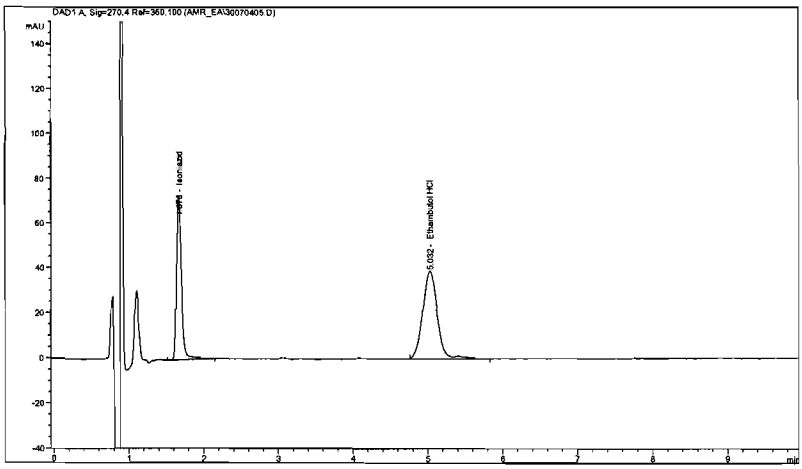


Figure B.6: Sample solution stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

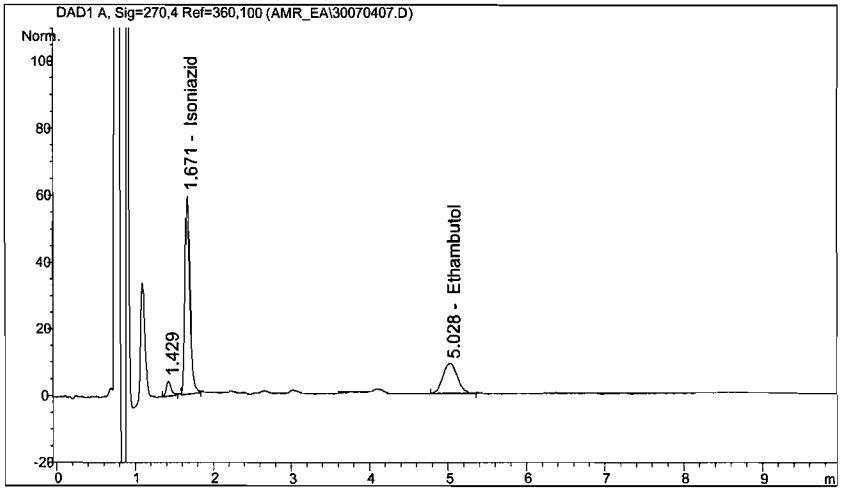


Figure B.7: Sample solution stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

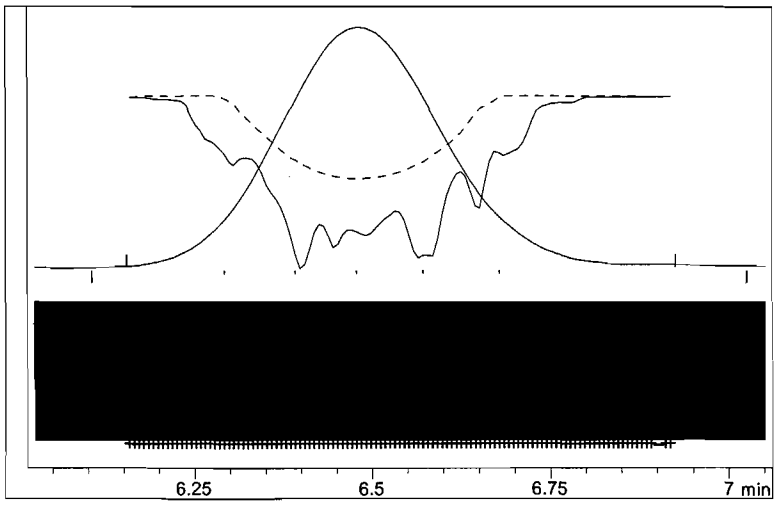


Figure B.8: Peak purity test results for ethambutol hydrochloride

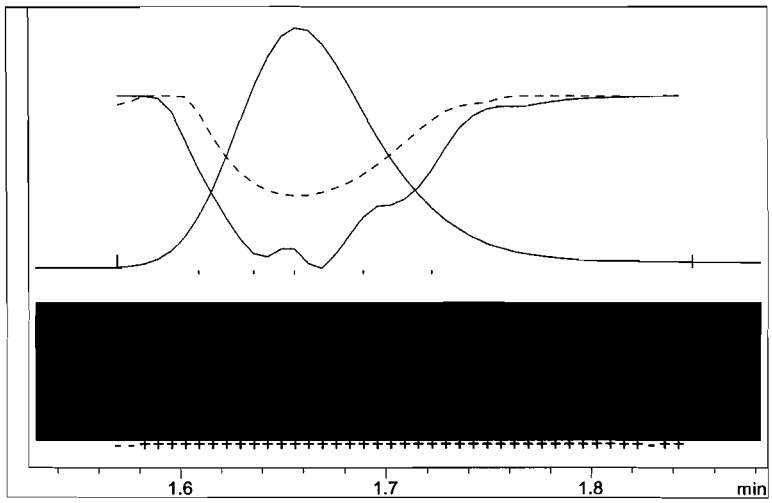


Figure B.9: Peak purity test results for isoniazid

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

None of the ingredients in the placebo interfered with the analyte peaks. Extra peaks formed during forced degradation did not interfere with any of the peaks of interest. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

B.10. ETHAMBUTOL HYDROCHLORIDE

B.10.1 Specificity

Specificity results for ethambutol hydrochloride are given in Table B.3.

Table B.3: Results for ethambutol hydrochloride to determine Specificity

Stress condition (40 °C)	% Degradation of ethambutol hydrochloride
Water	19.1
0.1 M Hydrochloric acid	18.4
0.1 M Sodium hydroxide	18.6
10 % Hydrogen peroxide	64.6

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 20 % occurred in sample solutions mixed with water, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. Degradation of about 65 % occurred in a sample solution mixed with 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

B.10.2 Linearity and Range

Linearity and Range results for ethambutol hydrochloride are given in Table B.4.

Table B.4: Results for ethambutol hydrochloride to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
40	191.2	214.1	202.6
60	292.0	291.7	291.8
80	394.2	407.4	400.8
100	489.2	487.1	488.1
120	596.1	587.3	591.7
140	670.4	675.9	673.1
160	772.8	772.1	772.4
180	869.2	862.6	865.9
200	963.6	965.3	964.5
220	1053.4	1054.8	1054.1
240	1152.8	1151.3	1152.0
260	1252.6	1256.6	1254.6
280	1343.0	1334.3	1338.6
300	1436.4	1436.0	1436.2

Regression statistics are given in Table B.5.

Table B.5: Regression statistics of ethambutol hydrochloride results

R squared	0.9999	Lower 95%	Upper 95%
Intercept	14.1385	7.5380	20.7390
Slope	4.7431	4.7081	4.7782

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 40 – 300 µg/ml (20 – 150 % of expected sample concentration) as shown in Figure B.10. The regression coefficient (R squared) is 0.9999. The method is suitable for single point calibration.

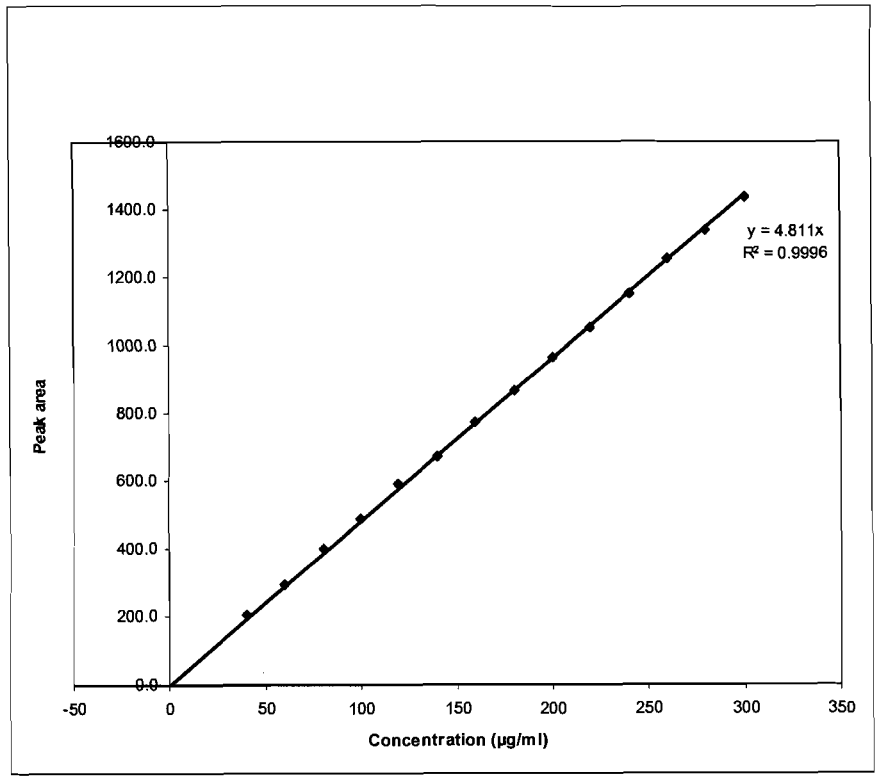


Figure B.10: Linear regression graph for ethambutol hydrochloride to determine Linearity and Range

B.10.3 Accuracy

Accuracy results for ethambutol hydrochloride and the statistical analysis thereof are given in Tables B.6 and B.7, respectively.

Table B.6: Results for ethambutol hydrochloride to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
160	784.1	783.1	783.6	160.8	100.50
160	780.3	777.5	778.9	159.8	99.90
160	772.3	780.9	776.6	159.4	99.60
200	975.7	983.8	979.7	202.1	101.04
200	943.2	959.0	951.1	196.2	98.09
200	975.1	980.9	978.0	201.7	100.87
240	1190.3	1175.0	1182.6	243.1	101.27
240	1152.4	1168.7	1160.5	238.5	99.38
240	1163.7	1156.6	1160.2	238.4	99.35

Table B.7: Statistical analysis results

Mean %	100.0
SD	1.0
% RSD	1.0
95% confidence intervals	
Lower limit	99.2
Upper limit	100.8
Estimated median	99.9
Confidence Level (95.0%)	0.8

Conclusion

(**Acceptance criterion:** Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

B.10.4 Precision

B.10.4.1 Intra-day Precision

Intra-day precision results for ethambutol hydrochloride are given in Table B.8.

Table B.8: Results for ethambutol hydrochloride to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.12951	781.2	770.2	775.7	160	99.48
0.12960	783.8	779.5	781.6	160	100.25
0.12955	798.0	781.9	790.0	160	101.32
0.16185	984.3	979.4	981.8	200	101.96
0.16190	979.6	983.3	981.4	200	101.92
0.16182	966.3	976.8	971.5	200	100.89
0.19425	1143.7	1147.1	1145.4	240	98.08
0.19422	1155.9	1146.5	1151.2	240	98.58
0.19438	1142.8	1150.5	1146.6	240	98.19
				Mean	100.1
				SD	1.5
				RSD %	1.5

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.5 %.

B.10.4.2 Inter-day Precision

Inter-day precision results for ethambutol hydrochloride and ANOVA single factor statistics thereof are given in Tables B.9 and B.10, respectively.

Table B.9: Results for ethambutol hydrochloride to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	981.83	976.48	964.93	
	981.41	971.49	953.09	
	971.52	958.66	981.26	
Mean	978.25	968.88	966.42	971.18
SD	4.77	7.51	11.55	7.94
RSD %	0.49	0.77	1.20	0.82

Table B.10: ANOVA single factor statistics for the determination of ethambutol hydrochloride

Groups	**Count**	**Sum**	**Average**	**Variance**	
Day 1	3	2934.76	978.25	34.06	
Day 2	3	2906.63	968.88	84.53	
Day 3	3	2899.27	966.42	200.16	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	233.94	2	116.97	1.10	0.39
Within Groups	637.50	6	106.25		
Total	871.45	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.8 %.

B.10.5 Ruggedness

B.10.5.1 Stability of sample solutions

A sample solution was left on the auto sampler tray in amber vials and re-analysed over several time intervals to determine the sample stability. The results are presented in Table B.11.

Table B.11: Results for ethambutol hydrochloride to determine Ruggedness

Time (hours)	Peak Area	%
0	999.64	100.00
1	999.50	99.99
2	999.49	99.98
3	999.06	99.94
4	996.95	99.73
5	986.68	98.70
6	985.32	98.57
7	980.76	98.11
8	977.53	97.79
Mean	991.66	99.20
SD	8.51	0.85
RSD %	0.86	0.86

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of less than 2.3 % was observed over an 8 hour period. Sample solutions are considered stable for 6 hours.

B.10.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table B.12.

Table B.12: Results for ethambutol hydrochloride to determine System Repeatability

	Area	Retention time (minutes)
	882.9	5.93
	903.6	5.93
	902.0	5.93
	886.0	5.93
	912.7	5.92
	879.7	5.92
Mean	894.5	5.93
SD	12.2	0.00
RSD %	1.4	0.06

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.4 % for peak area and 0.1 % for retention time respectively.

B.11. ISONIAZID

B.11.1 Specificity

Specificity results for isoniazid are given in Table B.13.

Table B.13: Results for isoniazid to determine Specificity

Stress condition (40 °C)	% Degradation of isoniazid
Water	12.6
0.1 M Hydrochloric acid	22.8
0.1 M Sodium hydroxide	33.6
10 % Hydrogen peroxide	44.9

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 12 to 23 % occurred in sample solutions mixed with water, and 0.1 M hydrochloric acid. Degradation of about 34 % occurred in a sample solution mixed with 0.1 M sodium hydroxide. Degradation of about 45 % occurred in a sample solution mixed with 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

B.11.2 Linearity and Range

Linearity and Range results for isoniazid are given in Table B.14.

Table B.14: Results for isoniazid to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
15	110.2	120.0	115.1
22.5	179.5	182.5	181.0
30.0	239.8	241.6	240.7
37.5	300.3	304.8	302.5
45.0	408.3	365.7	387.0
52.5	451.5	454.3	452.9
60.0	495.0	532.5	513.8
67.5	570.8	605.5	588.1
75.0	645.4	655.6	650.5
82.5	711.4	724.8	718.1
90.0	789.8	791.8	790.8
97.5	870.4	866.8	868.6
105.0	924.8	939.6	932.2
112.5	1015.7	1012.3	1014.0

Regression statistics are given in Table B.15.

Table B.15: Regression statistics of isoniazid results

R squared	0.9994	Lower 95%	Upper 95%
Intercept	-31.5307	-41.1371	-21.9243
Slope	9.1840	9.0478	9.3201

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 15.0 – 112.5 µg/ml (20 – 150 % of expected sample concentration) as shown in Figure B.11. The regression coefficient (R squared) is 0.9994. The method is suitable for single point calibration.

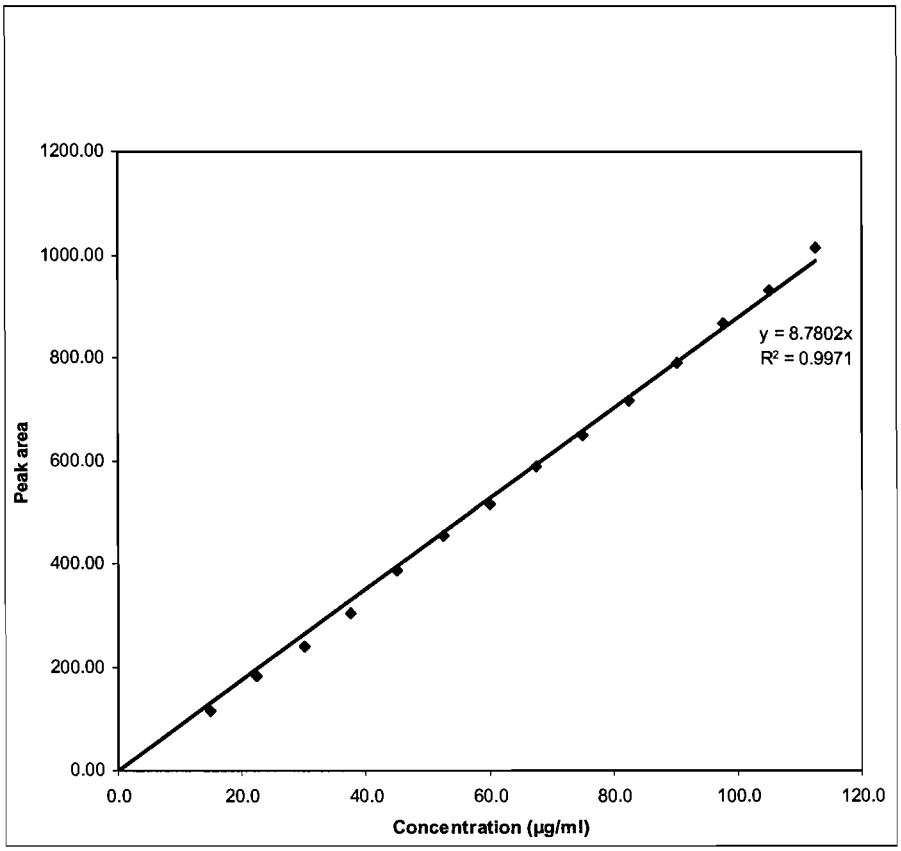


Figure B.11: Linear regression graph for isoniazid to determine Linearity and Range

B.11.3 Accuracy

Accuracy results for isoniazid and the statistical analysis thereof are given in Tables B.16 and B.17, respectively.

Table B.16: Results for isoniazid to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
60	470.3	472.3	471.3	59.2	98.65
60	488.0	474.4	481.2	60.4	100.72
60	486.4	475.0	480.7	60.4	100.62
75	581.4	597.7	589.6	75.1	100.14
75	582.0	580.9	581.5	74.1	98.76
75	593.3	597.3	595.3	75.8	101.11
90	716.9	723.2	720.1	89.1	99.05
90	728.5	736.3	732.4	90.7	100.74
90	729.0	728.0	728.5	90.2	100.21

Table B.17: Statistical analysis of isoniazid results

Mean %	100.0
SD	0.9
% RSD	0.9
95% confidence intervals	
Lower limit	99.5
Upper limit	100.9
Estimated median	100.2
Confidence Level (95.0%)	0.7

Conclusion

(**Acceptance criterion:** Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

B.11.4 Precision

B.11.4.1 Intra-day Precision

Intra-day precision results for isoniazid are given in Table B.18.

Table B.18: Results for isoniazid to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration. (µg/ml)	Recovery (%)
0.12951	469.78	472.33	471.05	60	98.60
0.12960	473.41	477.12	475.26	60	99.48
0.12955	489.00	476.21	482.61	60	101.02
0.16185	577.19	580.73	578.96	75	98.34
0.16190	590.96	596.17	593.57	75	100.82
0.16182	592.88	597.76	595.32	75	101.11
0.19425	719.63	718.06	718.84	90	98.88
0.19422	736.38	739.90	738.14	90	101.53
0.19438	736.95	740.17	738.56	90	101.59
				Mean	100.2
				SD	1.2
				RSD %	1.2

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.2 %.

B.11.4.2 Inter-day Precision

Inter-day precision results for isoniazid and ANOVA single factor statistics thereof are given in Tables B.19 and B.20, respectively.

Table B.19: Results for isoniazid to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	578.96	592.26	589.52	
	593.57	597.65	588.86	
	595.32	597.33	592.88	
Mean	589.28	595.74	590.42	591.82
SD	7.33	2.47	1.76	2.82
RSD %	1.24	0.41	0.30	0.48

Table B.20: ANOVA single factor statistics for the determination of isoniazid

Groups	**Count**	**Sum**	**Average**	**Variance**	
Day 1	3	1767.85	589.28	80.68	
Day 2	3	1787.23	595.74	9.15	
Day 3	3	1771.26	590.42	4.65	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	71.39	2	35.70	1.13	0.38
Within Groups	188.97	6	31.49		
Total	260.36	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.5 %.

B.11.5 Ruggedness

B.11.5.1 Stability of sample solutions

A sample solution was left in amber vials on the auto sampler tray and re-analysed over several time intervals to determine the sample stability. The results are presented in Table B.21.

Table B.21: Results for isoniazid to determine Ruggedness

Time (hours)	Peak Area	%
0	578.3	100.00
1	575.3	99.49
2	572.6	99.01
3	572.0	98.90
4	571.5	98.83
5	570.9	98.72
6	570.3	98.61
7	566.0	97.88
8	565.6	97.80
Mean	571.4	98.8
SD	3.8	0.65
RSD %	0.7	0.66

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of about 2.2 % was observed over an 8 hour period. Sample solutions are considered stable for 6 hours.

B.11.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table B.22.

Table B.22: Results for isoniazid to determine System Repeatability

	Area	Retention time (minutes)
	578.7	1.56
	573.4	1.56
	575.2	1.56
	586.3	1.56
	573.3	1.56
	585.3	1.56
Mean	578.7	1.56
SD	5.3	0.00
RSD %	0.9	0.04

Conclusion

(Acceptance criterion: The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 0.9 % for peak area and 0.1 % for retention time respectively.

B.12. ROBUSTNESS

The following changes in the chromatographic operating parameters were found to be acceptable:

Column: Luna C_{18}-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance, CA; and
Lichrospher 100-5 RP-1ec cartridge column, 125 x 4.0 mm, 5 µm, 100 10µm pores, 21.5 % carbon load, end capped, Machery-Nagel, Düren, Germany.
The Lichrospher gave different retention times, thus the flow rate must be altered.

Mobile phase pH: pH 4.8 – 5.0.

Flow rate: 1.9 – 2.1 ml/minute.

Wavelength: The wavelength can be altered by ± 2 nm without any ill effect.

Injection volume: 16 – 24 µl.

Conclusion

(**Acceptance criterion:** The method should be able to tolerate about 5.0 % variance in chromatographic conditions).

The method was able to tolerate small changes in the chromatographic conditions.

B.13. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Isoniazid	1.6 minutes
Ethambutol hydrochloride	5.9 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Isoniazid	1 841
Ethambutol hydrochloride	3 431

USP Tailing factor (T):

Isoniazid	1.421
Ethambutol hydrochloride	1.083

Capacity factor (k'):

Isoniazid	0.880
Ethambutol hydrochloride	6.510

Resolution between peaks (R):

Isoniazid	-
Ethambutol hydrochloride	16.460

B.14. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates and the resolution between the isoniazid and ethambutol hydrochloride peaks.
* Use the 5-sigma method to calculate the parameters in B.13.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 2.0 %.
* The column must have more than 1 500 theoretical plates for isoniazid and 3 000 plates for ethambutol hydrochloride.
* The resolution between the peaks of isoniazid and ethambutol hydrochloride must be at least 10.
* The USP tailing must be less than 1.5.

B.15. CONCLUSION

The method performed well and should be suitable to analyse isoniazid and ethambutol hydrochloride in products for stability testing, quality control and batch release purposes. No interference was encountered from stressed samples, thus the method can be regarded as being stability indicating.

APPENDIX C

Validation of an HPLC Assay Method:

Determination of Isoniazid and Pyrazinamide Content in a 3 Fixed Dose Combination product containing Isoniazid, Pyrazinamide and Rifampicin

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C.1. ORIGIN OF METHOD

The method was based in part on a method supplied by Manufacturer A (2004) for the determination of ethambutol hydrochloride in 4FDC tablets.

C.2. SPECIFICATION

The tablets must contain the following:

Isoniazid	75 mg (67.5 – 82.5 mg)
Pyrazinamide	400 mg (360 – 440 mg)

C.3. SUMMARY

In Table C.1 a summary of the validation results is given.

Table C.1: Summary of validation results

Parameter	Acceptance criteria	Results
Specificity	The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients.	Complies
Linearity and Range	The method is linear over the range: 10 – 150 % of the expected sample concentration. R^2 is not less than 0.99.	Complies Isoniazid: Linear from 5 – 82 µg/ml (10 – 150 % of the expected sample concentration) R^2: 0.9996 Pyrazinamide: Linear from 29 – 437 µg/ml (10 – 150 % of the expected sample concentration) R^2: 0.9997
Accuracy (Recovery)	Recovery must be between 98.0 – 102.0 %.	Complies Isoniazid: 100.0 % Pyrazinamide: 100.0 %
Precision (Repeatability)	RSD of 2.0 % or less.	Complies Isoniazid: Intra-day = 0.8 %, Inter-day = 1.2 % Pyrazinamide: Intra-day = 1.9 %, Inter-day = 1.1 %
Ruggedness (Stability)	The sample solution should not be used for a period longer than it takes to degrade by 2.0 %.	Isoniazid: Stable for 6 hours Pyrazinamide: Stable for 4 hours
Ruggedness (System repeatability)	The peak area and retention times should have an RSD of 2.0 % or less.	Complies Isoniazid: 0.5 % for peak area, 0.1 % for retention time Pyrazinamide: 0.6 % for peak area, 0.1 % for retention time
Robustness	The method should be able to tolerate about 5.0 % variance in chromatographic conditions.	Complies

C.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: Column L1, USP 29, 2006, p 2649, (Luna C_{18}-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 50 g ammonium acetate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 5.0 ± 0.05 with glacial acetic acid. Mix 940 ml of this solution with 60 ml methanol.

Flow rate: 2.0 ml/min.

Injection volume: 20 µl.

Detection: 240 nm (Diode Array Detection).

Retention times: About 1.8 and 2.2 minutes for isoniazid and pyrazinamide respectively.

Solvent: Water.

C.5. SAMPLE SOLUTION PREPARATION

1. Accurately weigh and powder 20 tablets and mix. Accurately weigh an amount of powder equivalent to 27.3 mg of isoniazid.
2. Transfer the powder into a 500 ml volumetric flask, and dissolve in about 400 ml of water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtrate.
5. Analyse by means of HPLC.

C.6. STANDARD SOLUTION PREPARATION

1. Accurately weigh about 27.3 mg of isoniazid reference standard[1] and 145.5 mg of pyrazinamide reference standard[2] into a 500 ml volumetric flask.
2. Dissolve in about 400 ml water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Analyse by means of HPLC (6 injections).

C.7. CALCULATIONS

Isoniazid

The tablet contains 75 mg isoniazid and 400 mg pyrazinamide. The prepared sample solution will contain 0.055 mg/ml isoniazid.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

Pyrazinamide

The tablet contains 75 mg isoniazid and 400 mg pyrazinamide. The prepared sample solution will contain 0.291 mg/ml pyrazinamide.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

[1] Isoniazid primary standard: USP, B/N 03452/INH

[2] Pyrazinamide primary standard: USP, B/N 032390281

C.8. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

C.8.1 Specificity

Method

1. Prepare a solution from the placebo powder[3] similar to preparing the sample solution.
2. Prepare a sample solution and dilute 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
3. Store the solutions prepared in point 2, overnight in closed test tubes at 40 °C/75 % RH to bring about degradation.
4. Analyse by means of HPLC.
5. Examine the chromatograms to determine whether any additional peaks were formed.

Acceptance criteria

The placebo should not generate any peaks that will interfere with the determination of the actives.

Extra peaks formed under stress conditions should be discernible from those of the active ingredients.

C.8.2 Linearity and Range

Method

1. Prepare a standard solution as described under standard preparation.
2. Inject variable volumes into the chromatograph to obtain standards from 10 – 150 % of the expected sample concentration.

Acceptance criterion

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

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[3] See Appendix B.8.1.

C.8.3 Accuracy

Method

1. Weigh amounts of the placebo equivalent to the amount of sample powder that would contain 80 %, 100 % and 120 % of 27.3 mg of isoniazid. Add 400 ml of solvent in triplicate as described under Sample Solution Preparation.
2. Spike, respectively with known amounts of the active ingredients at concentrations of approximately 80, 100 and 120 % of the expected sample concentration, make up to volume and filter.
3. Analyse in duplicate by means of HPLC.

Acceptance criterion

Recovery must be between 98.0 – 102.0 %.

C.8.4 Precision

C.8.4.1 Intra-day Precision

Method

1. Weigh amounts of tablet powder equivalent to 80 %, 100 % and 120 % of 27.3 mg of isoniazid. Prepare solutions thereof in triplicate as described under sample preparation.
2. Analyse in duplicate by means of HPLC.

C.8.4.2 Inter-day Precision

Method

1. Analyse the same homogenous sample in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more occasions on different days to determine the between-day variability of the method.

Acceptance criteria

Repeatability must be better than 2.0 % (n = 9).

Inter-day precision must be better than 2.0 % (n = 9).

C.8.5 Ruggedness

C.8.5.1 Stability of sample solution

Method

1. Prepare a sample solution as described under sample preparation in the method.
2. Analyse by means of HPLC.
3. Leave the sample solution in the auto sampler tray and re-analyse over a period of 8 hours to determine the stability.

Acceptance criterion

Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %, and in the case of degradation, special precautions should be followed to compensate for the loss.

C.8.5.2 System Repeatability

Method

1. Inject a sample six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area and retention times should have an RSD of 2.0 % or less.

C.8.6 Robustness

Method

1. Make deliberate changes to the chromatographic conditions to determine the method's tolerance towards changes.
2. Change the flow rate, wavelength, injection volume, mobile phase pH and use a similar column from a different manufacturer.

Acceptance criterion

The method should be able to tolerate about 5.0 % variance in the chromatographic conditions.

C.8.7 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between peaks and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 2.0 %. The resolution and plate count have to be determined through the validation for the specific system.

C.9. VALIDATION RESULTS

C.9.1 Specificity

A chromatogram of the solution containing the placebo is shown in Figure C.1. Figure C.2 shows a chromatogram obtained for a tablet sample solution. Figure C.3 shows a chromatogram of a standard solution. Figures C.4 – C.7 show chromatograms of sample solutions that have been stressed overnight as described under C.8.1. The analyte peaks that remained in the aqueous solution after being stressed were analysed by means of diode array peak purity testing to determine whether the peaks are still pure. The purity test results are given in Figures C.8 – C.9.

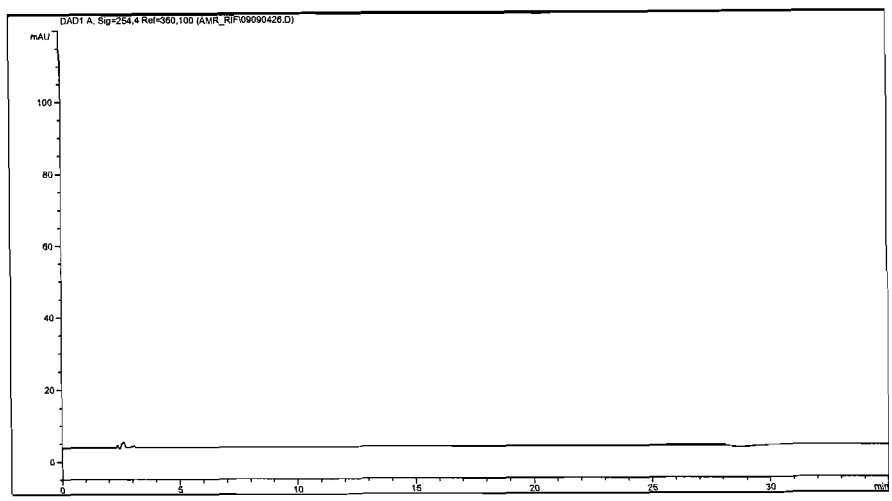


Figure C.1: Chromatogram of a solution containing the placebo

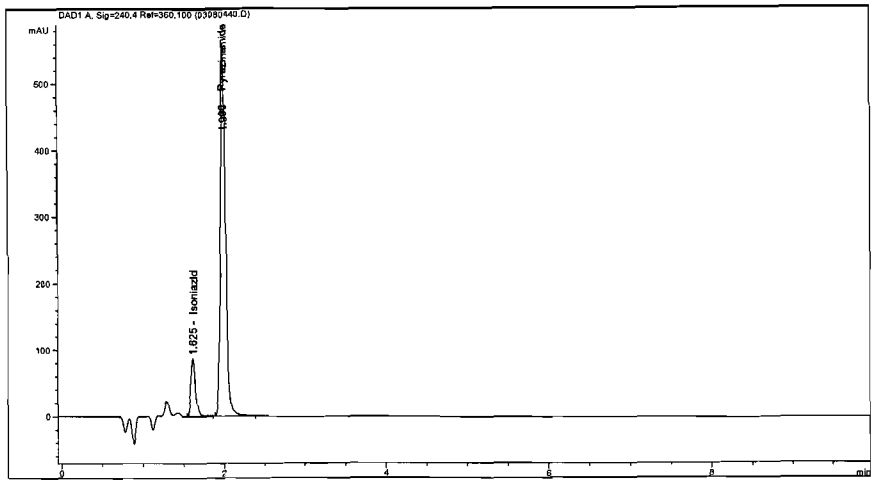


Figure C.2: Chromatogram of a tablet sample solution

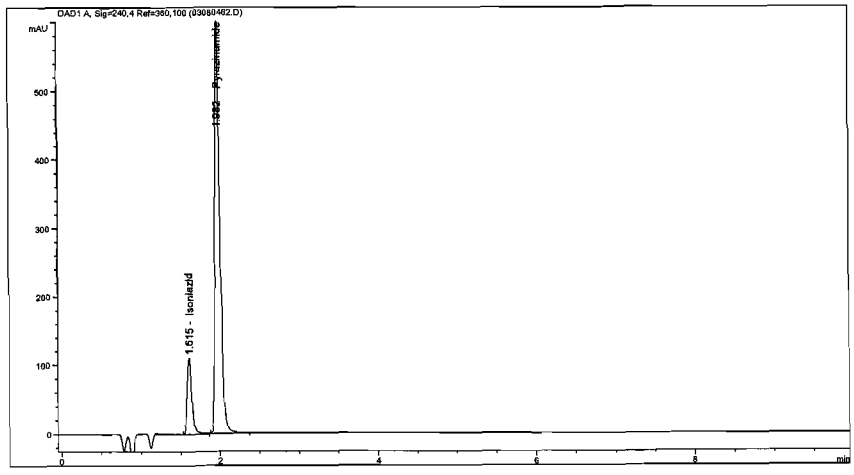


Figure C.3: Chromatogram of a standard solution

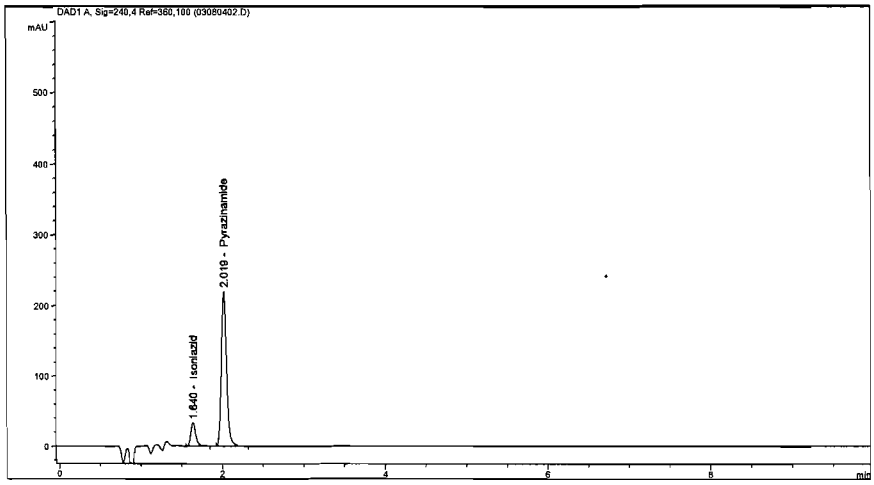


Figure C.4: Sample stressed overnight in water at 40 °C/75 % RH

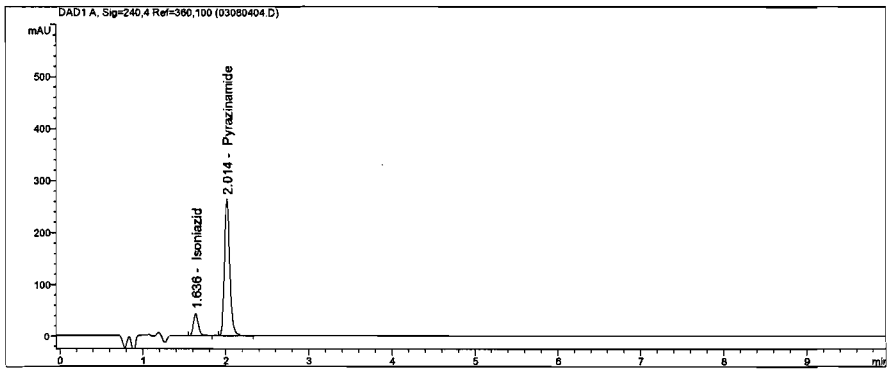


Figure C.5: Sample stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

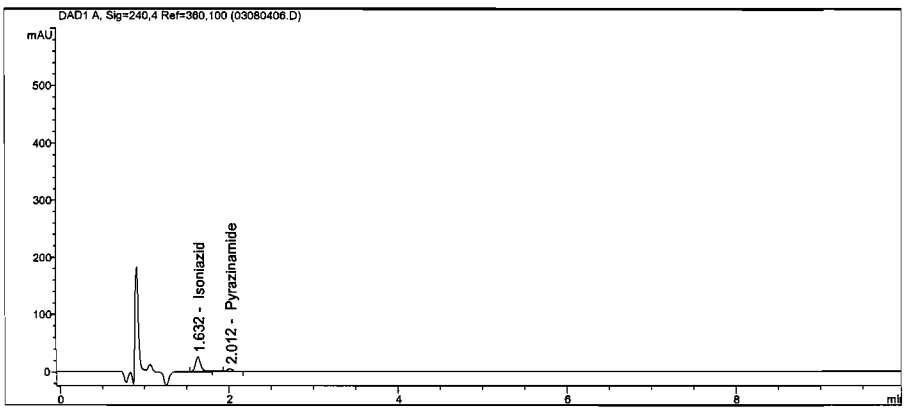


Figure C.6: Sample stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

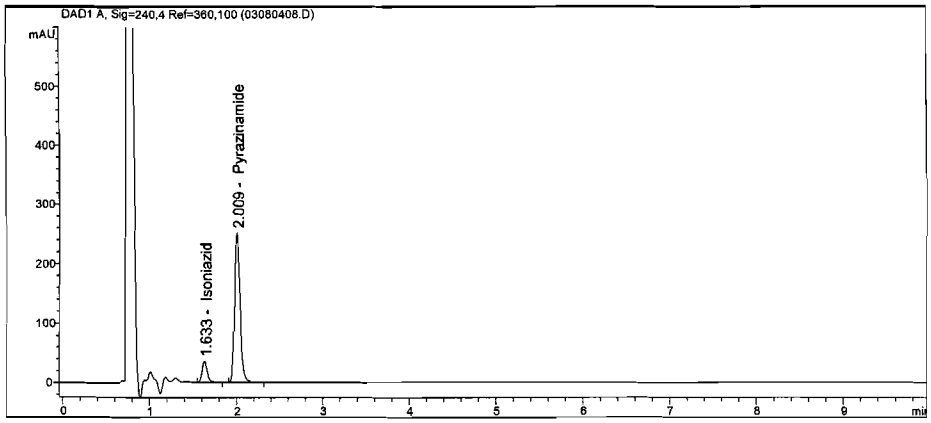


Figure C.7: Sample stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

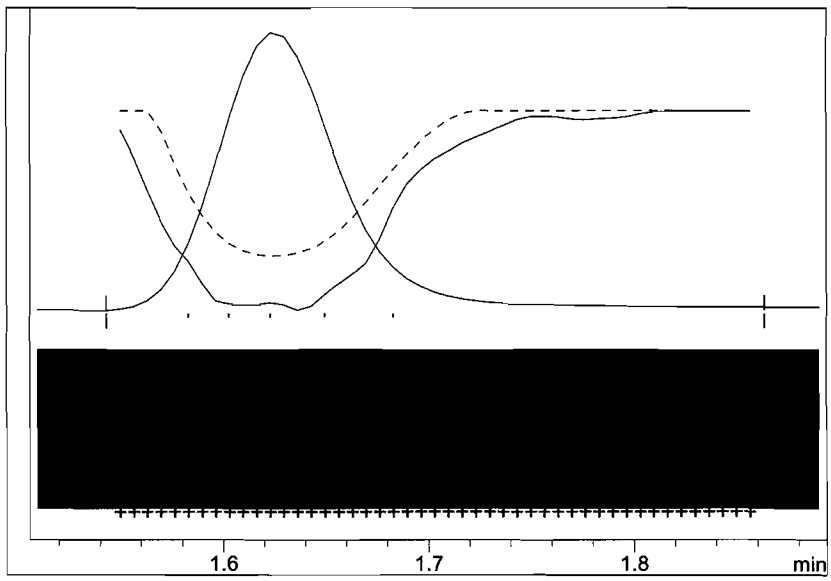


Figure C.8: Peak purity test results for isoniazid

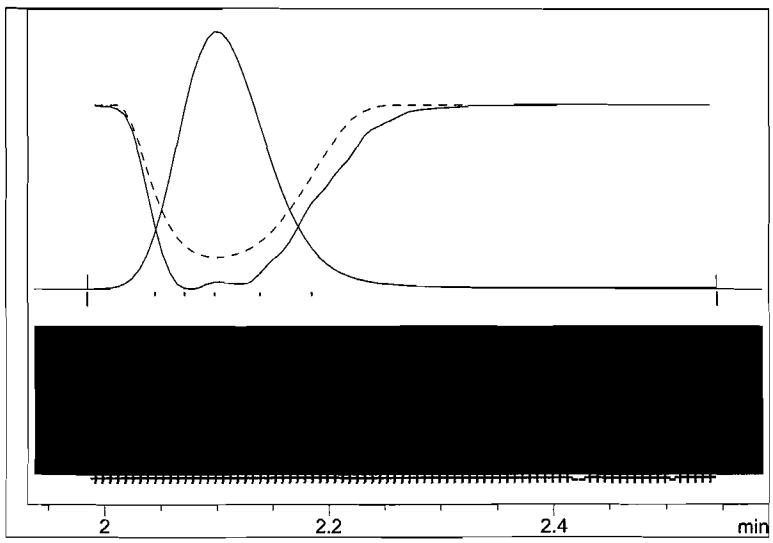


Figure C.9: Peak purity test results for pyrazinamide

Conclusion

(**Acceptance criterion:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

None of the ingredients in the placebo interfered with the analyte peaks. Extra peaks formed during forced degradation did not interfere with any of the peaks of interest. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

C.10. ISONIAZID

Specificity results for isoniazid are given in Table C.2.

C.10.1 Specificity

Table C.2: Results for isoniazid to determine Specificity

Stress condition (40 °C)	% Degradation of isoniazid
Water	15.7
0.1 M Hydrochloric acid	22.8
0.1 M Sodium hydroxide	37.0
10 % Hydrogen peroxide	42.6

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 15 % and 20 % occurred in sample solutions mixed with water and 0.1 M hydrochloric acid, respectively. Degradation of about 30 to 40 % occurred in sample solutions mixed with 0.1 M sodium hydroxide and 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

C.10.2 Linearity and Range

Linearity and Range results for isoniazid are given in Table C.3.

Table C.3: Results for isoniazid to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
5	48.8	48.9	48.8
11	97.2	96.9	97.1
16	144.3	141.5	142.9
22	188.9	187.3	188.1
27	235.4	237.1	236.3
33	288.5	285.7	287.1
38	340.6	332.4	336.5
44	387.2	380.1	383.7
49	429.3	429.5	429.4
55	481.2	482.1	481.6
60	534.6	534.6	534.6
66	591.2	582.2	586.7
71	629.8	634.3	632.0
76	681.9	680.7	681.3
82	729.9	733.7	731.8

Regression statistics are given in Table C.4.

Table C.4: Regression statistics of isoniazid results

R squared	0.9996	Lower 95%	Upper 95%
Intercept	-3.5104	-8.8078	1.7869
Slope	8.9321	8.8255	9.0388

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 5 – 82 µg/ml (10 – 150 % of expected sample concentration) as shown in Figure C.10. The regression coefficient (R squared) is 0.9996. The method is suitable for single point calibration.

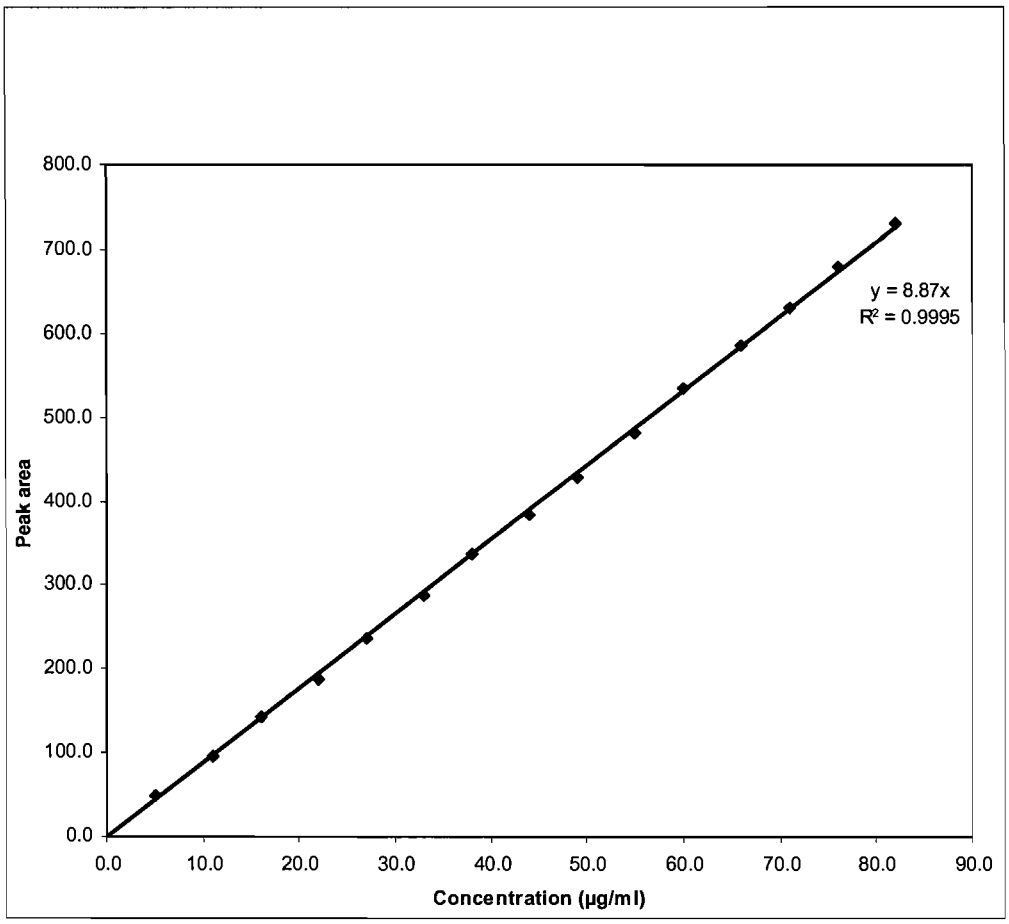


Figure C.10: Linear regression graph for isoniazid to determine Linearity and Range

C.10.3 Accuracy

Accuracy results for isoniazid and the statistical analysis thereof are given in Tables C.5 and C.6, respectively.

Table C.5: Results for isoniazid to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
44	388.2	375.7	382.0	43.2	98.22
44	390.0	397.1	393.6	44.5	101.20
44	389.7	392.6	391.2	44.3	100.58
55	481.6	485.0	483.3	54.9	99.79
55	486.9	482.6	484.7	55.0	100.08
55	486.4	483.6	485.0	55.1	100.14
66	600.6	605.7	603.1	65.7	99.54
66	607.4	604.3	605.9	66.0	99.99
66	607.4	610.0	608.7	66.3	100.46

Table C.6: Statistical analysis of isoniazid results

Mean %	100.0
SD	0.8
% RSD	0.8
95% confidence intervals	
Lower limit	99.4
Upper limit	100.7
Estimated median	100.1
Confidence Level (95.0%)	0.6

Conclusion

(**Acceptance criterion:** Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

C.10.4 Precision

C.10.4.1 Intra-day Precision

Intra-day precision results for isoniazid are given in Table C.7.

Table C.7: Results for isoniazid to determine Intermediate Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery %
0.06592	388.9	379.4	384.1	44	98.78
0.06595	383.3	388.5	385.9	44	99.23
0.06600	383.2	396.5	389.9	44	100.25
0.08233	485.7	481.3	483.5	55	99.83
0.08238	482.8	480.8	481.8	55	99.48
0.08237	487.3	492.1	489.7	55	101.10
0.09890	596.0	600.2	598.1	66	98.71
0.09888	600.2	597.0	598.6	66	98.80
0.09882	599.5	594.4	597.0	66	98.52
				Mean	99.4
				SD	0.8
				RSD %	0.8

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 0.8 %.

C.10.4.2 INTER-DAY PRECISION

Inter-day precision results for isoniazid and ANOVA single factor statistics thereof are given in Tables C.8 and C.9, respectively.

Table C.8: Results for isoniazid to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	483.50	476.54	472.50	
	481.80	493.41	474.48	
	489.68	490.17	474.97	
Mean	485.00	486.71	473.99	481.90
SD	3.39	7.31	1.07	5.64
RSD %	0.70	1.50	0.23	1.17

Table C.9: ANOVA single factor statistics for the determination of isoniazid

Groups	Count	Sum	Average	Variance	
Day 1	3	1454.99	485.00	17.20	
Day 2	3	1460.13	486.71	80.16	
Day 3	3	1421.96	473.99	1.71	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	286.09	2	143.04	4.33	0.07
Within Groups	198.15	6	33.02		
Total	484.23	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 1.17 %.

C.10.5 Ruggedness

C.10.5.1 Stability of sample solutions

A sample solution was left on the auto sampler tray in amber vials and re-analysed over several time intervals to determine the sample stability. The results are presented in Table C.10.

Table C.10: Results for isoniazid to determine Ruggedness

Time (hours)	Peak Area	%
0	429.16	100.00
1	427.22	99.55
2	425.92	99.25
3	425.80	99.22
4	424.73	98.97
5	423.62	98.71
6	421.93	98.32
7	420.52	97.99
8	418.61	97.54
Mean	424.17	98.84
SD	3.16	0.74
RSD %	0.74	0.74

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of less than 3.0 % was observed over an 8 hour period. Sample solutions are considered stable for 6 hours.

C.10.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table C.11.

Table C.11: Results for isoniazid to determine System Repeatability

	Area	Retention time (minutes)
	484.19	1.76
	485.92	1.76
	489.01	1.76
	491.10	1.76
	490.87	1.76
	490.58	1.76
Mean	488.61	1.76
SD	2.65	0.00
RSD %	0.54	0.14

Conclusion

(Acceptance criterion: The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 0.54 % for peak area and 0.14 % for retention time respectively.

C.11. PYRAZINAMIDE

C.11.1 Specificity

Specificity results for pyrazinamide are given in Table C.12.

Table C.12: Results for pyrazinamide to determine Specificity

Stress condition (40 °C)	% Degradation of pyrazinamide
Water	6.4
0.1 M Hydrochloric acid	7.6
0.1 M Sodium hydroxide	7.7
10 % Hydrogen peroxide	8.5

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 6 to 9 % occurred in sample solutions mixed with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

C.11.2 Linearity and Range

Linearity and Range results for isoniazid are given in Table C.13.

Table C.13: Results for pyrazinamide to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
29	714.4	712.2	713.3
58	1597.9	1586.1	1592.0
87	2356.8	2343.5	2350.1
116	3065.4	3071.8	3068.6
146	3734.4	3758.4	3746.4
175	4526.0	4515.3	4520.7
204	5284.1	5239.9	5262.0
233	6020.5	6055.7	6038.1
262	6790.3	6810.3	6800.3
291	7401.6	7487.5	7444.6
320	8217.8	8251.5	8234.7
349	8969.0	8992.4	8980.7
378	9662.4	9635.0	9648.7
407	10469.5	10486.7	10478.1
437	11076.7	11053.2	11064.9

Regression statistics are given in Table C.14.

Table C.14: Regression statistics of pyrazinamide results

R squared	0.9997	Lower 95%	Upper 95%
Intercept	88.5082	23.1401	153.8763
Slope	25.3767	25.1296	25.6238

Conclusion

(**Acceptance criterion:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 29 – 437 µg/ml (10 – 150 % of expected sample concentration) as shown in Figure C.11. The regression coefficient (R squared) is 0.9997. The method is suitable for single point calibration.

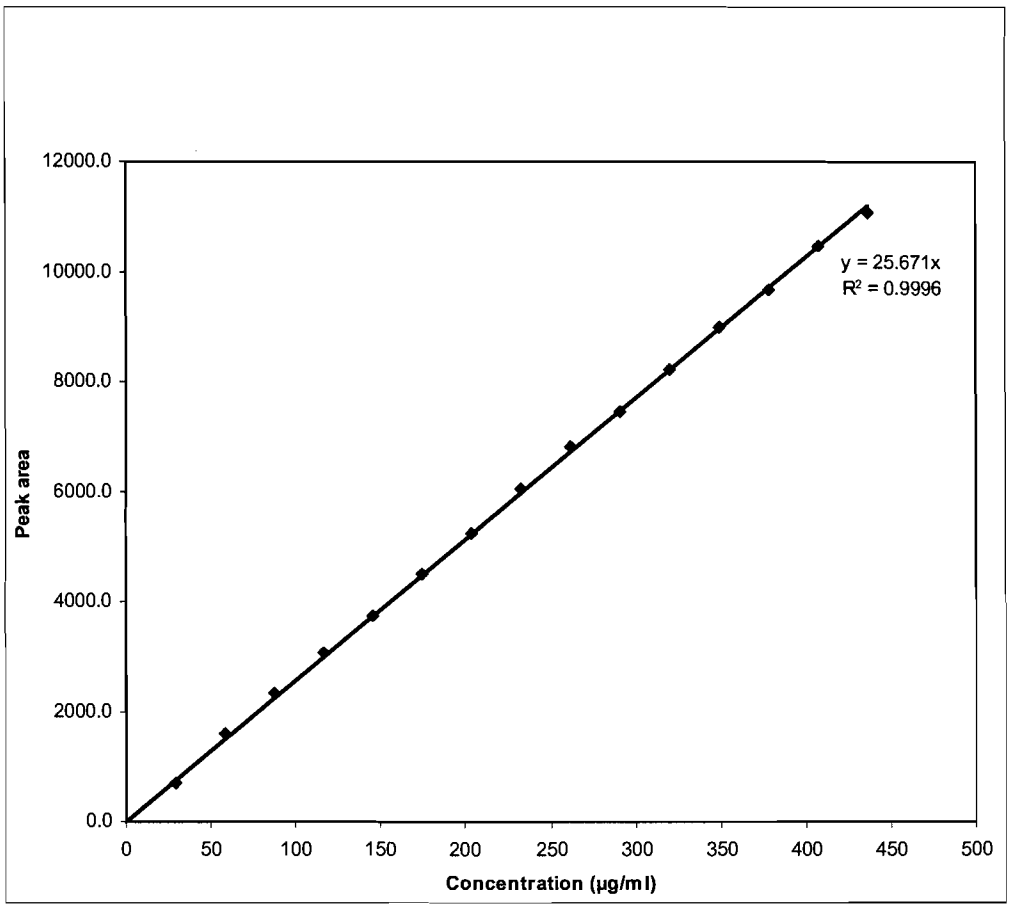


Figure C.11: Linear regression graph for pyrazinamide to determine Linearity and Range

C.11.3 Accuracy

Accuracy results for pyrazinamide and the statistical analysis thereof are given in Tables C.15 and C.16, respectively.

Table C.15: Results for pyrazinamide to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
230	6906.1	6813.4	6859.8	229.2	99.67
230	6895.0	6893.9	6894.5	230.4	100.18
230	6923.2	6862.2	6892.7	230.3	100.15
290	7789.9	7821.3	7805.6	291.2	100.42
290	7821.0	7756.1	7788.6	290.6	100.20
290	7696.4	7752.6	7724.5	288.2	99.38
350	8569.8	8555.6	8562.7	350.8	100.22
350	8517.3	8533.9	8525.6	349.3	99.79
350	8553.7	8531.5	8542.6	350.0	99.99

Table C.16: Statistical analysis of pyrazinamide results

Mean %	100.0
SD	0.3
% RSD	0.3
95% confidence intervals	
Lower limit	99.9
Upper limit	100.4
Estimated median	100.2
Confidence Level (95.0%)	0.3

Conclusion

(Acceptance criterion: Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

C.11.4 Precision

C.11.4.1 Intra-day Precision

Intra-day precision results for pyrazinamide are given in Table C.17.

Table C.17: Results for pyrazinamide to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.06592	6990.9	6928.4	6959.7	230	101.12
0.06595	6891.2	6867.7	6879.4	230	99.96
0.06600	6702.2	6718.0	6710.1	230	97.50
0.08233	7909.9	7928.7	7919.3	290	101.88
0.08238	7469.2	7451.8	7460.5	290	95.98
0.08237	7718.9	7754.0	7736.4	290	99.53
0.09890	8720.7	8679.1	8699.9	350	101.83
0.09888	8509.1	8359.0	8434.0	350	98.72
0.09882	8632.6	8620.5	8626.6	350	100.97
				Mean	99.7
				SD	1.9
				RSD %	1.9

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.9 %.

C.11.4.2 INTER-DAY PRECISION

Inter-day precision results for pyrazinamide and ANOVA single factor statistics thereof are given in Tables C.18 and C.19, respectively.

Table C.18: Results for pyrazinamide to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	7919.27	7779.10	7593.60	
	7460.47	7657.18	7540.71	
	7736.43	7853.95	7573.41	
Mean	7705.39	7763.41	7569.24	7679.35
SD	188.59	81.09	21.79	81.38
RSD %	2.45	1.04	0.29	1.06

Table C.19: ANOVA single factor statistics for the determination of pyrazinamide

Groups	Count	Sum	Average	Variance	
Day 1	3	23116.17	7705.39	53346.52	
Day 2	3	23290.23	7763.41	9864.16	
Day 3	3	22707.72	7569.24	712.46	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	59604.60	2	29802.30	1.40	0.32
Within Groups	127846.28	6	21307.71		
Total	187450.88	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 1.06 %.

C.11.5 Ruggedness

C.11.5.1 Stability of sample solutions

A sample solution was left in amber vials on the auto sampler tray and re-analysed over several time intervals to determine the sample stability. The results are presented in Table C.20.

Table C.20: Results for pyrazinamide to determine Ruggedness

Time (hours)	Peak Area	%
0	7897.7	100.00
1	7812.4	98.92
2	7803.8	98.81
3	7788.3	98.62
4	7759.5	98.25
5	7755.0	98.19
6	7738.6	97.99
7	7728.2	97.85
8	7657.2	96.95
Mean	7771.2	98.40
SD	62.7	0.79
RSD %	0.8	0.81

Conclusion

(Acceptance criterion: The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of about 3.1 % was observed over an 8 hour period. Sample solutions are considered stable for 4 hours.

C.11.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table C.21.

Table C.21: Results for pyrazinamide to determine System Repeatability

	Area	Retention time (minutes)
	7927.8	2.21
	7879.1	2.21
	7852.2	2.21
	7825.4	2.21
	7868.4	2.21
	7773.8	2.20
Mean	7854.4	2.21
SD	47.5	0.00
RSD %	0.6	0.14

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 0.6 % for peak area and 0.14 % for retention time respectively.

C.12. ROBUSTNESS

The following changes in the chromatographic operating parameters were found to be acceptable:

Column: Luna C18-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance, CA; and
Lichrospher 100-5 RP-1ec cartridge column, 125 x 4.0 mm, 5 µm, 100 10µm pores, 21.5 % carbon load, end capped, Machery-Nagel, Düren, Germany.
The Lichrospher gave different retention times, thus the flow rate must be altered.

Mobile phase pH: pH 4.8 – 5.0.

Flow rate: 1.9 – 2.1 ml/minute.

Wavelength: The wavelength can be altered by ± 2 nm without any ill effect.

Injection volume: 16 – 24 µl.

Conclusion

(**Acceptance criterion:** The method should be able to tolerate about 5.0 % variance in chromatographic conditions).

The method was able to tolerate small changes in the chromatographic conditions.

C.13. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Isoniazid	1.8 minutes
Pyrazinamide	2.2 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Isoniazid	2 899
Pyrazinamide	4 178

USP Tailing factor (T):

Isoniazid	1.247
Pyrazinamide	1.208

Capacity factor (k'):

Isoniazid	0.906
Pyrazinamide	1.339

Resolution between peaks (R):

Isoniazid	-
Pyrazinamide	3.025

C.14. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates and the resolution between the isoniazid and pyrazinamide peaks.
* Use the 5-sigma method to calculate the parameters in C.13.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 2.0 %.
* The column must have more than 2 000 and 3 500 theoretical plates for isoniazid and pyrazinamide respectively.
* The resolution between the peaks of isoniazid and pyrazinamide must be at least 2.0.
* The USP tailing must be less than 1.5.

C.15. CONCLUSION

The method performed well and should be suitable to analyse isoniazid and pyrazinamide in products for stability testing, quality control and batch release purposes. No interference was encountered from stressed samples, thus the method can be regarded as being stability indicating.

APPENDIX D

Validation of an HPLC Assay Method:

Determination of Ethambutol HCl, Isoniazid and Pyrazinamide Content in a 4 Fixed Dose Combination product containing Ethambutol HCl, Isoniazid, Pyrazinamide and Rifampicin

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D.1. ORIGIN OF METHOD

The method was based in part on a method supplied by Manufacturer A (2004) for the determination of ethambutol hydrochloride in 4FDC tablets.

D.2. SPECIFICATION

The tablets must contain the following:

Ethambutol hydrochloride	275 mg (247.5 – 302.5 mg)
Isoniazid	75 mg (67.5 – 90.0 mg)
Pyrazinamide	400 mg (360 – 440 mg)

D.3. SUMMARY

In Table D.1 a summary of the validation results is given.

Table D.1: Summary of validation results (Continue on next page)

Parameter	Acceptance criteria	Results
Specificity	The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients.	Complies
Linearity and Range	The method is linear over the range: 10 – 150 % of the expected sample concentration. R^2 is not less than 0.99.	Complies Ethambutol hydrochloride: Linear from 20 – 300 µg/ml (10 – 150 % of the expected sample concentration) R^2: 0.9999 Isoniazid: Linear from 5.5 – 81.9 µg/ml (10 – 150 % of the expected sample concentration) R^2: 0.9998 Pyrazinamide: Linear from 29 – 437 µg/ml (10 – 150 % of the expected sample concentration) R^2: 0.9997
Accuracy (Recovery)	Recovery must be between 98.0 – 102.0 %.	Complies Ethambutol hydrochloride:100.0 % Isoniazid: 100.0 % Pyrazinamide: 100.0 %
Precision (Repeatability)	RSD of 2.0 % or less.	Complies Ethambutol hydrochloride: Intra-day = 1.1 %, Inter-day = 0.2 % Isoniazid: Intra-day = 0.8 %, Inter-day = 1.2 % Pyrazinamide: Intra-day = 1.9 %, Inter-day = 1.1 %
Ruggedness (Stability)	The sample solution should not be used for a period longer than it takes to degrade by 2.0 %.	Ethambutol hydrochloride: Stable for 6 hours Isoniazid: Stable for 6 hours Pyrazinamide: Stable for 4 hours

Table D.1: Summary of validation results (Continued from previous page)

Parameter	Acceptance criteria	Results
Ruggedness (System repeatability)	The peak area and retention times should have an RSD of 2.0 % or less.	Complies Ethambutol hydrochloride: 1.2 % for peak area, 0.2 % for retention time Isoniazid: 0.5 % for peak area, 0.1 % for retention time Pyrazinamide: 0.6 % for peak area, 0.1 % for retention time
Robustness	The method should be able to tolerate about 5.0 % variance in chromatographic conditions.	Complies

D.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: Column L1, USP 29, 2006, p 2649, (Luna C_{18}-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 50 g ammonium acetate and 0.2 g copper(II) acetate monohydrate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 5.0 ± 0.05 with glacial acetic acid. Mix 940 ml of this solution with 60 ml methanol.

Flow rate: 2.0 ml/min.

Injection volume: 20 µl.

Detection: 270 nm (Diode Array Detection).

Retention times: About 1.8, 2.2 and 5.6 minutes for isoniazid, pyrazinamide and ethambutol hydrochloride respectively.

Solvent: Water.

D.5. SAMPLE SOLUTION PREPARATION

1. Accurately weigh and powder 20 tablets and mix. Accurately weigh an amount of powder equivalent to 100 mg ethambutol hydrochloride.
2. Transfer the powder into a 500 ml volumetric flask, and dissolve in about 400 ml of water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtrate.
5. Analyse by means of HPLC.

D.6. STANDARD SOLUTION PREPARATION

1. Accurately weigh about 100 mg of ethambutol hydrochloride reference standard[1], 27.3 mg of isoniazid reference standard[2] and 145.5 mg of pyrazinamide reference standard[3] into a 500 ml volumetric flask.
2. Dissolve in about 400 ml water.
3. Shake well for about 10 minutes and make up to volume with water.
4. Analyse by means of HPLC (6 injections).

D.7. CALCULATIONS

Ethambutol hydrochloride

The tablet contains 275 mg ethambutol hydrochloride, 75 mg isoniazid and 400 mg pyrazinamide. The prepared sample solution will contain 0.2 mg/ml ethambutol hydrochloride.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

[1] Ethambutol hydrochloride primary standard: USP, B/N 032501195

[2] Isoniazid primary standard: USP, B/N 03452/INH

[3] Pyrazinamide primary standard: USP, B/N 032390281

Isoniazid

The tablet contains 275 mg ethambutol hydrochloride, 75 mg isoniazid and 400 mg pyrazinamide. The prepared sample solution will contain 0.055 mg/ml isoniazid.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

Pyrazinamide

The tablet contains 275 mg ethambutol hydrochloride, 75 mg isoniazid and 400 mg pyrazinamide. The prepared sample solution will contain 0.291 mg/ml pyrazinamide.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

D.8. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

D.8.1 Specificity

Method

1. Prepare a solution from the placebo powder[4] similar to preparing the sample solution.
2. Prepare a sample solution and dilute 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
3. Store the solutions prepared in point 2, overnight in closed test tubes at 40 °C/75 % RH to bring about degradation.
4. Analyse by means of HPLC.
5. Examine the chromatograms to determine whether any additional peaks were formed.

[4] See Appendix B.8.1.

Acceptance criteria

The placebo should not generate any peaks that will interfere with the determination of the actives.

Extra peaks formed under stress conditions should be discernible from those of the active ingredients.

D.8.2 Linearity and Range

Method

1. Prepare a standard solution as described under Standard Solution Preparation.
2 Inject variable volumes into the chromatograph to obtain standards from 10 – 150 % of the expected sample concentration.

Acceptance criterion

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

D.8.3 Accuracy

Method

1. Weigh amounts of the placebo equivalent to the amount of sample powder that would contain 80 %, 100 % and 120 % of 100 mg of ethambutol hydrochloride. Add 400 ml of solvent in triplicate as described under Sample Solution Preparation.
2. Spike, respectively with known amounts of the active ingredients at concentrations of approximately 80, 100 and 120 % of the expected sample concentration, make up to volume and filter.
3. Analyse in duplicate by means of HPLC.

Acceptance criterion

Recovery must be between 98.0 – 102.0 %.

D.8.4 Precision

D.8.4.1 Intra-day Precision

Method

1. Weigh amounts of tablet powder equivalent to 80 %, 100 % and 120 % of 100 mg of ethambutol hydrochloride. Prepare solutions thereof in triplicate as described under Sample Solution Preparation.
2. Analyse in duplicate by means of HPLC.

D.8.4.2 Inter-day Precision

Method

1. Analyse the same homogenous sample in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more occasions on different days to determine the between-day variability of the method.

Acceptance criteria

Repeatability must be better than 2.0 % (n = 9).

Inter-day precision must be better than 2.0 % (n = 9).

D.8.5 Ruggedness

D.8.5.1 Stability of sample solution

Method

1. Prepare a sample solution as described under Sample Solution Preparation.
2. Analyse by means of HPLC.
3. Leave the sample solution in the auto sampler tray and re-analyse over a period of 8 hours to determine the stability.

Acceptance criterion

Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %, and in the case of degradation, special precautions should be followed to compensate for the loss.

D.8.5.2 SYSTEM REPEATABILITY

Method

1. Inject a sample solution six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area and retention times should have an RSD of 2.0 % or less.

D.8.6 Robustness

Method

1. Make deliberate changes to the chromatographic conditions to determine the method's tolerance towards changes.
2. Change the flow rate, wavelength, injection volume, mobile phase pH and use a similar column from a different manufacturer.

Acceptance criterion

The method should be able to tolerate about 5.0 % variance in the chromatographic conditions.

D.8.7 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between peaks and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 2.0 %. The resolution and plate count have to be determined through the validation for the specific system.

D.9. VALIDATION RESULTS

D.9.1 Specificity

A chromatogram of the solution containing the placebo is shown in Figure D.1. Figure D.2 shows a chromatogram obtained for a sample solution. Figure D.3 shows a chromatogram of a standard solution. Figures D.4 – D.7 show chromatograms of sample solutions that have been stressed overnight as described under D.8.1. The analyte peaks that remained in the aqueous solution after being stressed were analysed by means of diode array peak purity testing to determine whether the peaks are still pure. The purity test results are given in Figures D.8 – D.10.

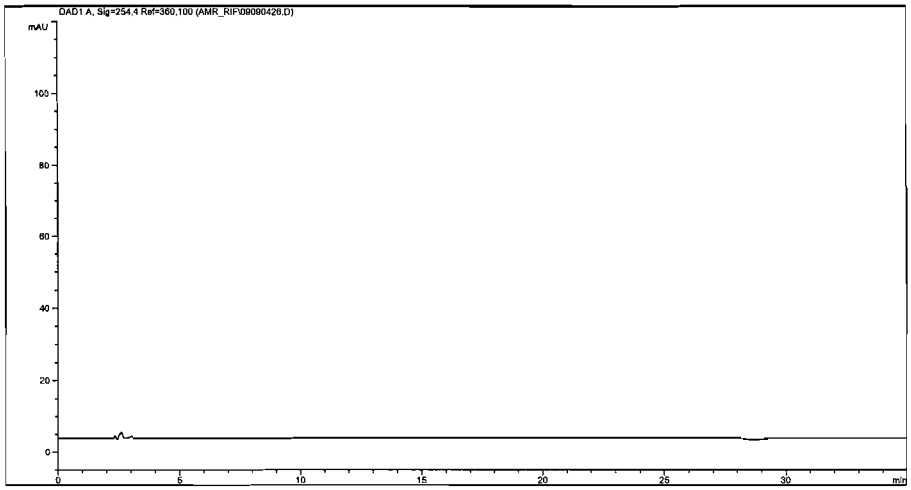


Figure D.1: Chromatogram of a solution containing the placebo

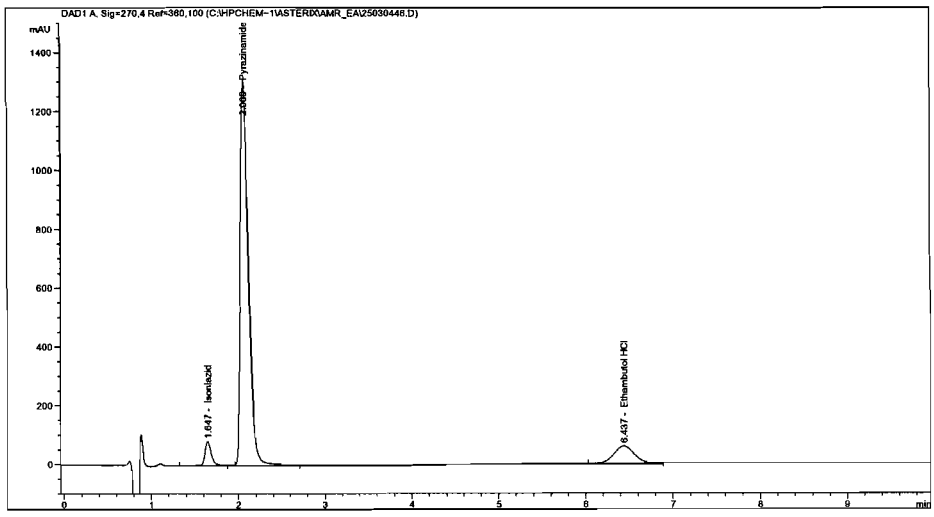


Figure D.2: Chromatogram of a sample solution

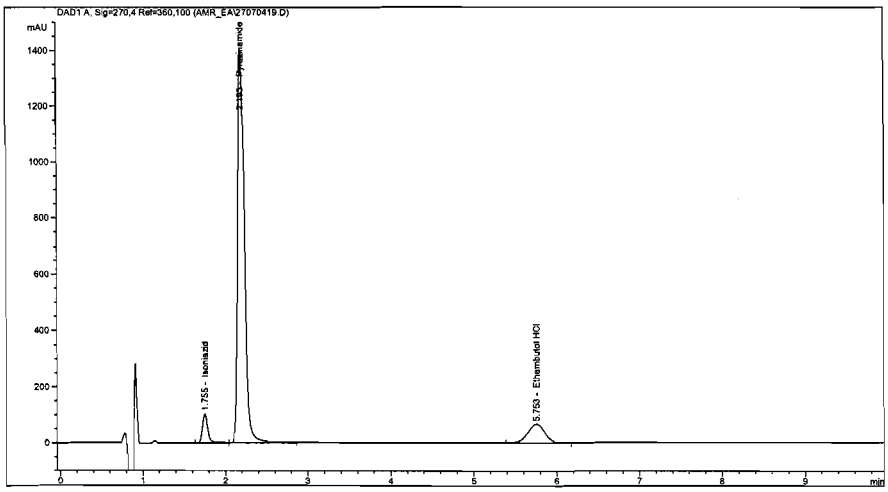


Figure D.3: Chromatogram of a standard solution

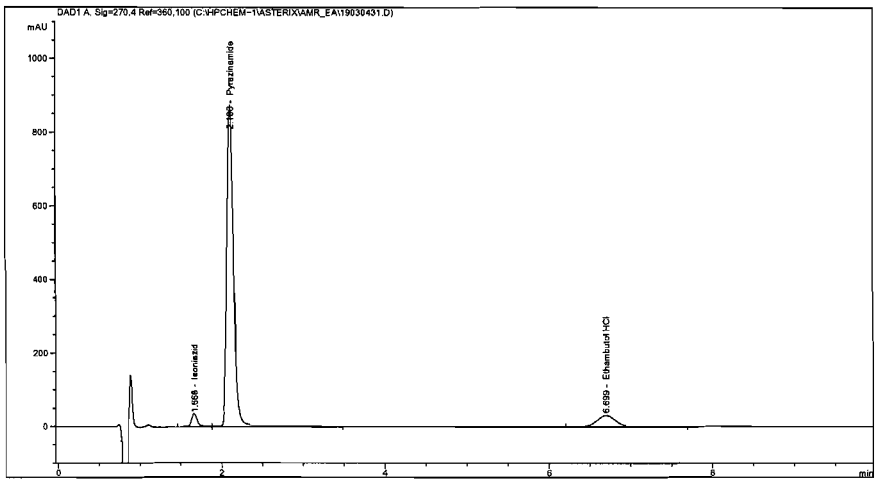


Figure D.4: Sample solution stressed overnight in water at 40 °C/75 % RH

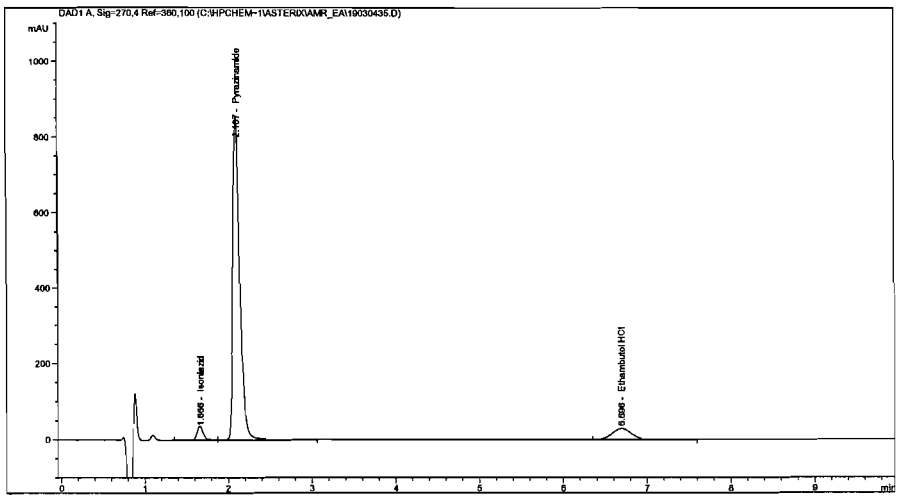


Figure D.5: Sample solution stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

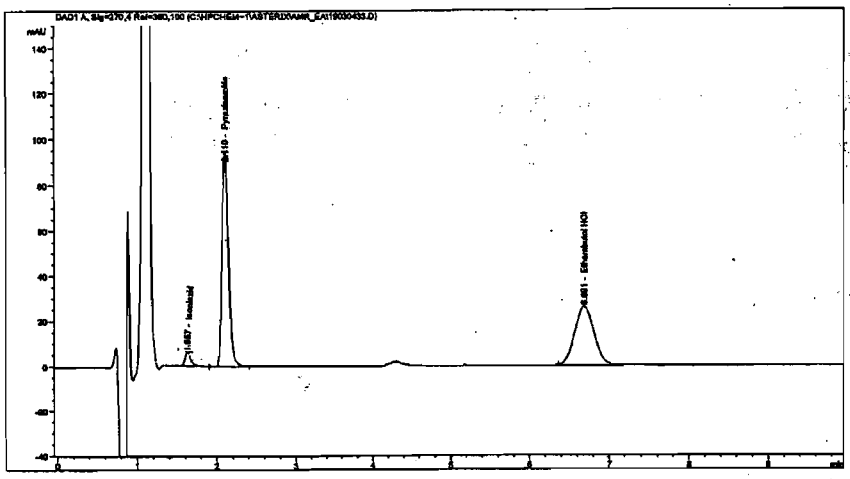


Figure D.6: Sample solution stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

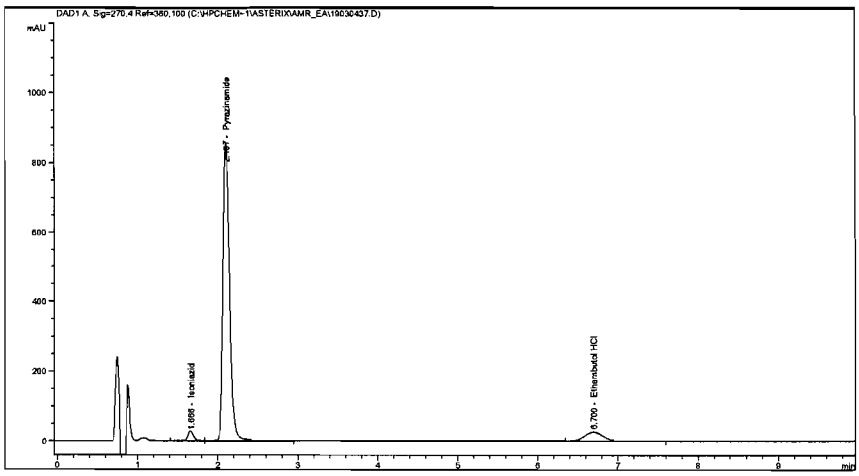


Figure D.7: Sample solution stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

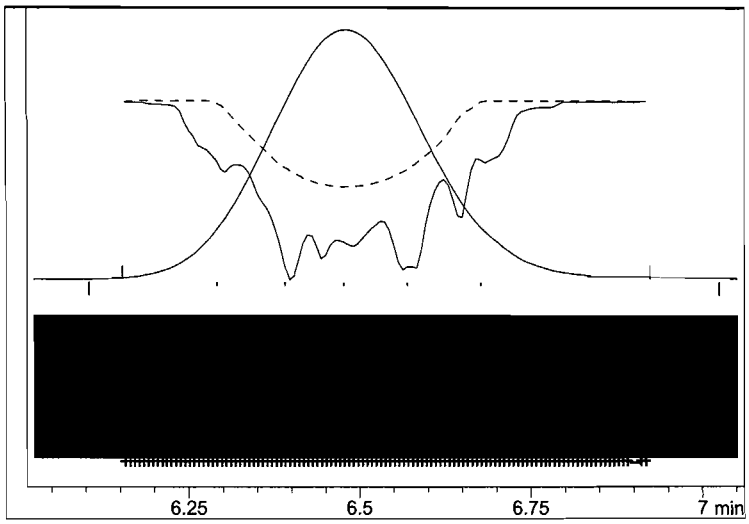


Figure D.8: Peak purity test results for ethambutol hydrochloride

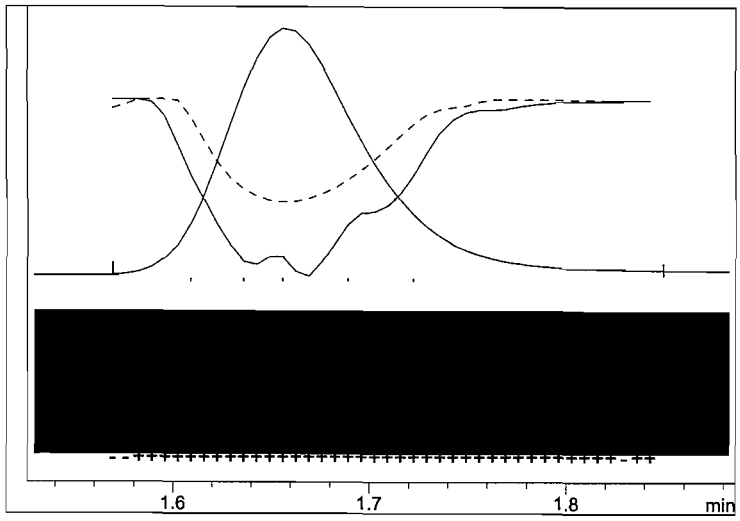


Figure D.9: Peak purity test results for isoniazid

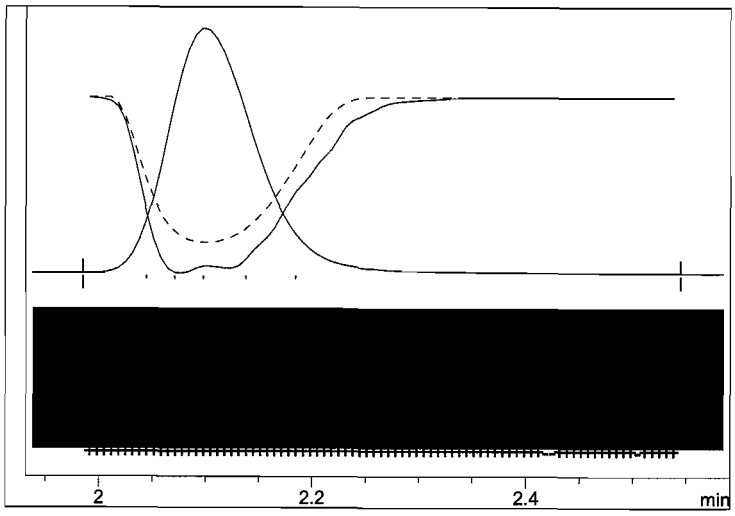


Figure D.10: Peak purity test results for pyrazinamide

Conclusion

(**Acceptance criterion:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

None of the ingredients in the placebo interfered with the analyte peaks. Extra peaks formed during forced degradation did not interfere with any of the peaks of interest. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

D.10. ETHAMBUTOL HYDROCHLORIDE

D.10.1 Specificity

Specificity results for ethambutol hydrochloride are given in Table D.2.

Table D.2: Results for ethambutol hydrochloride to determine Specificity

Stress condition (40 °C)	% Degradation of ethambutol hydrochloride
Water	17.1
0.1 M Hydrochloric acid	22.3
0.1 M Sodium hydroxide	28.8
10 % Hydrogen peroxide	59.8

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 20 to 30 % occurred in sample solutions mixed with water, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. Degradation of about 60 % occurred in a sample solution mixed with 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

D.10.2 Linearity and Range

Linearity and Range results for ethambutol hydrochloride are given in Table D.3.

Table D.3: Results for ethambutol hydrochloride to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
20	99.1	102.7	100.9
40	192.5	197.9	195.2
60	294.8	298.0	296.4
80	395.4	390.7	393.1
100	487.2	491.4	489.3
120	585.9	591.1	588.5
140	681.4	694.8	688.1
160	780.3	785.0	782.7
180	878.3	884.6	881.4
200	982.0	976.3	979.1
220	1084.3	1070.2	1077.2
240	1183.1	1177.7	1180.4
260	1286.7	1265.5	1276.1
280	1356.7	1376.1	1366.4
300	1483.0	1480.5	1481.7

Regression statistics are given in Table D.4.

Table D.4: Regression statistics of ethambutol hydrochloride results

R squared	0.9999	Lower 95%	Upper 95%
Intercept	-0.0153	-4.5490	4.5184
Slope	4.9070	4.8820	4.9319

Conclusion

(Acceptance criteria: The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 20 – 300 µg/ml (10 – 150 % of expected sample concentration) as shown in Figure D.11. The regression coefficient (R squared) is 0.9999. The method is suitable for single point calibration.

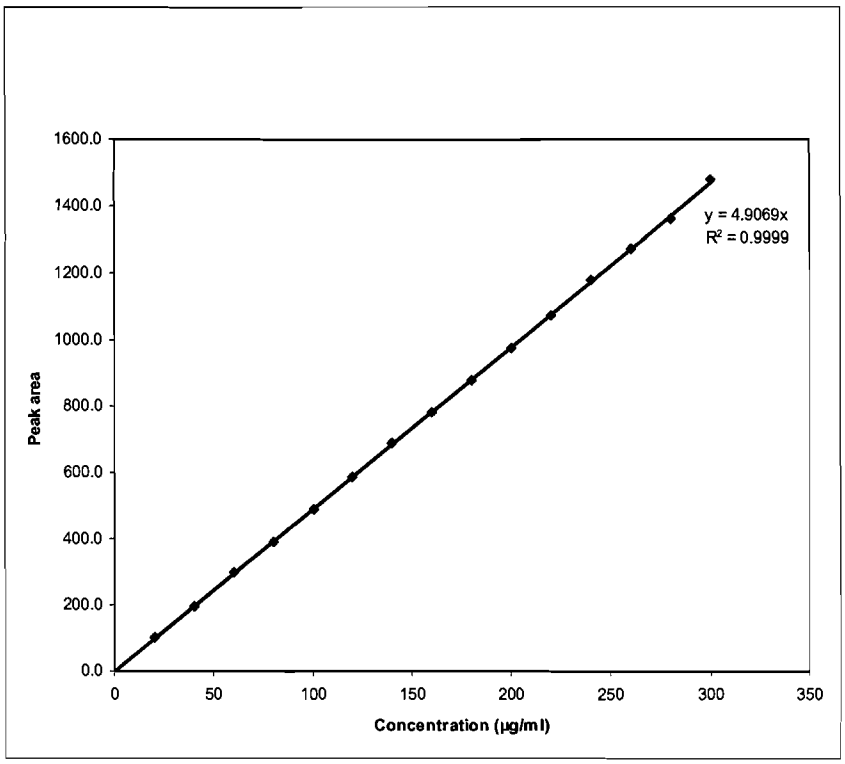


Figure D.11: Linear regression graph for ethambutol hydrochloride to determine Linearity and Range

D.10.3 Accuracy

Accuracy results for ethambutol hydrochloride and the statistical analysis thereof are given in Tables D.5 and D.6, respectively.

Table D.5: Results for ethambutol hydrochloride to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
160	760.2	747.4	753.8	158.9	99.33
160	763.5	775.6	769.6	162.2	101.41
160	755.6	750.9	753.3	158.8	99.26
200	971.6	943.5	957.5	199.7	99.87
200	942.3	957.6	949.9	198.2	99.08
200	971.5	966.3	968.9	202.1	101.05
240	1154.6	1160.5	1157.5	238.4	99.31
240	1162.2	1178.3	1170.2	241.0	100.40
240	1174.6	1163.1	1168.9	240.7	100.29

Table D.6: Statistical analysis of ethambutol hydrochloride results

Mean %	100.0
SD	0.8
% RSD	0.8
95% confidence intervals	
Lower limit	98.8
Upper limit	101.2
Estimated median	99.3
Confidence Level (95.0%)	1.2

Conclusion

(Acceptance criterion: Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

D.10.4 Precision

D.10.4.1 INTRA-DAY PRECISION

Intra-day precision results for ethambutol hydrochloride are given in Table D.7.

Table D.7: Results for ethambutol hydrochloride to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.06592	718.0	760.7	739.4	160	97.43
0.06595	748.1	761.0	754.5	160	99.43
0.06600	740.6	754.3	740.6	160	97.60
0.08233	944.3	935.8	944.3	200	98.49
0.08238	929.4	944.1	936.8	200	97.71
0.08237	932.8	931.5	932.1	200	97.22
0.09890	1126.4	1200.5	1163.4	240	99.82
0.09888	1127.8	1213.0	1170.4	240	100.42
0.09882	1148.3	1166.0	1157.1	240	99.28
				Mean	98.6
				SD	1.1
				RSD %	1.1

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.1 %.

D.10.4.2 INTER-DAY PRECISION

Inter-day precision results for ethambutol hydrochloride and ANOVA single factor statistics thereof are given in Tables D.8 and D.9, respectively.

Table D.8: Results for ethambutol hydrochloride to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	944.33	940.56	942.95	
	936.77	964.33	941.88	
	932.14	952.14	952.16	
Mean	937.75	952.34	945.66	945.25
SD	5.02	9.70	4.61	2.31
RSD %	0.54	1.02	0.49	0.24

Table D.9: ANOVA single factor statistics for the determination of ethambutol hydrochloride

Groups	Count	Sum	Average	Variance	
Day 1	3	2813.25	937.75	37.85	
Day 2	3	2857.04	952.34	141.28	
Day 3	3	2836.99	945.66	31.89	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	320.28	2	160.14	2.28	0.18
Within Groups	422.04	6	70.34		
Total	742.32	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.2 %.

D.10.5 Ruggedness

D.10.5.1 Stability of sample solutions

A sample solution was left on the auto sampler tray in amber vials and re-analysed over several time intervals to determine the sample stability. The results are presented in Table D.10.

Table D.10: Results for ethambutol hydrochloride to determine Ruggedness

Time (hours)	Peak Area	%
0	892.13	100.00
1	890.91	99.86
2	888.01	99.54
3	883.45	99.03
4	880.13	98.66
5	879.90	98.63
6	875.52	98.14
7	869.79	97.50
8	866.52	97.13
Mean	880.71	98.72
SD	8.46	0.95
RSD %	0.96	0.96

Conclusion

(Acceptance criterion: The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of less than 3.0 % was observed over an 8 hour period. Sample solutions are considered stable for 6 hours.

D.10.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table D.11.

Table D.11: Results for ethambutol hydrochloride to determine System Repeatability

	Area	Retention time (minutes)
	962.22	5.61
	963.12	5.61
	964.51	5.61
	963.80	5.61
	995.17	5.61
	973.87	5.58
Mean	970.45	5.60
SD	11.72	0.01
RSD %	1.21	0.22

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.2 % for peak area and 0.22 % for retention time respectively.

D.11. ISONIAZID

D.11.1 Specificity

Specificity results for isoniazid are given in Table D.12.

Table D.12: Results for isoniazid to determine Specificity

Stress condition (40 °C)	% Degradation of isoniazid
Water	15.7
0.1 M Hydrochloric acid	22.8
0.1 M Sodium hydroxide	37.0
10 % Hydrogen peroxide	42.6

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 15 to 23 % occurred in sample solutions mixed with water and 0.1 M hydrochloric acid. Degradation of about 37 % occurred in a sample solution mixed with 0.1 M sodium hydroxide. Degradation of about 43 % occurred in a sample solution mixed with 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

D.11.2 Linearity and range

Linearity and Range results for isoniazid are given in Table D.13.

Table D.13: Results for isoniazid to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
5.4	48.75	48.90	48.83
11.0	97.24	96.89	97.06
16.4	144.29	141.51	142.90
21.8	188.85	187.25	188.05
27.4	235.35	237.15	236.25
32.6	288.49	285.69	287.09
38.2	340.60	332.36	336.48
43.6	387.23	380.12	383.68
49.2	429.30	429.51	429.40
54.6	481.16	482.11	481.64
60.6	534.59	534.57	534.58
65.6	591.24	582.20	586.72
71.8	629.81	634.26	632.03
77.0	681.93	680.74	681.33
82.0	729.94	733.75	731.85

Regression statistics are given in Table D.14.

Table D.14: Regression statistics of isoniazid results

R squared	0.9998	Lower 95%	Upper 95%
Intercept	-3.5915	-7.6973	0.5144
Slope	17.8082	17.6434	17.9730

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 5.5 – 81.9 µg/ml (10 – 150 % of expected sample concentration) as shown in Figure D.12. The regression coefficient (R squared) is 0.9998. The method is suitable for single point calibration.

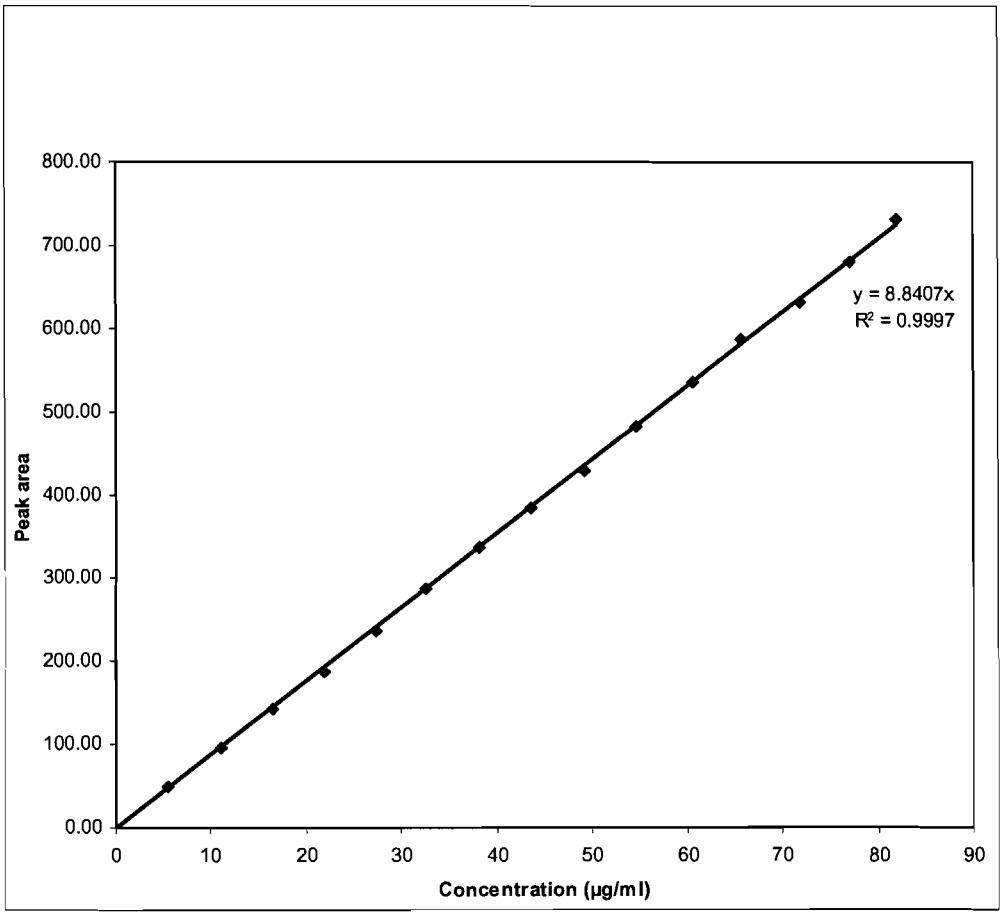


Figure D.12: Linear regression graph for isoniazid to determine Linearity and Range

D.11.3 Accuracy

Accuracy results for isoniazid and the statistical analysis thereof are given in Tables D.15 and D.16, respectively.

Table D.15: Results for isoniazid to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
44	388.2	375.7	382.0	42.9	98.22
44	390.0	397.1	393.6	44.2	101.20
44	389.7	392.6	391.2	43.9	100.58
55	481.6	485.0	483.3	54.5	99.79
55	486.9	482.6	484.7	54.6	100.08
55	486.4	483.6	485.0	54.7	100.14
66	600.6	605.7	603.2	65.2	99.54
66	607.4	604.3	605.9	65.5	99.99
66	607.42	610.0	608.7	65.8	100.46

Table D.16: Statistical analysis of isoniazid results

Mean %	100.0
SD	0.8
% RSD	0.8
95% confidence intervals	
Lower limit	99.4
Upper limit	100.7
Estimated median	100.1
Confidence Level (95.0%)	0.6

Conclusion

(Acceptance criterion: Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

D.11.4 Precision

D.11.4.1 Intra-day Precision

Intra-day precision results for isoniazid are given in Table D.17.

Table D.17: Results for isoniazid to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.06592	388.91	379.37	384.14	44	98.78
0.06595	383.35	388.46	385.90	44	99.23
0.06600	383.23	396.50	389.86	44	100.25
0.08233	485.67	481.33	483.50	55	99.83
0.08238	482.81	480.79	481.80	55	99.48
0.08237	487.26	492.10	489.68	55	101.10
0.09890	595.96	600.22	598.09	66	98.71
0.09888	600.19	597.02	598.61	66	98.80
0.09882	599.52	594.38	596.95	66	98.52
				Mean	99.4
				SD	0.8
				RSD %	0.8

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 0.8 %.

D.11.4.2 Inter-day Precision

Inter-day precision results for isoniazid and ANOVA single factor statistics thereof are given in Tables D.18 and D.19, respectively.

Table D.18: Results for isoniazid to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	483.50	476.54	472.50	
	481.80	493.41	474.48	
	489.68	490.17	474.97	
Mean	485.00	486.71	473.99	481.90
SD	3.39	7.31	1.07	5.64
RSD %	0.70	1.50	0.23	1.17

Table D.19: ANOVA single factor statistics for the determination of ioniazid

Groups	Count	Sum	Average	Variance	
Day 1	3	1454.99	485.00	17.20	
Day 2	3	1460.13	486.71	80.16	
Day 3	3	1421.96	473.99	1.708	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	286.09	2	143.04	4.33	0.07
Within Groups	198.15	6	33.02		
Total	484.23	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 1.2 %.

D.11.5 Ruggedness

D.11.5.1 Stability of sample solutions

A sample solution was left in amber vials on the auto sampler tray and re-analysed over several time intervals to determine the sample stability. The results are presented in Table D.20.

Table D.20: Results for isoniazid to determine Ruggedness

Time (hours)	Peak Area	%
0	429.2	100.00
1	427.2	99.55
2	425.9	99.25
3	425.8	99.22
4	424.7	98.97
5	423.6	98.71
6	421.9	98.32
7	420.5	97.99
8	418.6	97.54
Mean	424.2	98.84
SD	3.2	0.74
RSD %	0.7	0.74

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of about 2.5 % was observed over an 8 hour period. Sample solutions are considered stable for 6 hours.

D.11.5.2 Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table D.21.

Table D.21: Results for isoniazid to determine System Repeatability

	Area	Retention time (minutes)
	484.2	1.76
	485.9	1.76
	489.0	1.76
	491.1	1.76
	490.9	1.76
	490.6	1.76
Mean	488.6	1.76
SD	2.6	0.00
RSD %	0.5	0.14

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 0.5 % for peak area and 0.14 % for retention time respectively.

D.12. PYRAZINAMIDE

D.12.1 Specificity

Specificity results for pyrazinamide are given in Table D.22.

Table D.22: Results for pyrazinamide to determine Specificity

Stress condition (40 °C)	% Degradation of pyrazinamide
Water	6.4
0.1 M Hydrochloric acid	7.7
0.1 M Sodium hydroxide	7.6
10 % Hydrogen peroxide	8.5

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

Degradation of about 6 to 8 % occurred in sample solutions mixed with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

D.12.2 Linearity and range

Linearity and Range results for pyrazinamide are given in Table D.23.

Table D.23: Results for pyrazinamide to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
29	714.37	712.21	713.29
58	1597.94	1586.11	1592.02
87	2356.78	2343.51	2350.14
116	3065.42	3071.83	3068.62
146	3734.43	3758.39	3746.41
175	4526.01	4515.31	4520.66
204	5284.08	5239.90	5261.99
233	6020.47	6055.68	6038.08
262	6790.30	6810.28	6800.29
291	7401.58	7487.54	7444.56
320	8217.77	8251.54	8234.65
349	8969.02	8992.38	8980.70
378	9662.35	9635.02	9648.69
407	10469.47	10486.68	10478.07
438	11076.65	11053.16	11064.90

Regression statistics are given in Table D.24.

Table D.24: Regression statistics of pyrazinamide results

R squared	0.9997	Lower 95%	Upper 95%
Intercept	88.5082	23.1404	153.8763
Slope	25.3767	25.1296	25.6238

Conclusion

(Acceptance criteria: The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 29 – 437 µg/ml (10 – 150 % of expected sample concentration) as shown in Figure D.13. The regression coefficient (R squared) is 0.9997. The method is suitable for single point calibration.

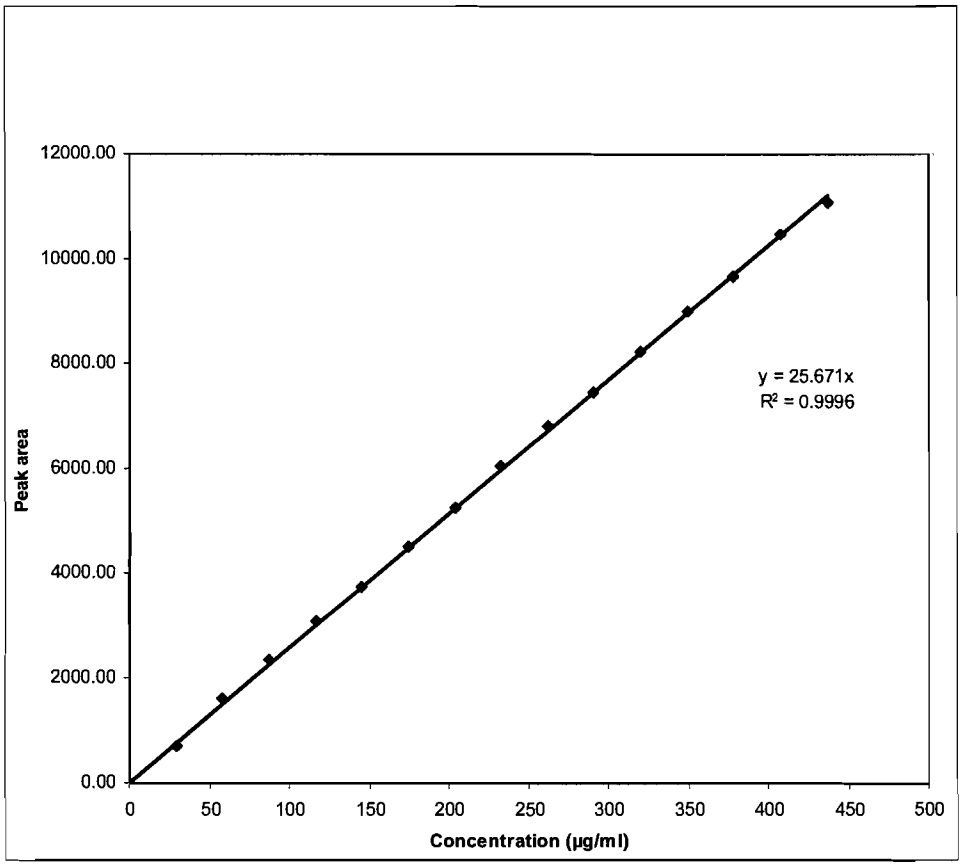


Figure D.13: Linear regression graph for pyrazinamide to determine Linearity and Range

D.12.3 Accuracy

Accuracy results for pyrazinamide and the statistical analysis thereof are given in Tables D.25 and D.26, respectively.

Table D.25: Results for pyrazinamide to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (mg/ml)	Recovery (%)
232	6906.1	6813.4	6859.8	231.2	99.67
232	6895.0	6893.9	6894.5	232.4	100.18
232	6923.2	6862.2	6892.7	232.4	100.15
290	7789.9	7821.3	7805.6	291.2	100.42
290	7821.0	7756.1	7788.6	290.6	100.20
290	7696.4	7752.6	7724.5	288.2	99.38
348	8569.8	8555.6	8562.7	348.8	100.22
348	8517.3	8533.9	8525.6	347.3	99.79
348	8553.7	8531.5	8542.6	348.0	99.99

Table D.26: Statistical analysis of pyrazinamide results

Mean %	100.0
SD	0.3
% RSD	0.3
95% confidence intervals	
Lower limit	99.9
Upper limit	100.4
Estimated median	100.2
Confidence Level (95.0%)	0.3

Conclusion

(Acceptance criterion: Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

D.12.4 Precision

D.12.4.1 Intra-day Precision

Intra-day precision results for pyrazinamide are given in Table D.27.

Table D.27: Results for pyrazinamide to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (mg/ml)	Recovery (%)
0.06592	6990.93	6928.43	6959.68	232	101.12
0.06595	6891.19	6867.70	6879.45	232	99.96
0.06600	6702.17	6718.01	6710.09	232	97.50
0.08233	7909.85	7928.69	7919.27	290	101.88
0.08238	7469.19	7451.75	7460.47	290	95.98
0.08237	7718.88	7753.98	7736.43	290	99.53
0.09890	8720.74	8679.11	8699.92	348	101.83
0.09888	8509.13	8358.96	8434.05	348	98.72
0.09882	8632.58	8620.53	8626.55	348	100.97
				Mean	99.7
				SD	1.9
				RSD %	1.9

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.9 %.

D.12.4.2 Inter-day precision

Inter-day precision results for pyrazinamide and ANOVA single factor statistics thereof are given in Tables D.28 and D.29, respectively.

Table D.28: Results for pyrazinamide to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	7919.27	7779.10	7593.60	
	7460.47	7657.18	7540.71	
	7736.43	7853.95	7573.41	
Mean	7705.39	7763.41	7569.24	7679.35
SD	188.59	81.09	21.79	81.38
RSD %	2.45	1.04	0.29	1.06

Table D.29: ANOVA single factor statistics for the determination of pyrazinamide

Groups	Count	Sum	Average	Variance	
Day 1	3	23116.17	7705.39	53346.52	
Day 2	3	23290.23	7763.41	9864.16	
Day 3	3	22707.72	7569.24	712.46	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	59604.60	2	29802.30	1.40	0.32
Within Groups	127846.28	6	21307.71		
Total	187450.88	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 1.1 %.

D.12.5 Ruggedness

D.12.5.1 Stability of sample solutions

A sample solution was left in amber vials on the auto sampler tray and re-analysed over several time intervals to determine the sample stability. The results are presented in Table D.30.

Table D.30: Results for pyrazinamide to determine Ruggedness

Time (hours)	Peak Area	%
0	7897.7	100.00
1	7812.4	98.92
2	7803.8	98.81
3	7788.3	98.62
4	7759.5	98.25
5	7755.0	98.19
6	7738.6	97.99
7	7728.2	97.85
8	7657.2	96.95
Mean	7755.4	98.40
SD	62.7	0.79
RSD %	0.8	0.81

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of about 3.1 % was observed over an 8 hour period. Sample solutions are considered stable for 4 hours.

D.12.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table D.31.

Table D.31: Results for pyrazinamide to determine System Repeatability

	Area	Retention time (minutes)
	7927.8	2.21
	7879.1	2.21
	7852.2	2.21
	7825.4	2.21
	7868.4	2.21
	7773.8	2.20
Mean	7854.4	2.21
SD	47.5	0.00
RSD %	0.6	0.14

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 0.6 % for peak area and 0.14 % for retention time respectively.

D.13. ROBUSTNESS

The following changes in the chromatographic operating parameters were found to be acceptable:

Column: Luna C18-2 column, 150 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance, CA; and
Lichrospher 100-5 RP-1ec cartridge column, 125 x 4.0 mm, 5 µm, 100 10µm pores, 21.5 % carbon load, end capped, Machery-Nagel, Düren, Germany.
The Lichrospher gave different retention times, thus the flow rate must be altered.

Mobile phase pH: pH 4.8 – 5.0.

Flow rate: 1.9 – 2.1 ml/minute.

Wavelength: The wavelength can be altered by ± 2 nm without any ill effect.

Injection volume: 16 – 24 µl.

Conclusion

(**Acceptance criterion:** The method should be able to tolerate about 5.0 % variance in chromatographic conditions).

The method was able to tolerate small changes in the chromatographic conditions.

D.14. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Isoniazid	1.8 minutes
Pyrazinamide	2.2 minutes
Ethambutol hydrochloride	5.6 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Isoniazid	2 166
Pyrazinamide	3 390
Ethambutol hydrochloride	1 220

USP Tailing factor (T):

Isoniazid	1.185
Pyrazinamide	1.200
Ethambutol hydrochloride	1.023

Capacity factor (k'):

Isoniazid	1.087
Pyrazinamide	1.610
Ethambutol hydrochloride	5.621

Resolution between peaks (R):

Isoniazid	-
Pyrazinamide	2.920
Ethambutol hydrochloride	8.555

D.15. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates and the resolution between the isoniazid and pyrazinamide peaks, and the pyrazinamide and ethambutol hydrochloride peaks.
* Use the 5-sigma method to calculate the parameters in D.14.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 2.0 %.
* The column must have more than 2 000, 3 000 and 1 000 theoretical plates for isoniazid, pyrazinamide and ethambutol hydrochloride respectively.
* The resolution between the peaks of isoniazid and pyrazinamide must be at least 2.0.
* The USP tailing must be less than 1.5.

D.16. CONCLUSION

The method performed well and should be suitable to analyse isoniazid, pyrazinamide and ethambutol hydrochloride in tablets for stability testing, quality control and batch release purposes. No interference was encountered from stressed samples, thus the method can be regarded as being stability indicating.

APPENDIX E

Validation of an HPLC Assay Method:

Determination of Rifampicin Content in Tablets and Capsules containing Rifampicin

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E.1. ORIGIN OF METHOD

The method was based in part on an article by Sankar *et al.* (2003:733) discussing the decomposition of rifampicin in the presence of isoniazid in the pH range 1 – 3.

E.2. SPECIFICATION

The sample must contain the following:

Rifampicin	150 mg (135 – 165 mg)
	or 300 mg (270 – 330 mg)

E.3. SUMMARY

In Table E.1 a summary of the validation results is given.

Table E.1: Summary of validation results

Parameter	Acceptance criteria	Results
Specificity	The placebo should not generate any peaks that will interfere with the determination of the active ingredient. Extra peaks formed under stress conditions should be discernible from those of the active ingredients.	Capsules: Complies Tablets: Complies
Linearity and Range	The method is linear over the range: 20 – 200 % of the expected sample concentration. R^2 is not less than 0.99.	Complies Rifampicin: Linear from 150 - 1500 µg/ml (20 – 200 % of the expected sample concentration) R^2: 0.9998
Accuracy (Recovery)	Recovery must be between 98.0 – 102.0 %	Complies Rifampicin: 100.0 %
Precision (Repeatability)	RSD of 2.0 % or less.	Complies Rifampicin (Capsules): Intra-day = 1.2 %, Inter-day = 0.4 % Rifampicin (Tablets): Intra-day = 0.8 %, Inter-day = 0.2 %
Ruggedness (Stability)	The sample solution should not be used for a period longer than it takes to degrade by 2.0 %.	Rifampicin: Stable for 4 hours
Ruggedness (System repeatability)	The peak area and retention times should have an RSD of 2.0 % or less.	Complies Rifampicin: 1.8 % for peak area, 1.5 % for retention time
Robustness	The method should be able to tolerate about 5.0 % variance in chromato-graphic conditions.	Complies

E.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: L1, USP 29, 2006, p 2649, (Luna C18 column, 250 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 1.361 g potassium dihydrogen orthophosphate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 7.00 ± 0.05 with 0.01 M NaOH. Mix 400 ml of this solution with 600 ml methanol.

Flow rate: 1.0 ml/min.

Injection volume: 10 µl.

Detection: 254 nm (Diode Array Detection).

Retention time: About 25 to 30 minutes for rifampicin.

Solvent: Methanol.

E.5. SAMPLE SOLUTION PREPARATION

1. Accurately weigh and powder 20 tablets and mix, or empty the contents of 20 capsules and mix. Accurately weigh amounts of powder equivalent to 150 mg rifampicin.
2. Transfer the powder into 100 ml volumetric flasks, and dissolve in about 50 ml of methanol.
3. Shake well and make up to volume with methanol.
4. Dilute 25 ml to 50 ml with methanol.
5. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtrate.
6. Analyse by means of HPLC.

E.6. STANDARD SOLUTION PREPARATION

1. Accurately weigh about 150 mg of rifampicin reference standard[1] into a 100 ml volumetric flask.
2. Dissolve in about 50 ml methanol.
3. Shake well and make up to volume with methanol.
4. Dilute 25 ml to 50 ml with methanol.
5. Analyse by means of HPLC (6 injections).

E.7. CALCULATIONS

Rifampicin

The capsule contains 150 or 300 mg rifampicin. The tablet contains 150 or 300 mg rifampicin. The prepared sample solutions will contain 0.75 mg/ml rifampicin.

The equations for the calculation of content:

$$\text{mg/capsule} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Mass of Capsule Content(g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

[1] Rifampicin primary standard: USP, B/N 0420400006

E.8. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

E.8.1 Specificity

Method

1. Prepare a solution from the placebo powder[2] similar to preparing the sample solution.
2. Prepare a capsule sample and tablet sample and dilute the sample solutions 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
3. Store the solutions prepared in point 2, overnight in closed test tubes at 40 °C/75 % RH to bring about degradation.
4. Analyse by means of HPLC.
5. Examine the chromatograms to determine whether any additional peaks were formed.

Acceptance criteria

The placebo should not generate any peaks that will interfere with the determination of the actives.

Extra peaks formed under stress conditions should be discernible from those of the active ingredients.

E.8.2 Linearity and Range

Method

1. Prepare a standard solution as described under Standard Solution Preparation.
2. Inject variable volumes into the chromatograph to obtain standards from 20 – 200 % of the expected sample concentration.

Acceptance criterion

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

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[2] See Appendix B.8.1.

E.8.3 Accuracy

Method

1. Weigh amounts of the placebo equivalent to the amount of sample powder that would contain 80 %, 100 % and 120 % of 150 mg rifampicin. Prepare these solutions in triplicate as described under Sample Solution Preparation.
2. Spike, respectively with known amounts of rifampicin at concentrations of approximately 80, 100 and 120 % of the expected sample concentration, make up to volume and filter.
3. Analyse in duplicate by means of HPLC.

Acceptance criterion

Recovery must be between 98.0 – 102.0 %.

E.8.4 Precision

E.8.4.1 Intra-day Precision

Method

1. Weigh amounts of sample powder that equivalent 80 %, 100 % and 120 % of 150 mg rifampicin. Prepare solutions thereof in triplicate as described under Sample Solution Preparation.
2. Analyse in duplicate by means of HPLC.

E.8.4.2 Inter-day Precision

Method

1. Analyse the same homogenous sample in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more occasions on different days to determine the between-day variability of the method.

Acceptance criteria

Repeatability must be better than 2.0 % (n = 9).

Inter-day precision must be better than 2.0 % (n = 9).

E.8.5 Ruggedness

E.8.5.1 Stability of sample solutions

Method

1. Prepare samples solutions as described under Sample Solution Preparation.
2. Analyse by means of HPLC.
3. Leave the sample solutions in the auto sampler tray and re-analyse over a period of 8 hours to determine the stability.

Acceptance criterion

Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %, and in the case of degradation, special precautions should be followed to compensate for the loss.

E.8.5.2 System Repeatability

Method

1. Inject the sample solutions six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area and retention times should have an RSD of 2.0 % or less.

E.8.6 Robustness

Method

1. Make deliberate changes to the chromatographic conditions to determine the method's tolerance towards changes.
2. Change the flow rate, wavelength, injection volume and use a similar column from a different manufacturer.

Acceptance criterion

The method should be able to tolerate about 5.0 % variance in the chromatographic conditions.

E.8.7 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between 3-formyl rifamycin and rifampicin peaks (should be at least 1.5 - see Appendix G.14) and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 2.0 %. The resolution and plate count has to be determined through the validation for the specific system.

E.9. VALIDATION RESULTS

E.9.1 Specificity

A chromatogram of the solution containing the placebo is shown in Figure E.1. Figures E.2 and E.3 show chromatograms obtained for sample solutions. Figure E.4 shows a chromatogram of a standard solution. Figures E.5 – E.12 shows chromatograms of sample solutions that have been stressed as described under E.8.1. The analyte peaks that remained in the aqueous solution after being stressed were analysed by means of diode array peak purity testing to determine whether the peaks are still pure. The purity test results are given in Figure E.13.

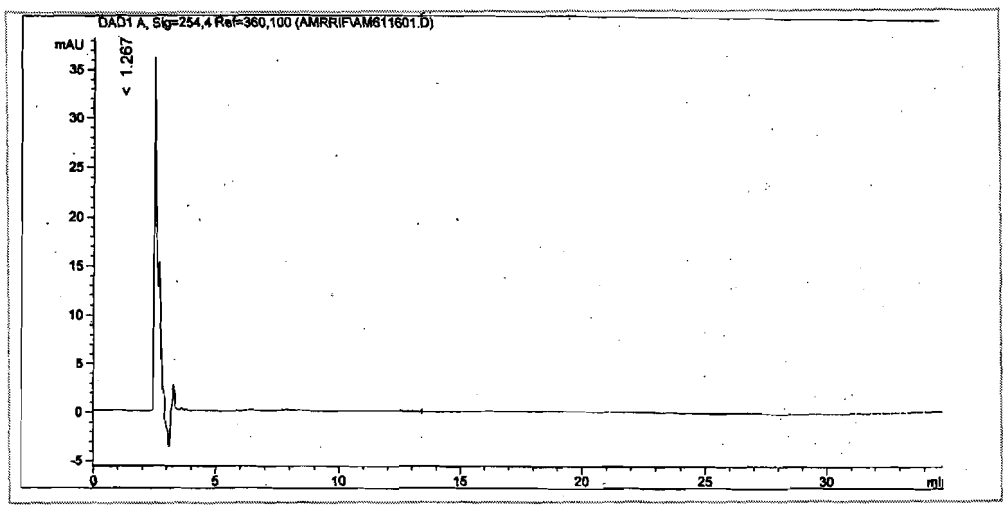


Figure E.1: Chromatogram of a solution containing the placebo

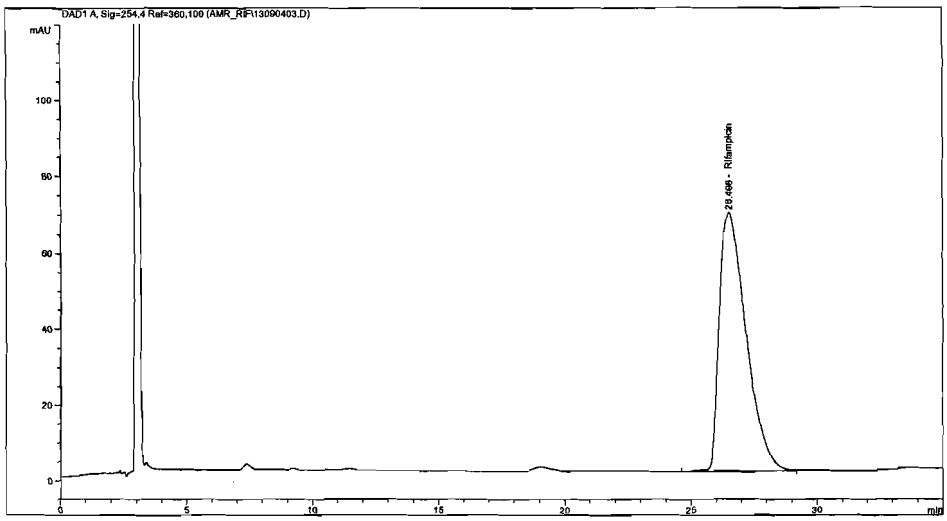


Figure E.2: Chromatogram of a sample solution (capsule)

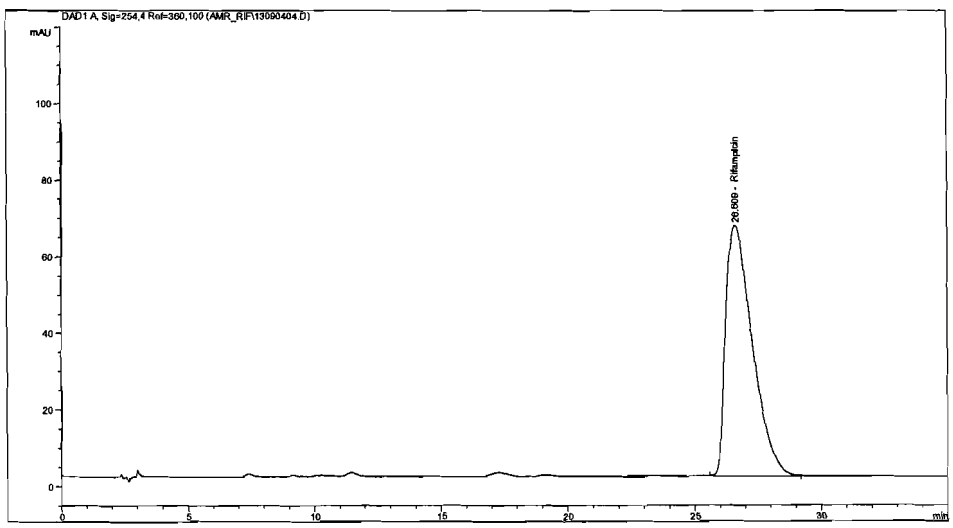


Figure E.3: Chromatogram of a sample solution (tablet)

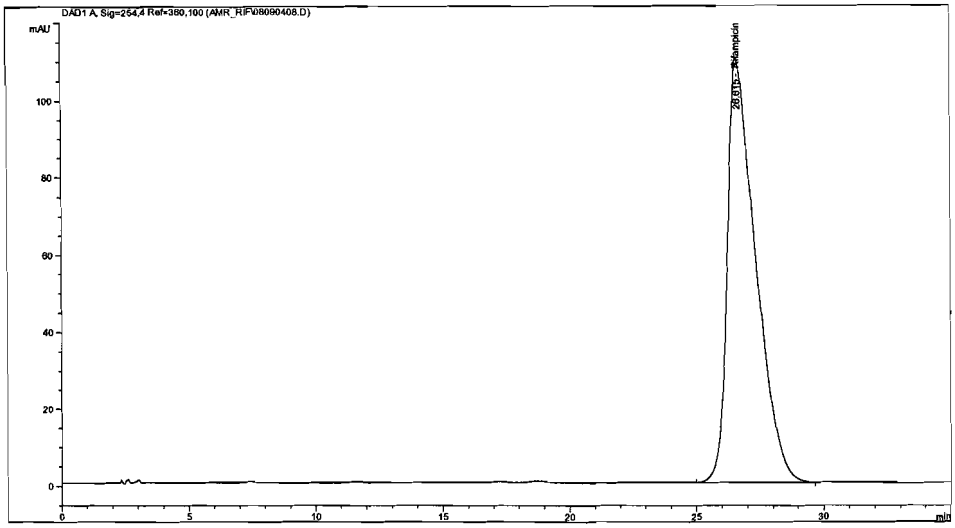


Figure E.4: Chromatogram of a standard solution

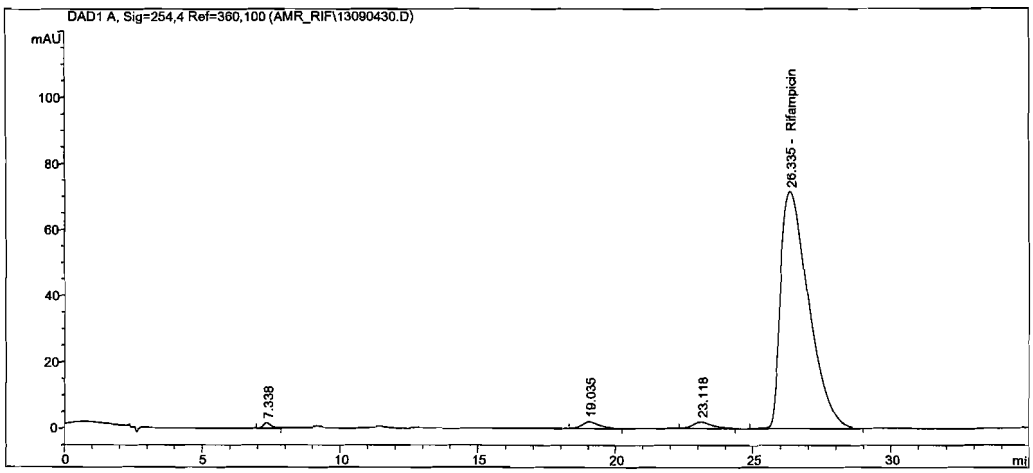


Figure E.5: Sample solution (capsule) stressed overnight in water at 40 °C/75 % RH

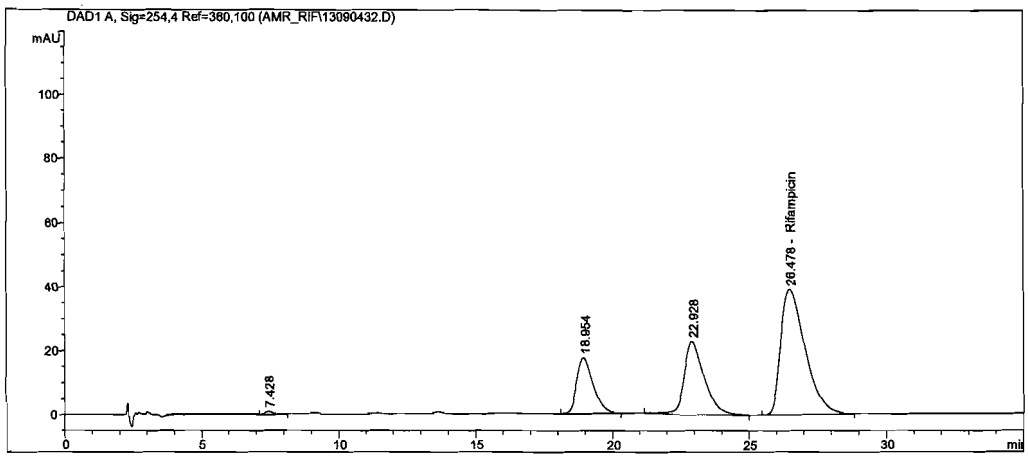


Figure E.6: Sample solution (capsule) stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

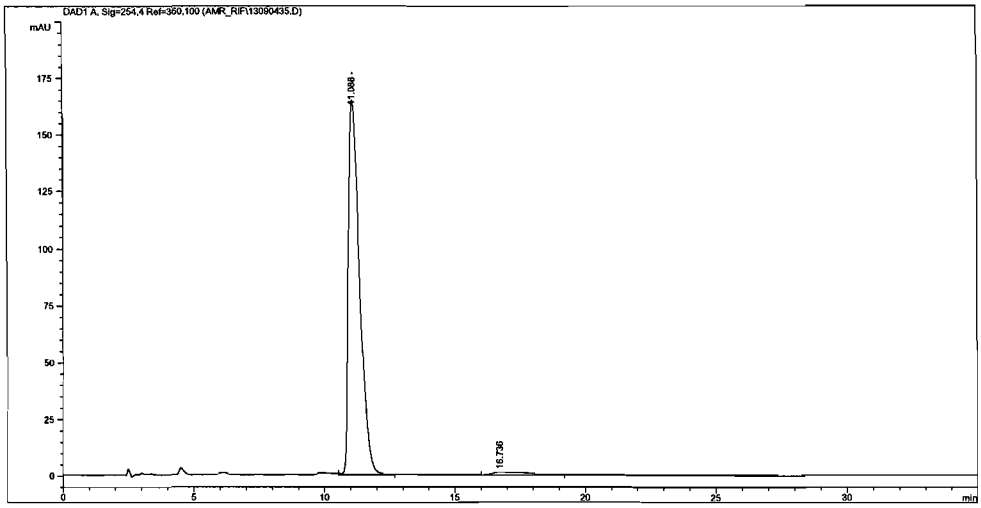


Figure E.7: Sample solution (capsule) stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

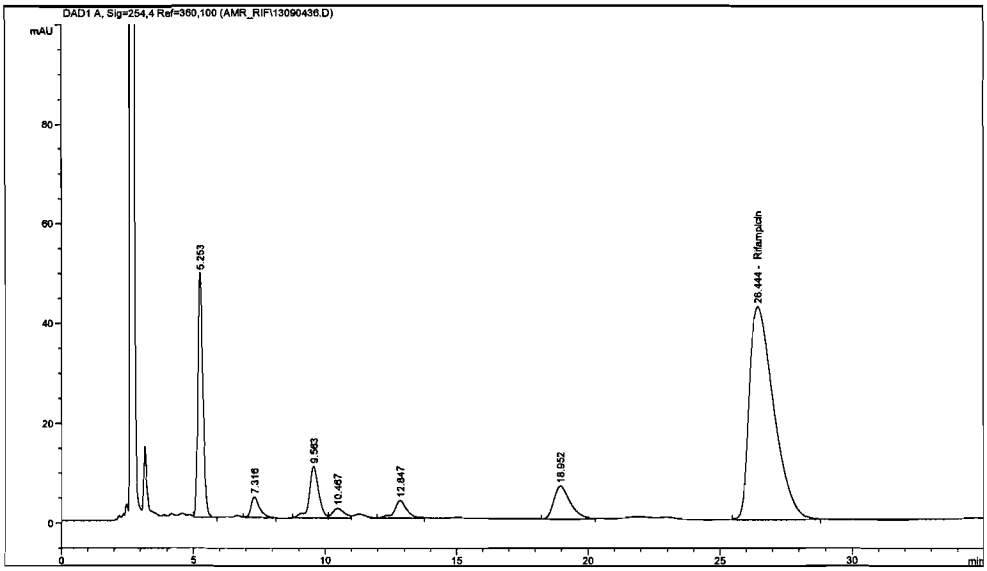


Figure E.8: Sample solution (capsule) stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

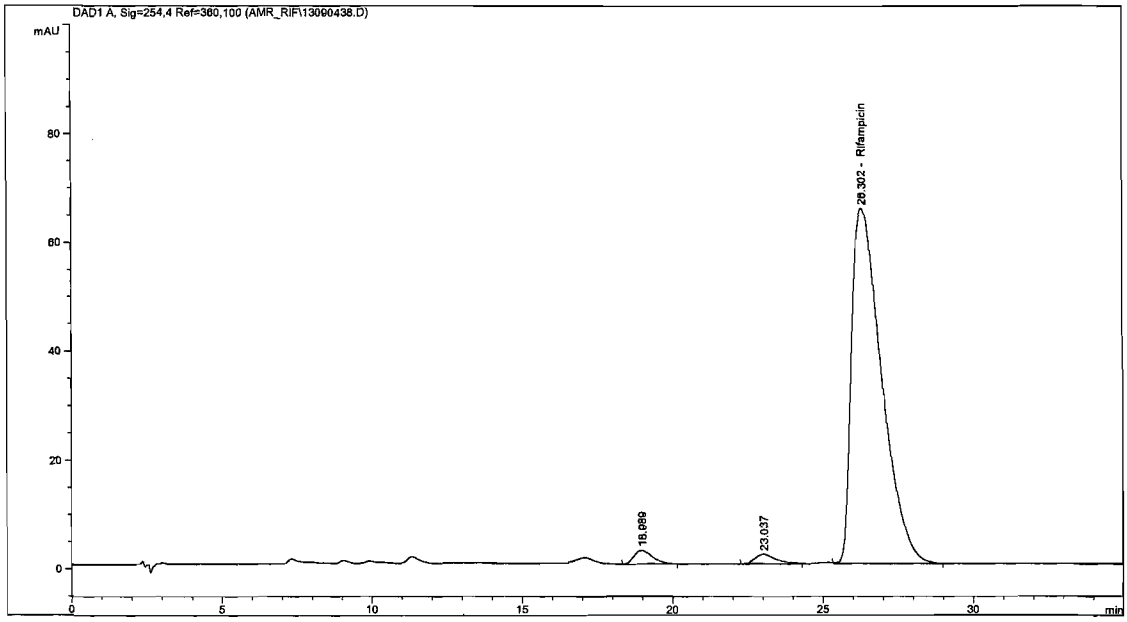


Figure E.9: Sample solution (tablet) stressed overnight in water at 40 °C/75 % RH

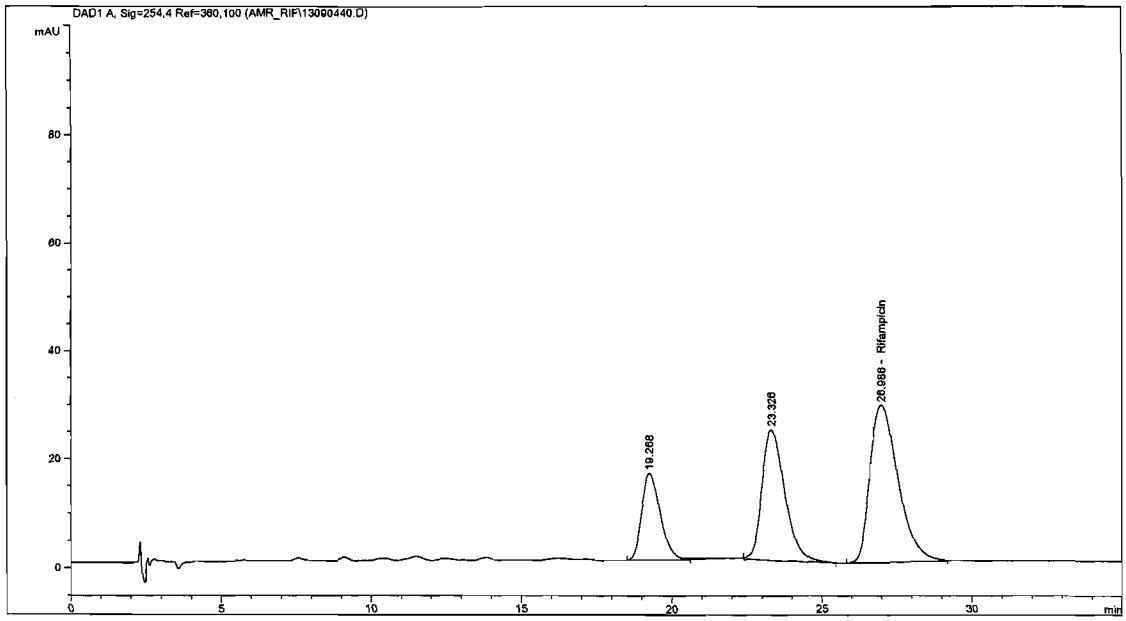


Figure E.10: Sample solution (tablet) stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

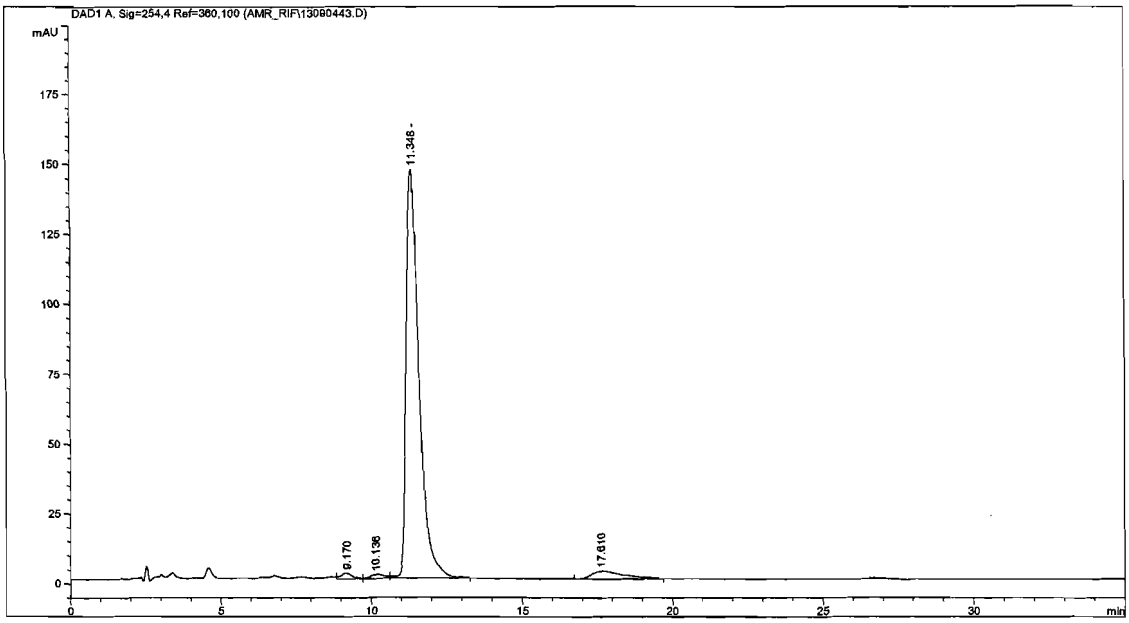


Figure E.11: Sample solution (tablet) stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

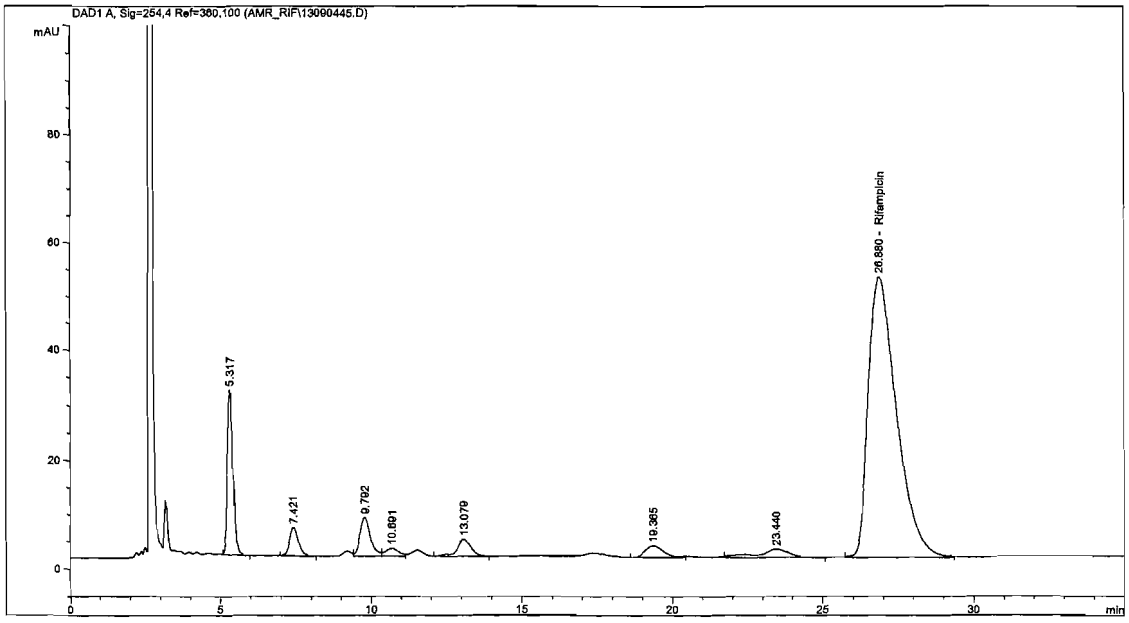


Figure E.12: Sample solution (tablet) stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

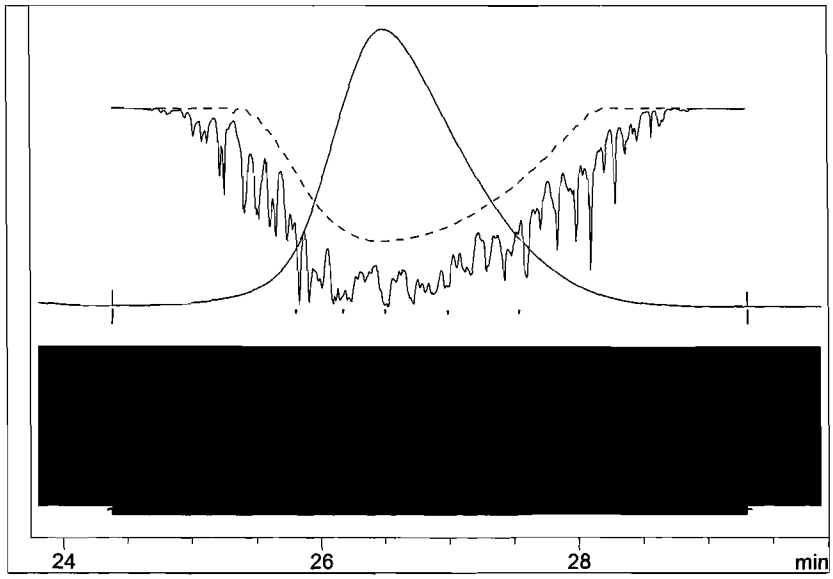


Figure E.13: Peak purity test results for rifampicin

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredient. Extra peaks formed under stress conditions should be discernible from those of the active ingredient).

None of the ingredients in the placebo interfered with the analyte peak. Extra peaks formed during forced degradation did not interfere with any of the peaks of interest. The same results were found with both the tablet and capsule samples. In all cases the area for rifampicin decreased while the areas for the related substances increased. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

Specificity results for rifampicin for capsules and tablets are given in Tables E.2 and E.3, respectively.

Table E.2: Results for rifampicin (in capsules) to determine Specificity

Stress condition (40 °C)	% Degradation of rifampicin
Water	0.3
0.1 M Hydrochloric acid	50.9
0.1 M Sodium hydroxide	> 90 %
10 % Hydrogen peroxide	41.4

Table E.3: Results for rifampicin (in tablets) to determine Specificity

Condition	% Degradation of rifampicin
Water	1.8
0.1 M Hydrochloric acid	59.0
0.1 M Sodium hydroxide	> 90 %
10 % Hydrogen peroxide	37.8

Degradation of about 0.3 to 1.8 % occurred in sample solutions mixed with water. Degradation of about 51 to 59 % occurred in sample solutions mixed with 0.1 M hydrochloric acid. Degradation of more than 90 % occurred in sample solutions mixed with 0.1 M sodium hydroxide. In sample solutions mixed with 10 % hydrogen peroxide, degradation of 37.8 to 41.4 % occurred. Additional peaks that formed during stress conditions were discernible from those of the active ingredient.

E.9.2 Linearity and Range

Linearity and Range results for rifampicin are given in Table E.4.

Table E.4: Results for rifampicin to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
150	1278.2	1274.9	1276.5
300	2759.5	2756.9	2758.2
450	4235.6	4130.8	4183.2
600	5501.0	5412.7	5456.9
750	6893.9	6885.1	6889.5
900	8265.6	8266.8	8266.2
1050	9440.9	9551.3	9496.1
1200	11000.7	11019.3	11010.0
1350	12389.5	12400.9	12395.2
1500	13971.0	13598.4	13784.7

Regression statistics are given in Table E.5.

Table E.5: Regression statistics of rifampicin results

R squared	0.9998	Lower 95%	Upper 95%
Intercept	-37.0797	-134.7448	60.5854
Slope	9.1984	9.0935	9.3034

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 150 – 1500 µg/ml (20 – 200 % of expected sample concentration) as shown in Figure E.14. The regression coefficient (R squared) is 0.9998. The method is suitable for single point calibration.

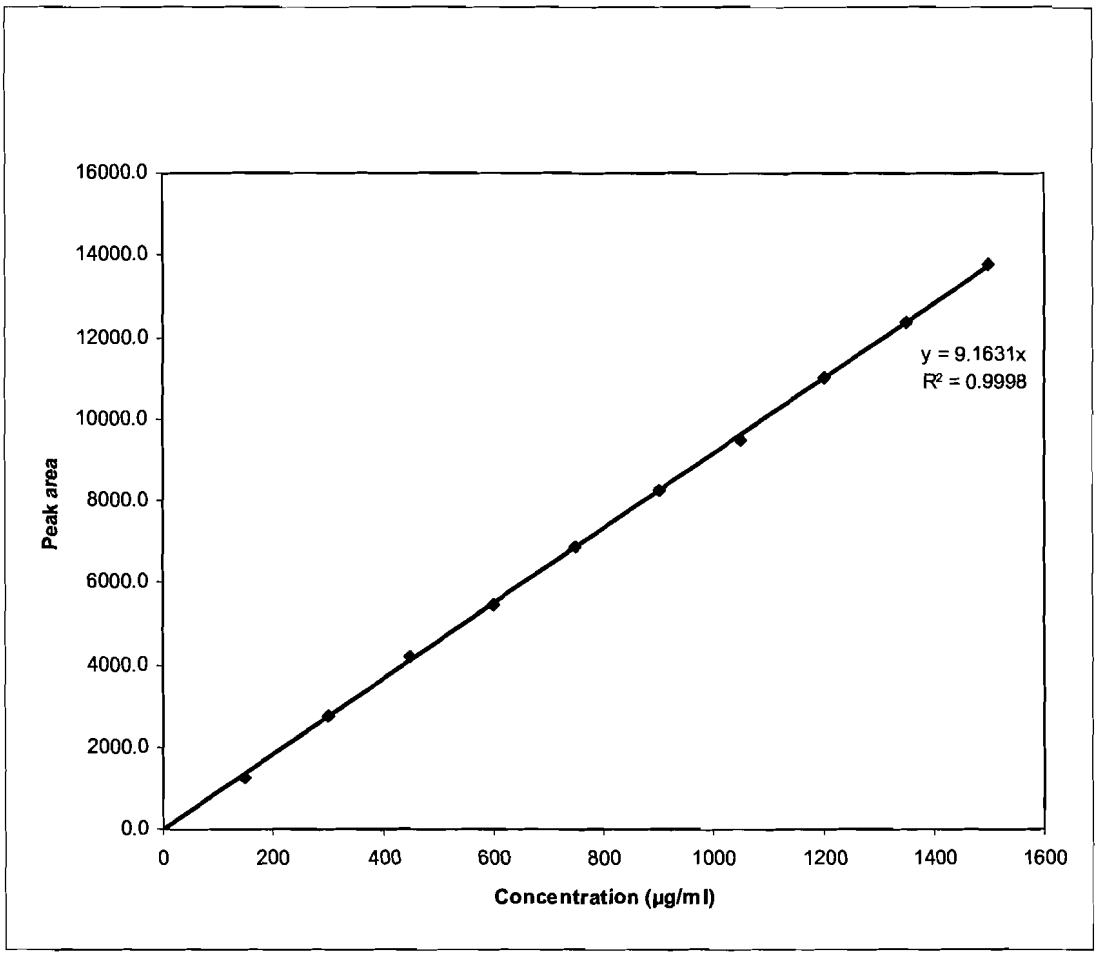


Figure E.14: Linear regression graph for rifampicin to determine Linearity and Range

E.9.3 Accuracy

Accuracy results for rifampicin and the statistical analysis thereof are given in Tables E.6 and E.7, respectively.

Table E.6: Results for rifampicin to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
600	5394.6	5509.7	5452.2	596.6	99.43
600	5522.4	5561.9	5542.2	606.4	101.07
600	5459.8	5453.2	5456.5	597.0	99.51
750	6984.8	6989.0	6986.9	745.1	99.35
750	7116.4	7132.3	7124.4	759.8	101.30
750	6995.7	6979.2	6987.5	745.2	99.35
900	8436.1	8463.6	8449.9	895.1	99.46
900	8398.2	8397.5	8397.9	889.6	98.84
900	8675.2	8605.4	8640.3	915.3	101.70

Table E.7: Statistical analysis of rifampicin results

Mean %	100.0
SD	1.0
% RSD	1.0
95% confidence intervals	
Lower limit	99.2
Upper limit	100.8
Estimated median	99.5
Confidence Level (95.0%)	0.8

Conclusion

(**Acceptance criterion:** Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 100.0 %.

E.9.4 Precision

E.9.4.1 INTRA-DAY PRECISION

Intra-day precision results for rifampicin in capsules and tablets are given in Tables E.8 and E.9, respectively.

Table E.8: Results for rifampicin (in capsules) to determine Intra-day Precision

Capsule contents powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.1055	5552.9	5463.3	5508.1	600	100.45
0.1054	5509.3	5541.6	5525.5	600	100.76
0.1056	5525.1	5614.7	5569.9	600	101.57
0.1320	7196.9	7088.9	7142.9	750	101.56
0.1319	7148.0	7145.1	7146.5	750	101.62
0.1319	7136.6	7099.9	7118.2	750	101.21
0.1584	8425.3	8447.1	8436.2	900	99.30
0.1581	8355.9	8346.5	8351.2	900	98.30
0.1583	8342.8	8450.5	8396.6	900	98.83
				Mean	100.4
				SD	1.2
				RSD %	1.2

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.2 %.

Table E.9: Results for rifampicin (in tablets) to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.0750	6092.3	6056.7	6074.5	600	99.92
0.0751	6022.4	6022.9	6022.7	600	99.07
0.0749	6057.7	6035.8	6046.8	600	99.46
0.0939	7518.8	7527.5	7523.1	750	100.76
0.0945	7519.4	7558.2	7538.8	750	100.97
0.0942	7535.0	7512.1	7523.5	750	100.76
0.1127	9332.1	9412.6	9372.3	900	99.07
0.1125	9331.1	9432.1	9381.6	900	99.17
0.1128	9348.8	9401.6	9375.2	900	99.10
				Mean	99.8
				SD	0.8
				RSD %	0.8

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 0.8 %.

E.9.4.2 INTER-DAY PRECISION

Inter-day precision results for rifampicin in capsules and tablets are given in Tables E.10 and E.12, respectively. ANOVA single factor statistics thereof are given in Tables E.11 and E.13.

Table E.10: Results for rifampicin (in capsules) to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	7142.89	7095.91	7168.93	
	7146.51	7125.50	7078.12	
	7118.25	7139.53	7072.75	
Mean	7135.88	7120.31	7106.60	7120.93
SD	12.56	18.18	44.13	24.96
RSD %	0.18	0.26	0.62	0.35

Table E.11: ANOVA single factor statistics for the determination of rifampicin (in capsules)

Groups	Count	Sum	Average	Variance	
Day 1	3	21407.64	7135.88	236.49	
Day 2	3	21360.94	7120.31	495.97	
Day 3	3	21319.81	7106.60	2921.18	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	1287.58	2.00	643.79	0.53	1287.58
Within Groups	7307.28	6.00	1217.88		7307.28
Total	8594.85	8.00			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(**Acceptance criterion:** RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.4 %.

Table E.12: Results for rifampicin (in tablets) to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	7523.15	7517.94	7469.64	
	7538.79	7478.67	7460.45	
	7523.55	7473.82	7456.45	
Mean	7528.49	7490.14	7462.18	7493.61
SD	7.28	19.75	5.52	10.85
RSD %	0.10	0.26	0.07	0.14

Table E.13: ANOVA single factor statistics for the determination of rifampicin (in tablets)

Groups	Count	Sum	Average	Variance	
Day 1	3	22585.48	7528.49	79.52	
Day 2	3	22470.43	7490.14	585.37	
Day 3	3	22386.55	7462.18	45.75	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	6649.76	2.00	3324.88	14.04	0.01
Within Groups	1421.27	6.00	236.88		
Total	8071.03	8.00			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.1 %.

E.9.5 Ruggedness

E.9.5.1 Stability of sample solutions

A sample solution was left on the auto sampler tray in amber vials and re-analysed over several time intervals to determine the sample stability. The results are presented in Table E.14.

Table E.14: Results for rifampicin to determine Ruggedness

Time (hours)	Peak Area	%
0	7445.71	100.00
1	7409.15	99.51
2	7392.65	99.29
3	7309.07	98.16
4	7297.11	98.00
5	7253.16	97.41
6	7157.33	96.13
7	7115.13	95.56
8	7103.45	95.40
Mean	7275.86	97.72
SD	121.08	1.63
RSD %	1.66	1.66

Conclusion

(**Acceptance criterion:** The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of nearly 5.0 % was observed over an 8 hour period. Sample solutions are considered stable for 4 hours.

E.9.5.2 System Repeatability

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table E.15.

Table E.15: Results for rifampicin to determine System Repeatability

	Area	Retention time (minutes)
	7811.0	30.06
	7797.6	30.03
	8107.5	30.02
	7730.6	29.90
	7878.0	30.12
	7679.7	31.25
Mean	7834.1	30.23
SD	137.3	0.46
RSD %	1.8	1.53

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.8 % for peak area and 1.5 % for retention time respectively.

E.10. ROBUSTNESS

The following changes in the chromatographic operating parameters were found to be acceptable:

Column: Luna C_{18} column, 250 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance, CA; and
µBondapak C_{18} column, 250 x 4.6 mm, 10 µm pores, Waters, Ireland.
The µBondapak gave different retention times, thus the flow rate must be altered.

Flow rate: 0.9 – 1.1 ml/minute.

Wavelength: The wavelength can be altered by ± 2 nm without any ill effect.

Injection volume: 8 – 12 µl.

Conclusion

(**Acceptance criterion:** The method should be able to tolerate about 5.0 % variance in chromatographic conditions).

The method was able to tolerate small changes in the chromatographic conditions.

E.11. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Rifampicin	25 to 30 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Rifampicin	2 225

USP Tailing factor (T):

Rifampicin	1.259

Capacity factor (k'):

Rifampicin	15.474

Resolution between peaks (R):

Rifampicin	-

E.12. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates.
* Use the 5-sigma method to calculate the parameters in E.11.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 2.0 %.
* The column must have more than 1 800 theoretical plates for rifampicin.
* The resolution between the peaks of 3-formyl rifamycin and rifampicin must be at least 1.5.
* The USP tailing must be less than 1.5.

E.13. CONCLUSION

The method performed well and should be suitable to analyse rifampicin in products for stability testing, quality control and batch release purposes. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

APPENDIX F

Validation of an HPLC Assay Method:

Determination of Rifampicin Content in a 4 Fixed Dose Combination product containing Ethambutol Hydrochloride, Isoniazid, Pyrazinamide and Rifampicin

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F.1. ORIGIN OF METHOD

The method was based in part on an article by Sankar *et al.* (2003:733) discussing the decomposition of rifampicin in the presence of isoniazid in the pH range 1 – 3.

F.2. SPECIFICATION

The sample must contain the following:

Rifampicin	150 mg (135 – 165 mg)

F.3. SUMMARY

In Table F.1 a summary of the validation results is given.

Table F.1: Summary of validation results

Parameter	Acceptance criteria	Results
Specificity	The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients.	Complies
Linearity and Range	The method is linear over the range: 20 – 200 % of the expected sample concentration. R^2 is not less than 0.99.	Complies Rifampicin: Linear from 150 - 1500 µg/ml (20 – 200 % of the expected sample concentration) R^2: 0.9998
Accuracy (Recovery)	Recovery must be between 98.0 – 102.0 %	Complies Rifampicin: 99.7 %
Precision (Repeatability)	RSD of 2.0 % or less.	Complies Rifampicin: Intra-day = 1.5 %, Inter-day = 0.3 %
Ruggedness (Stability)	The sample solution should not be used for a period longer than it takes to degrade by 2.0 %.	Rifampicin: Stable for 1 hour
Ruggedness (System repeatability)	The peak area and retention times should have an RSD of 2.0 % or less.	Complies Rifampicin: 1.4 % for peak area, 1.3 % for retention time
Robustness	The method should be able to tolerate about 5.0 % variance in chromato-graphic conditions.	Complies

F.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: L1, USP 29, 2006, p 2649, (Luna C18 column, 250 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 1.361 g potassium dihydrogen orthophosphate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 7.00 ± 0.05 with 0.01 M NaOH. Mix 400 ml of this solution with 600 ml methanol.

Flow rate: 1.0 ml/min.

Injection volume: 10 µl.

Detection: 254 nm (Diode Array Detection).

Retention time: About 25 to 30 minutes for rifampicin.

Solvent: Methanol.

F.5. SAMPLE SOLUTION PREPARATION

1. Accurately weigh and powder 20 tablets and mix. Accurately weigh amounts of powder equivalent to 150 mg rifampicin.
2. Transfer the powder into 100 ml volumetric flasks, and dissolve in about 50 ml of methanol.
3. Shake well and make up to volume with methanol.
4. Dilute 25 ml to 50 ml with methanol.
5. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtrate.
6. Analyse by means of HPLC.

F.6. STANDARD SOLUTION PREPARATION

1. Accurately weigh about 150 mg of rifampicin reference standard[1] into a 100 ml volumetric flask.
2. Dissolve in about 50 ml methanol.
3. Shake well and make up to volume with methanol.
4. Dilute 25 ml to 50 ml with methanol.
5. Analyse by means of HPLC (6 injections).

F.7. CALCULATIONS

Rifampicin:

The tablet contains 150 mg rifampicin. The prepared sample solutions will contain 0.75 mg/ml rifampicin.

The equation for the calculation of content:

$$\text{mg/tablet} = \frac{\text{Sample peak area x standard mass (mg) x std. potency (\%) x Average Tablet Mass (g)}}{\text{Standard peak area x sample mass (g) x 100}}$$

F.8. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

F.8.1 Specificity

Method

1. Prepare a solution from the placebo powder[2] similar to preparing the sample solution.
2. Prepare a tablet sample and dilute the sample solutions1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10 % hydrogen peroxide respectively.
3. Store the solutions prepared in point 2, overnight in closed test tubes at 40 °C/75 % RH to bring about degradation.

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[1] Rifampicin primary standard: USP, B/N 0420400006

4. Analyse by means of HPLC.
5. Examine the chromatograms to determine whether any additional peaks were formed.

Acceptance criteria:

The placebo should not generate any peaks that will interfere with the determination of the actives.

Extra peaks formed under stress conditions should be discernible from those of the active ingredients.

F.8.2 Linearity and Range

Method

1. Prepare a standard solution as described under Standard Solution Preparation.
2. Inject variable volumes into the chromatograph to obtain standards from 20 – 200 % of the expected sample concentration.

Acceptance criterion

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

F.8.3 Accuracy

Method

1. Weigh amounts of the placebo equivalent to the amount of sample powder that contains 80 %, 100 % and 120 % of 150 mg rifampicin. Add 50 ml of solvent in triplicate as described under Sample Solution Preparation.
2. Spike, respectively with a known amount of rifampicin at concentrations of approximately 80, 100 and 120 % of the expected sample concentration, make up to volume and filter.
3. Analyse in duplicate by means of HPLC.

Acceptance criterion

Recovery must be between 98.0 – 102.0 %.

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[2] See Appendix B.8.1.

F.8.4 Precision

F.8.4.1 Intra-day Precision

Method

1. Weigh amounts of sample powder that equivalent 80 %, 100 % and 120 % of 150 mg rifampicin. Prepare solutions thereof in triplicate as described under Sample Solution Preparation.
2. Analyse in duplicate by means of HPLC.

F.8.4.2 Inter-day Precision

Method

1. Analyse the same homogenous sample in triplicate as described above for intra-day precision (at 100 % of the sample concentration) on two more occasions on different days to determine the between-day variability of the method.

Acceptance criteria

Repeatability must be better than 2.0 % (n = 9).

Inter-day precision must be better than 2.0 % (n = 9).

F.8.5 Ruggedness

F.8.5.1 Stability of sample solution

Method

1. Prepare sample solution as described under Sample Solution Preparation.
2. Analyse by means of HPLC.
3. Leave the sample solution in the auto sampler tray and re-analyse over a period of 8 hours to determine the stability.

Acceptance criterion

Sample solutions should not be used for a period longer than it takes to degrade by 2.0 %, and in the case of degradation, special precautions should be followed to compensate for the loss.

F.8.5.2 SYSTEM REPEATABILITY

Method

1. Inject the sample solutions six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area and retention times should have an RSD of 2.0 % or less.

F.8.6 Robustness

Method

1. Make deliberate changes to the chromatographic conditions to determine the method's tolerance towards changes.
2. Change the flow rate, wavelength, injection volume and use a similar column from a different manufacturer.

Acceptance criterion

The method should be able to tolerate about 5.0 % variance in the chromatographic conditions.

F.8.7 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between 3-formyl rifamycin and rifampicin peaks (should be at least 1.5 - see Appendix G.14) and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 2.0 %. The resolution and plate count have to be determined through the validation for the specific system.

F.9. VALIDATION RESULTS

F.9.1 Specificity

A chromatogram of the solution containing the placebo is shown in Figure F.1. Figure F.2 shows a chromatogram obtained for a sample solution. Figure F.3 shows a chromatogram of a standard solution. Figures F.4 – F.7 show chromatograms of sample solutions that have been stressed as described under F.8.1. The analyte peaks that remained in the aqueous solution after being stressed were analysed by means of diode array peak purity testing to determine whether the peaks are still pure. The purity test results are given in Figure F.8.

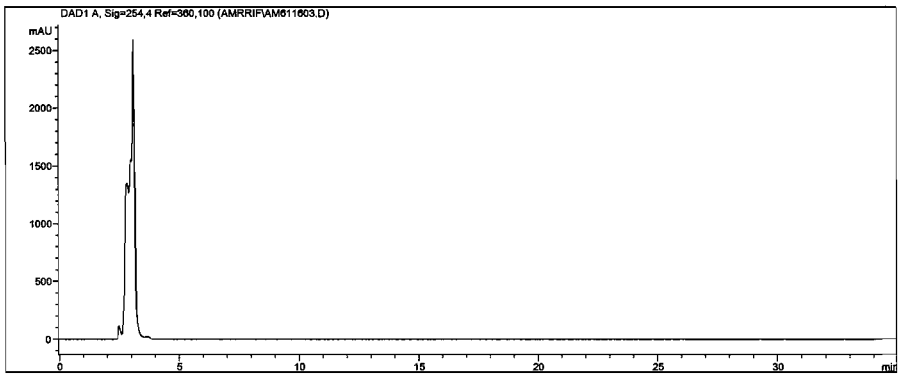


Figure F.1: Chromatogram of a solution containing the placebo

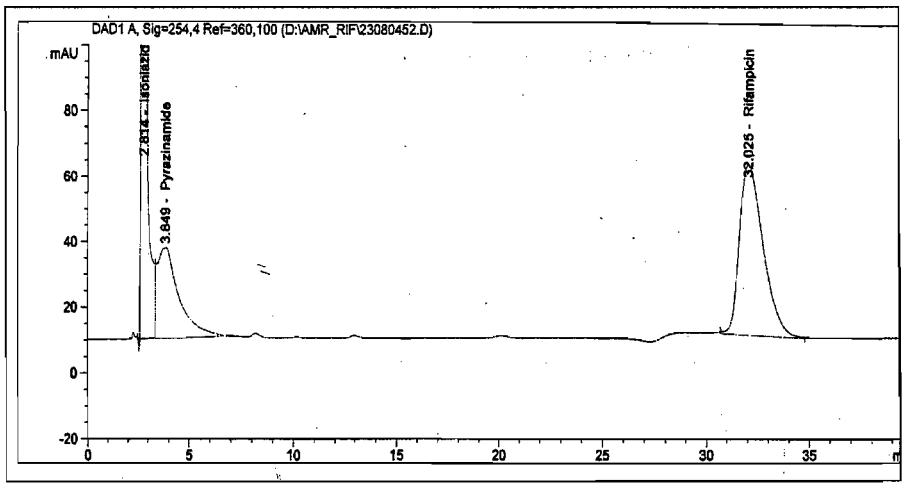


Figure F.2: Chromatogram of a sample solution

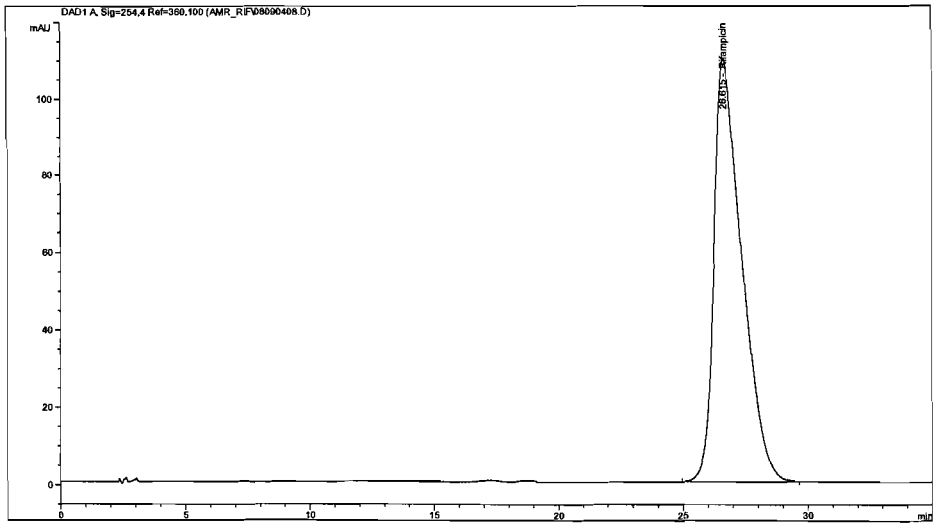


Figure F.3: Chromatogram of a standard solution

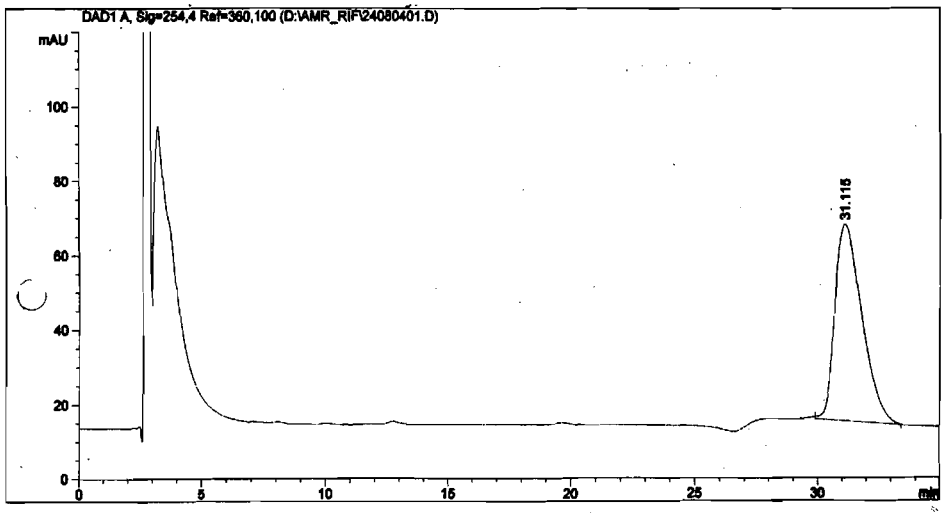


Figure F.4: Sample solution stressed overnight in water at 40 °C/75 % RH

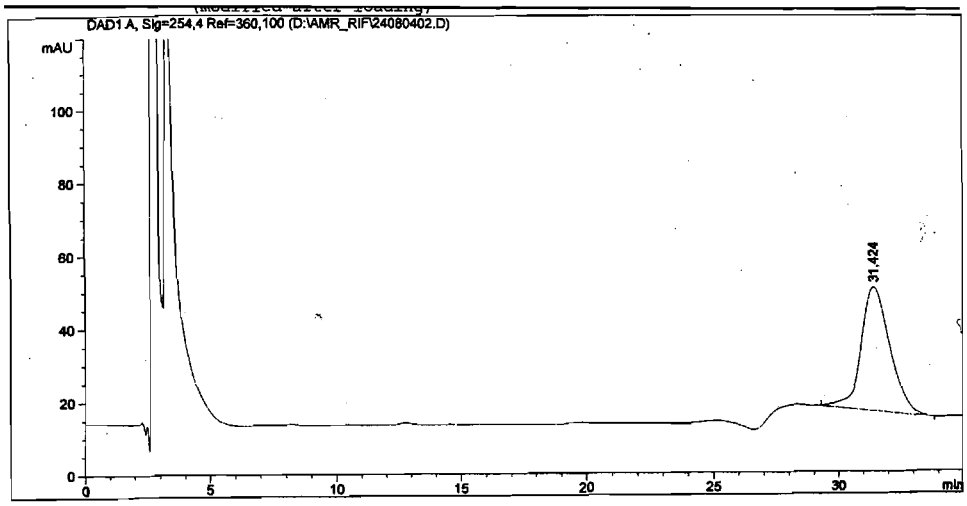


Figure F.5: Sample solution stressed overnight in 0.1 M hydrochloric acid at 40 °C/75 % RH

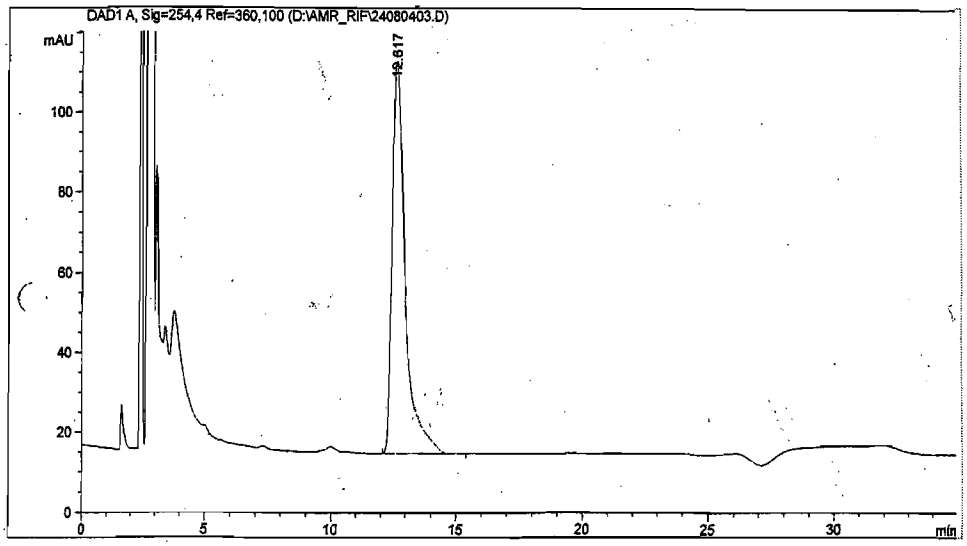


Figure F.6: Sample solution stressed overnight in 0.1 M sodium hydroxide at 40 °C/75 % RH

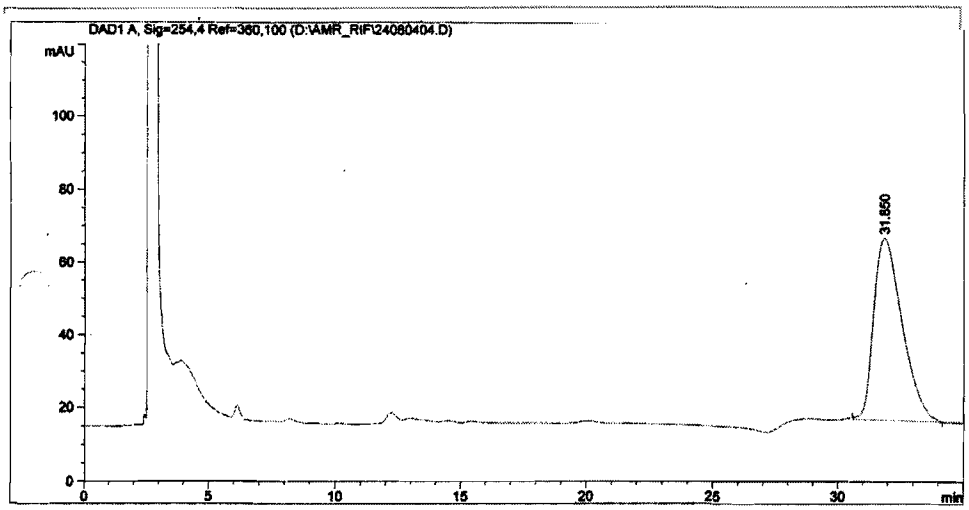


Figure F.7: Sample solution stressed overnight in 10 % hydrogen peroxide at 40 °C/75 % RH

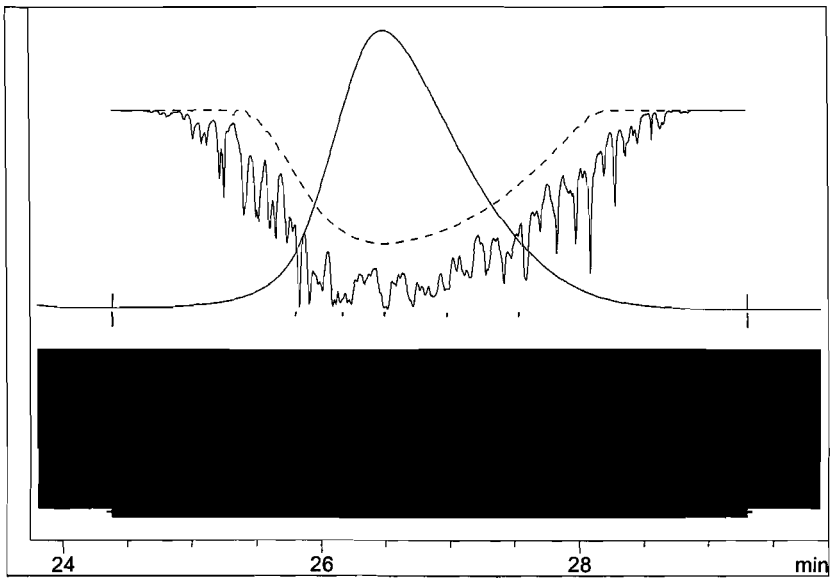


Figure F.8: Peak purity test results for rifampicin

Conclusion

(**Acceptance criteria:** The placebo should not generate any peaks that will interfere with the determination of the active ingredients. Extra peaks formed under stress conditions should be discernible from those of the active ingredients).

None of the ingredients in the placebo interfered with the analyte peaks. Extra peaks formed during forced degradation did not interfere with any of the peaks of interest. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

Specificity results for rifampicin are given in Table F.2.

Table F.2: Results for rifampicin to determine Specificity

Stress condition (40 °C)	% Degradation of rifampicin
Water	5.7
0.1 M Hydrochloric acid	36.9
0.1 M Sodium hydroxide	> 90 %
10 % Hydrogen peroxide	8.5

Degradation of about 5.7 % occurred in a sample solution mixed with water. Degradation of about 36.9 % occurred in a sample solution mixed with 0.1 M hydrochloric acid. Degradation of more than 90 % occurred in sample solutions mixed with 0.1 M sodium hydroxide. In a sample

solution mixed with 10 % hydrogen peroxide, degradation of 8.5 % occurred. Additional peaks that formed during stress conditions were discernible from those of the active ingredients.

F.9.2 Linearity and Range

Linearity and Range results for rifampicin are given in Table F.3.

Table F.3: Results for rifampicin to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
150	1278.2	1274.9	1276.5
300	2759.5	2756.9	2758.2
450	4235.6	4130.8	4183.2
600	5501.0	5412.7	5456.9
750	6893.9	6885.1	6889.5
900	8265.6	8266.8	8266.2
1050	9440.9	9551.3	9496.1
1200	11000.7	11019.3	11010.0
1350	12389.5	12400.9	12395.2
1500	13971.0	13598.4	13784.7

Regression statistics are given in Table F.4.

Table F.4: Regression statistics of rifampicin results

R squared	0.9998	Lower 95%	Upper 95%
Intercept	-37.0797	-134.7448	60.5854
Slope	9.1984	9.0935	9.3034

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 150– 1500 µg/ml (20 – 200 % of expected sample concentration) as shown in Figure F.9. The regression coefficient (R squared) is 0.9998. The method is suitable for single point calibration.

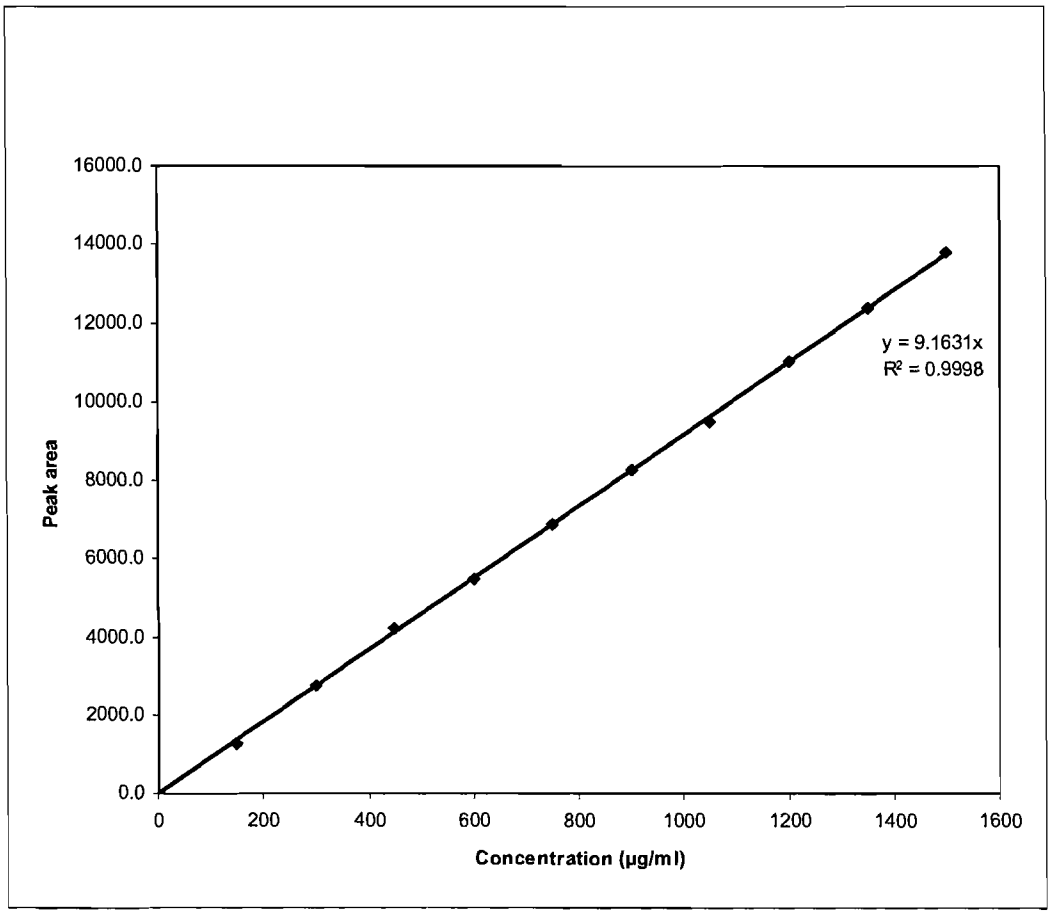


Figure F.9: Linear regression graph for rifampicin to determine Linearity and Range

F.19.3 Accuracy

Accuracy results for rifampicin and the statistical analysis thereof are given in Tables F.5 and F.6, respectively.

Table F.5: Results for rifampicin to determine Accuracy

Conc. spiked (µg/ml)	Area 1	Area 2	Mean	Recovery (µg/ml)	Recovery (%)
600	7715.9	7601.6	7658.8	585.7	97.62
600	7653.9	7686.2	7670.0	586.6	97.76
600	7561.2	7765.5	7663.4	586.1	97.68
750	7950.8	7856.0	7903.4	755.5	100.74
750	7991.2	8006.4	7998.8	764.7	101.95
750	7988.4	8001.2	7994.8	764.3	101.90
900	8036.3	7863.5	7949.9	912.0	101.33
900	7664.4	7995.7	7830.0	898.2	99.80
900	7776.0	7733.8	7754.9	889.6	98.84

Table F.6: Statistical analysis of rifampicin results

Mean %	99.7
SD	1.7
% RSD	1.7
95% confidence intervals	
Lower limit	98.3
Upper limit	101.1
Estimated median	99.8
Confidence Level (95.0%)	1.4

Conclusion

(Acceptance criterion: Recovery must be between 98.0 – 102.0 %).

Over the range of 80 – 120 % of the sample concentration, the method yielded a mean recovery of 99.7 %.

F.9.4 Precision

F.9.4.1 Intra-day Precision

Intra-day precision results for rifampicin are given in Table F.7.

Table F.7: Results for rifampicin to determine Intra-day Precision

Tablet powder (g)	Area 1	Area 2	Mean Area	Concentration (µg/ml)	Recovery (%)
0.450	6534.3	6580.7	6557.5	600	104.48
0.455	6654.3	6570.5	6612.4	600	105.35
0.455	6516.2	6668.7	6592.4	600	105.04
0.567	8122.8	7952.5	8037.6	750	102.45
0.566	8160.9	7910.7	8035.8	750	102.43
0.560	7621.7	8293.8	7957.7	750	101.43
0.674	9913.9	9777.0	9845.5	900	104.58
0.670	9411.4	9741.2	9576.3	900	101.72
0.676	9413.0	9599.4	9506.2	900	100.97
				Mean	103.2
				SD	1.6
				RSD %	1.5

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Intra-day precision was satisfactory with an RSD of 1.5 %.

F.9.4.2 INTER-DAY PRECISION

Inter-day precision results for rifampicin are given in Table F.8. ANOVA single factor statistics thereof are given in Table F.9.

Table F.8: Results for rifampicin to determine Inter-day Precision

	Day 1	Day 2	Day 3	Between days
	8037.61	8066.93	7864.60	
	8035.80	8015.39	7893.33	
	7957.73	8016.37	7870.09	
Mean	8010.38	8032.90	7876.01	7973.09
SD	37.24	24.07	12.45	24.58
RSD %	0.46	0.30	0.16	0.31

Table F.9: ANOVA single factor statistics for the determination of rifampicin

Groups	Count	Sum	Average	Variance	
Day 1	3	24031.14	8010.38	2079.94	
Day 2	3	23998.69	7999.56	5854.87	
Day 3	3	23628.02	7876.01	232.54	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	33438.39	2	16719.19	6.14	0.04
Within Groups	16334.70	6	2722.45		
Total	49773.08	8			

SS = sum of squares

df = degrees of freedom

MS = mean squares

F = F ratio

Conclusion

(Acceptance criterion: RSD of 2.0 % or less).

Inter-day precision was satisfactory with an RSD of 0.3 %.

F.9.5 Ruggedness

F.9.5.1 Stability of sample solutions

A sample solution was left on the auto sampler tray in amber vials and re-analysed over several time intervals to determine the sample stability. The results are presented in Table F.10.

Table F.10: Results for rifampicin to determine Ruggedness

Time (hours)	Peak Area	%
0	10134.2	100.00
1	9936.79	98.05
2	9835.76	97.06
3	9537.65	94.11
4	9236.46	91.14
5	9007.16	88.88
6	8767.59	86.51
7	8490.43	83.78
8	8353.33	82.43
Mean	9255.48	91.33
SD	6.02	6.02
RSD %	0.07	6.59

Conclusion

(Acceptance criterion: The sample solution should not be used for a period longer than it takes to degrade by 2.0 %).

Degradation of nearly 18.0 % was observed over an 8 hour period. Sample solutions are considered stable for about 1 hour.

F.9.5.2 SYSTEM REPEATABILITY

A sample solution was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table F.11.

Table F.11: Results for rifampicin to determine System Repeatability

	Area	Retention time (minutes)
	8531.0	26.50
	8310.2	26.20
	8532.9	26.80
	8431.8	25.90
	8274.2	26.80
	8235.5	26.70
Mean	8385.9	26.48
SD	119.4	0.33
RSD %	1.4	1.26

Conclusion

(**Acceptance criterion:** The peak area and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.4 % for peak area and 1.3 % for retention time respectively.

F.10. ROBUSTNESS

The following changes in the chromatographic operating parameters were found to be acceptable:

Column: Luna C_{18} column, 250 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance, CA; and
µBondapak C_{18} column, 250 x 4.6 mm, 10 µm pores, Waters, Ireland.
The µBondapak gave different retention times, thus the flow rate must be altered.

Flow rate: 0.9 – 1.1 ml/minute.

Wavelength: The wavelength can be altered by ± 2 nm without any ill effect.

Injection volume: 8 – 12 µl.

Conclusion

(**Acceptance criterion:** The method should be able to tolerate about 5.0 % variance in chromatographic conditions).

The method was able to tolerate small changes in the chromatographic conditions.

F.11. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Rifampicin	25 to 30 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Rifampicin	1 924

USP Tailing factor (T):

Rifampicin	0.999

Capacity factor (k'):

Rifampicin	12.4857

Resolution between peaks (R):

Rifampicin	-

F.12. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates.
* Use the 5-sigma method to calculate the parameters in F.11.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 2.0 %.
* The column must have more than 1 800 theoretical plates for rifampicin.
* The resolution between the peaks of 3-formyl rifamycin and rifampicin must be at least 1.5.
* The USP tailing must be less than 1.5.

F.13. CONCLUSION

The method performed well and should be suitable to analyse rifampicin in products for stability testing, quality control and batch release purposes. Peak purity testing of all the remaining peaks after forced degradation in the water sample resulted in purity values of more than 999 out of 1 000, which showed that the peaks were still pure, thus proving that the method is stability-indicating.

APPENDIX G

Validation of an HPLC Assay Method:

Determination of Known Related Substances of Rifampicin in Rifampicin Capsules / Tablets and in a Fixed Dose Combination product containing Rifampicin

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G.1. ORIGIN OF METHOD

The method was based in part on an article by Sankar *et al.* (2003:733) discussing the decomposition of rifampicin in the presence of isoniazid in the pH range 1 – 3.

G.2. SPECIFICATIONS

The related substance specifications set for a formulation are given in Table G.1.

TableG.1: Related substance specifications for a formulation

Related substances	Capsules/Tablets (containing rifampicin)[1]	Tablets (Any combination of rifampicin, isoniazid, pyrazinamide or ethambutol)[2]
Rifampicin N-oxide	1.5 %	No specific limit, thus 1 %
Rif-iso hydrazone*	Not applicable	5 %
Rifampicin-quinone	4 %	4 %
3-Formyl rifamycin	0.5 %	Not applicable (Converted to rif-iso hydrazone)
Any other impurity	1 %	1 %

*Rif-iso hydrazone is an alternative name for the isonicotinyl hydrazone.

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[1] Taken from BP (2005)

[2] Taken from specifications of a number of 4FDC tablet products obtained from manufacturers

G.3 SUMMARY

In Table G.2 a summary of the validation results are given.

Table G.2: Summary of validation results (Continue on next page)

Parameter	Acceptance criteria	Results
Specificity	No peaks should interfere with the determination of the peaks of interest.	Complies
Linearity and Range	The method is linear for the rif-iso hydrazone over the range: 0 – 10.0 % of the expected rifampicin concentration. The method is linear for the other related substances over the range: 0 – 5.0 % of the expected rifampicin concentration. R^2 is not less than 0.99.	Complies Rifampicin N-oxide: Linear from 0 – 37.5 µg/ml (0 – 5.0 % of the expected rifampicin concentration) R^2: 0.9999 Rif-iso hydrazone: Linear from 0 – 75.0 µg/ml (0 – 10 % of the expected rifampicin concentration) R^2: 0.9990 Rifampicin-quinone: Linear from 0 – 37.5 µg/ml (0 – 5.0 % of the expected rifampicin concentration) R^2: 0.9992 3-Formyl rifamycin: Linear from 0 – 37.5 µg/ml (0 – 5.0 % of the expected rifampicin concentration) R^2: 0.9989
Precision (Repeatability)	RSD of 5.0 % or less.	Complies Rifampicin N-oxide: 1.1 % Rif-iso hydrazone: 3.7 % Rifampicin-quinone: 3.9 % 3-Formyl rifamycin: 1.8 %

Table G.2: Summary of validation results (Continued from previous page)

Parameter	Acceptance criteria	Results
Ruggedness (System repeatability)	The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less.	Complies Rifampicin N-oxide: 0.6 % for peak area, 0.3 % for retention time Rif-iso hydrazone: 1.1 % for peak area, 0.1 % for retention time Rifampicin-quinone: 1.4 % for peak area, 0.3 % for retention time 3-Formyl rifamycin: 2.9 % for peak area, 0.7 % for retention time
Limit of Detection	The concentration determined at the LOD has a signal-to-noise ratio of 3:1.	Rifampicin N-oxide: 0.3 µg/ml (0.04 % of expected rifampicin concentration) Rif-iso hydrazone: 0.3 µg/ml (0.04 % of expected rifampicin concentration) Rifampicin-quinone: 0.5 µg/ml (0.07 % of expected rifampicin concentration) 3-Formyl rifamycin: 1.0 µg/ml (0.13 % of expected rifampicin concentration)
Limit of Quantitation	The concentration determined at the LOQ has a signal-to-noise ratio of 10:1.	Rifampicin N-oxide: 0.9 µg/ml (0.12 % of expected rifampicin concentration) Rif-iso hydrazone: 1.0 µg/ml (0.13 % of expected rifampicin concentration) Rifampicin-quinone: 1.0 µg/ml (0.13 % of expected rifampicin concentration) 3-Formyl rifamycin: 5.0 µg/ml (0.7 % of expected rifampicin concentration)

G.4. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: HP1100 series HPLC equipped with a HP1100 quaternary gradient pump, HP1100 auto sampler, HP1100 diode array detector and Chemstation Rev. A.09.03 (1417) data acquisition and analysis software.

Column: Column L1, USP 29, 2006, p 2649, (Luna C_{18} column, 250 x 4.6 mm, 5 µm, 17.8 % carbon load, end capped, Phenomenex, Torrance was used).

Mobile phase: 1.361 g potassium dihydrogen orthophosphate weighed into a 1 000 ml volumetric flask, dissolved and made up to volume with water. pH adjusted to 7.00 ± 0.05 with 0.01 M NaOH. Mix 400 ml of this solution with 600 ml methanol.

Flow rate: 1.0 ml/min.

Injection volume: 10 µl.

Detection: 254 nm (Diode Array Detection).

Retention times: About 7.3 minutes for rifampicin N-oxide
About 14.4 minutes for rif-iso hydrazone
About 18.8 minutes for rifampicin-quinone
About 23.7 minutes for 3-formyl rifamycin
About 25 to 30 minutes for rifampicin

Relative retention times with respect to rifampicin were found to be about 0.7; 0.9, 0.3 and 0.5 for rifampicin-quinone, 3-formyl rifamycin, rifamycin N-oxide and the isonicotinyl hydrazone, respectively.

Solvent: Methanol.

G.5. RIFAMPICIN STANDARD SOLUTION PREPARATION

1. Accurately weigh about 150 mg of rifampicin reference standard[3] into a 100 ml volumetric flask.

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[3] Rifampicin primary standard: USP, B/N 0420400006

2. Dissolve in about 50 ml methanol.
3. Shake well and make up to volume with methanol.
4. Dilute 5 ml to 10 ml with methanol.
5. Analyse by means of HPLC (6 injections).

G.6. RELATED SUBSTANCE SOLUTION PREPARATION

1. For the rif-iso hydrazone solution accurately weigh 7.5 mg of rif-iso hydrazone[4] into a 50 ml volumetric flask. Dissolve in about 25 ml methanol and make up to volume with methanol. Dilute 5 ml of this solution to 10 ml with methanol (this is equal to 10.0 % of the rifampicin standard solution concentration).
2. For the rifampicin N-oxide, rifampicin-quinone and 3-formyl rifamycin solutions accurately weigh 7.5 mg of rifampicin N-oxide[5], rifampicin-quinone[6] and 3-formyl rifamycin[7] into separate 200 ml volumetric flasks. Dissolve in about 25 ml methanol and make up to volume with methanol (this is equal to 5.0 % of the rifampicin standard solution concentration).
3. Shake well and make up to volume with methanol.
4. Analyse by means of HPLC (6 injections).

G.7. VALIDATION TEST PROCEDURES AND ACCEPTANCE CRITERIA

G.7.1 Specificity

Method

1. Prepare a standard solution as described under Standard Solution Preparation.
2. Prepare related substances solutions as described under Related Substance Solution Preparation.
3. Spike the related substances solutions with standard solution.

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[4] Rif-iso hydrazone: Donated by Doctor Sarangjit Singh, unknown potency

[5] Rifampicin N-oxide primary standard: BP, B/N 1948

[6] Rifampicin-quinone primary standard: EP, Lot 3

4. Analyse by means of HPLC.
5. Examine the chromatograms to determine whether the peaks are well separated from each other.

Acceptance criterion

All the peaks should be well separated from each other.

G.7.2 Linearity and Range

Method

1. Prepare the solutions as described under Related Substance Solution Preparation. Dilute the rif-iso hydrazone solution to prepare solutions that represent the following rifampicin standard solution concentrations: 10.0 %; 5.0 %; 2.0 %; 0.5 % and 0.2 %. Dilute the other related substance solutions to prepare solutions that represent the following rifampicin standard solution concentrations: 5.0 %; 2.5 %; 1.0 %; 0.5 % and 0.25 %. Analyse these solutions by means of HPLC.

Acceptance criteria

The method is linear over the range 0 – 10.0 % for rif-iso hydrazone, and 0 – 5.0 % for the other solutions.

Linear regression analysis should yield a regression coefficient (R squared) of not less than 0.99.

G.7.3 Precision

Method

1. Prepare the solutions as described under Related Substance Solution Preparation. Prepare a standard solution as described under Standard Solution Preparation. Separately mix equal amounts of the rifampicin standard solution and related substance solutions.
2. Analyse by means of HPLC (6 injections).

Acceptance criterion

The Percentage Relative Area must have an RSD of 5.0 % or less.

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[7] 3-Formyl rifamycin primary standard: BP, B/N 2084

G.7.4 Ruggedness

G.7.4.1 System Repeatability

Method

1. For rif-iso hydrazone, inject the solution (representing 10 % of the related substance solution) six times consecutively in order. For the other related substances, inject the solutions (representing a concentration of 5 % of the related substance solutions) six times consecutively in order to test the repeatability of the peak area as well as the retention time.

Acceptance criterion

The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less.

G.7.5 Limit of Detection and Quantitation

The Limit of Detection (LOD) is the smallest concentration that can be detected reliably. The LOD is usually defined as a peak with a signal-to-noise ratio of at least 3:1. Signal refers to the baseline-corrected absorbance of the analyte peak, and noise refers to the width of the baseline. The Limit of Quantitation (LOQ) is the concentration that can be quantitated reliably with a specified level of accuracy and precision (Snyder *et al.*, 1997:645). The LOD and LOQ values determined during method validation are affected by separation conditions such as: columns, reagents, and especially instrumentation and data systems. Instrumental changes (particularly pumping systems and detectors), or the use of contaminated reagents can result in large changes in signal-to-noise ratio (especially affecting baseline noise and drift) (Snyder *et al.*, 1997:695).

G.7.5.1 Limit of Detection

According to the ICH (2005:11) several approaches for determining Limit of Detection are possible. Approaches other than those listed may be acceptable:

1. **Based on visual evaluation**

   The Limit of Detection is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

2. **Based on signal-to-noise**

   This approach can only be applied to analytical procedures which exhibit baseline noise. Compare measured signals from samples with known low concentrations of analyte with those of blank samples and establish the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating Limit of Detection.

3. **Based on the standard deviation of the response and the slope**

   The slope may be estimated from the calibration curve of the analyte. The estimate of the slope may be based on the standard deviation of the blank, or on the calibration curve.

Method

1. Prepare and inject the solutions as described under Linearity and Range.
2. Calculate the signal-to-noise ratio for each data point of the related substance peak of interest as described by Snyder *et al.* (1997:646).
3. With the use of linear regression, extrapolate the concentration values to obtain a signal-to-noise ratio of 3:1.
4. This concentration is the Limit of Detection.

Acceptance criterion

The concentration determined as the Limit of Detection has a signal-to-noise ratio of 3:1.

G.7.5.2 Limit of Quantitation

According to the ICH (2005:12) several approaches for determining Limit of Quantitation are possible. Approaches other than those listed may be acceptable:

1. **Based on visual evaluation**

   The Limit of Quantitation is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

2. **Based on signal-to-noise**

   This approach can only be applied to analytical procedures which exhibit baseline noise. Compare measured signals from samples with known low concentrations of analyte with those of blank samples and establish the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

3. **Based on the standard deviation of the response and the slope**

   The slope may be estimated from the calibration curve of the analyte. The estimate of the slope may be based on the standard deviation of the blank, or on the calibration curve.

Method

1. Prepare and inject the solutions as described under Linearity and Range.
2. Calculate the signal-to-noise ratio for each data point of the related substance peak of interest as described by Snyder *et al.* (1997:646).
3. With the use of linear regression, extrapolate the concentration values to obtain a signal-to-noise ratio of 10:1.
4. This concentration is the Limit of Quantitation.

Acceptance criterion

The concentration determined as the Limit of Quantitation has a signal-to-noise ratio of 10:1 as illustrated in Figure G.1 using the rif-iso hydrazone solution as an example.

G.7.6 System and method performance characteristics (System Suitability)

Method

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP peak tailing factor, capacity factor, resolution between peaks and repeatability of multiple injections.
2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

Acceptance criteria

The USP tailing factor must be less than 1.5 and the RSD must be less than 5.0 %. The resolution and plate count have to be determined through the validation for the specific system.

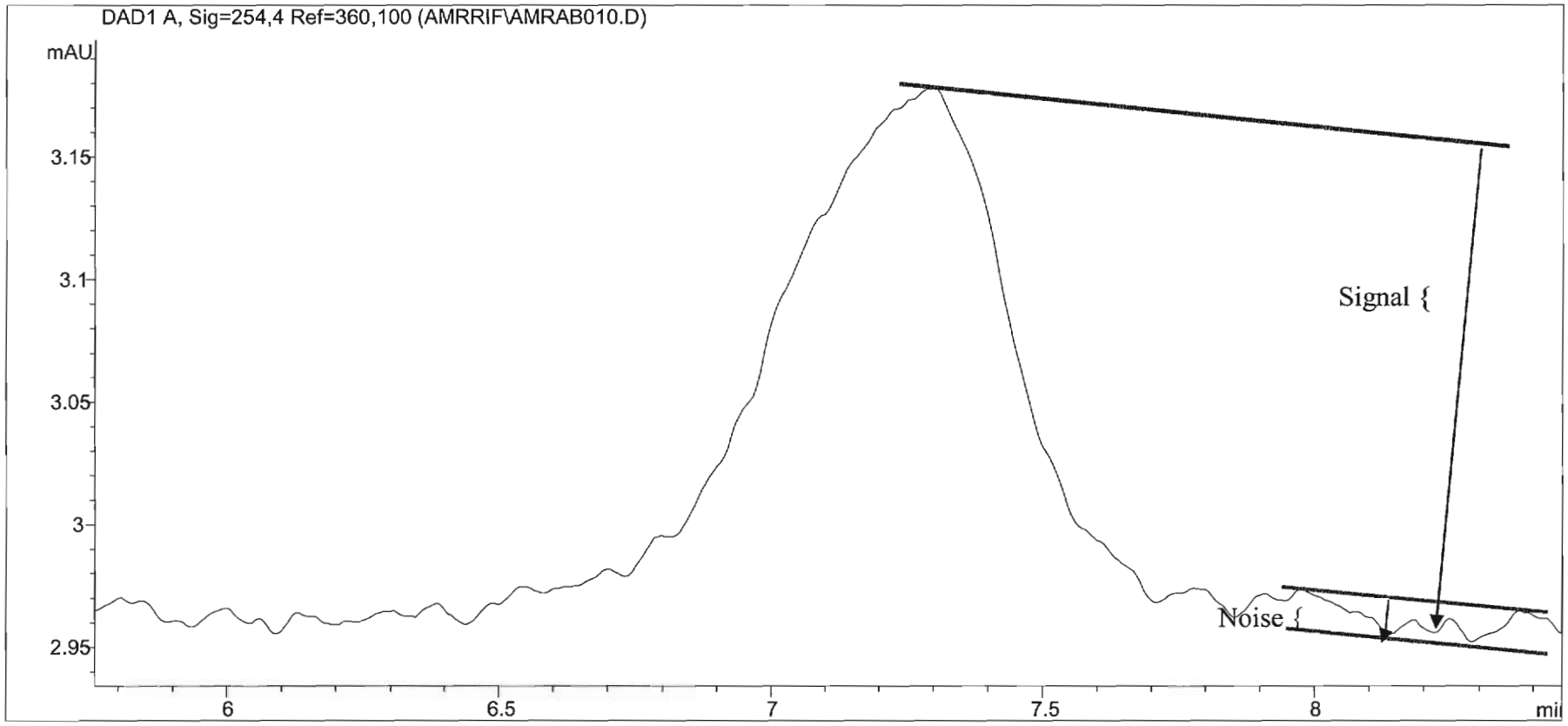


Figure G.1: Representation of a signal-to-noise ratio of about 10:1 for the rif-iso hydrazone solution

G.8. VALIDATION RESULTS

G.8.1 Specificity

A solution containing rifampicin and the related substances is shown in Figure G.2 and a chromatogram of a standard solution is shown in Figure G.3.

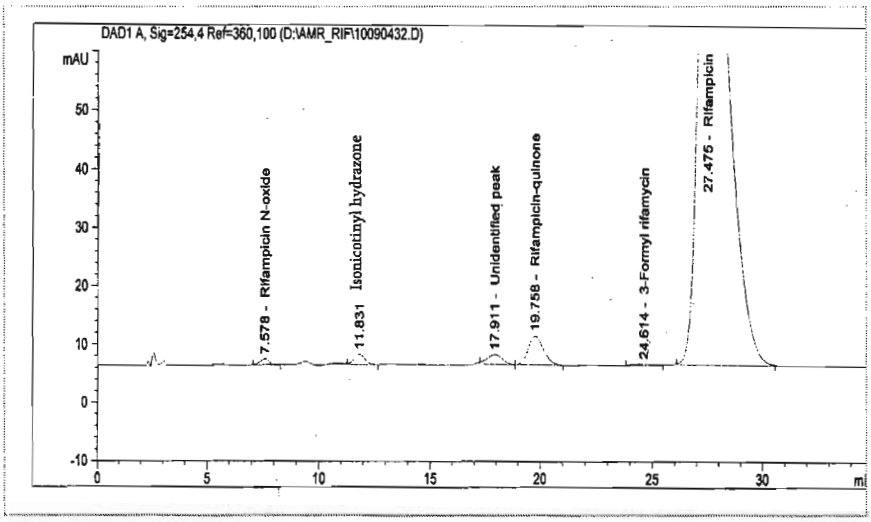


Figure G.2: Chromatogram of a solution containing rifampicin, rif-iso hydrazone, rifampicin N-oxide, rifampicin-quinone and 3-formyl rifamycin

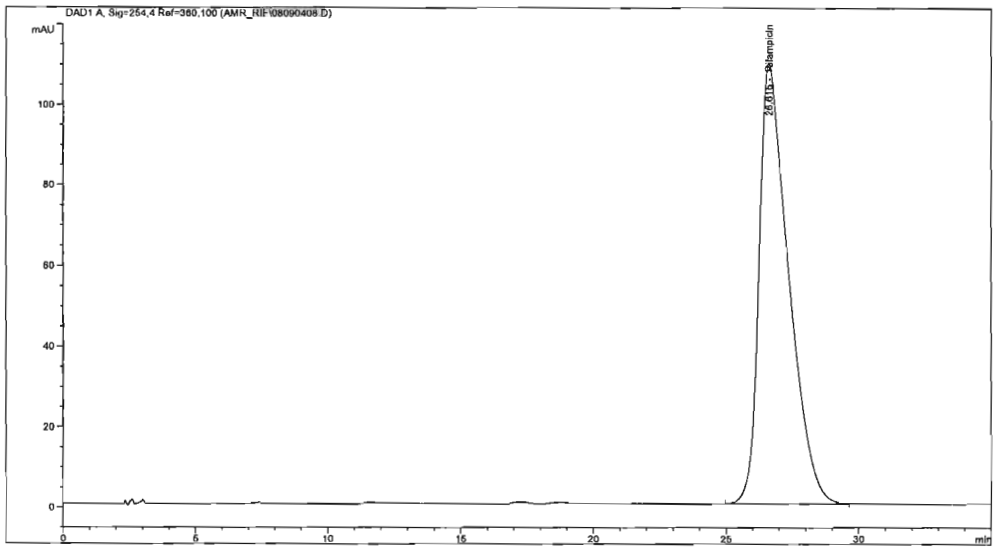


Figure G.3: Chromatogram of a standard solution

Conclusion

(**Acceptance criterion:** All peaks should be well separated from each other).

All the peaks were well separated from each other.

G.9. RIFAMPICIN N-OXIDE

G.9.1 Linearity and Range

Linearity and Range results for rifampicin N-oxide are given in Table G.3.

Table G.3: Results for rifampicin N-oxide to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
0	0	0	0
1.9	24.4	22.6	23.5
3.8	44.4	44.6	44.5
7.5	94.6	90.7	92.6
18.8	238.6	239.2	238.9
37.5	486.2	494.6	490.4

Regression statistics are given in Table G.4.

Table G.4: Regression statistics of rifampicin N-oxide results

R squared	0.9999	Lower 95%	Upper 95%
Intercept	-3.4474	-8.6016	1.7068
Slope	13102.0490	12807.2415	13396.8564

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 0 – 37.5 µg/ml (0 – 5.0 % of the expected rifampicin concentration) as shown in Figure G.4.

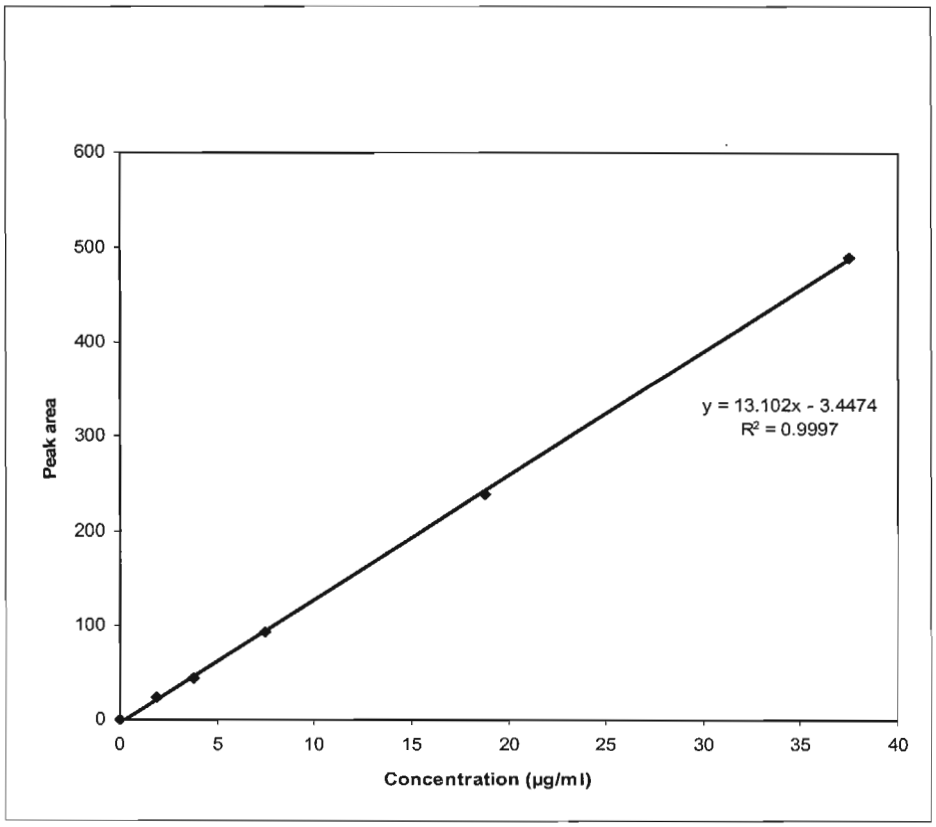


Figure G.4: Linear regression graph for rifampicin N-oxide to determine Linearity and Range

G.9.2 Precision

Precision results for a 37.5 µg/ml rifampicin N-oxide solution are given in Table G.5.

Table G.5: Results for rifampicin N-oxide to determine Precision

Rifampicin N-oxide	Rifampicin	%
250.80	4900.85	5.1
251.86	4872.41	5.2
247.60	4867.13	5.1
249.68	4897.28	5.1
252.09	4827.47	5.2
251.70	4808.86	5.2
	Mean	5.2
	SD	0.1
	% RSD	1.1

Conclusion

(**Acceptance criterion:** The Percentage Relative Area must have an RSD of 5.0 % or less).

Precision was satisfactory with an RSD of 1.1 %.

G.9.3 Ruggedness

G.9.3.1 System Repeatability

A solution (representing 50 % of the related substance solution for rifampicin N-oxide) was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table G.6.

Table G.6: Results for rifampicin N-oxide to determine System Repeatability

	Area	Retention time (minutes)
	237.4	7.26
	236.1	7.28
	238.1	7.30
	236.7	7.30
	234.3	7.31
	234.0	7.34
Mean	236.1	7.30
SD	1.5	0.02
RSD %	0.6	0.33

Conclusion

(**Acceptance criterion:** The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less.)

System performance proved well with RSD values of 0.6 % for peak area and 0.3 % for retention time respectively.

G.9.4 Limit of Detection and Quantitation

G.9.4.1 Limit of Detection

Experimental signal-to-noise ratios for rifampicin N-oxide and the regression statistics thereof, are given in Tables G.7 and G.8, respectively. A linear regression graph is shown in Figure G.5.

Table G.7: Experimental signal-to-noise ratios for rifampicin N-oxide at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
1.9	20
3.8	35
7.5	60

Table G.8: Experimental regression statistics of rifampicin N-oxide results

R squared	0.9984	Lower 95%	Upper 95%
Intercept	7.1373	-10.8691	25.1437
Slope	7090.0123	3471.8129	10708.2117

Theoretical signal-to-noise ratios for rifampicin N-oxide and the regression statistics thereof, are given in Tables G.9 and G.10, respectively. A linear regression graph is shown in Figure G.6.

Table G.9: Theoretical signal-to-noise ratios for rifampicin N-oxide at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
0.3	3
0.9	10
1.9	20
3.8	35
7.5	60

Table G.10: Theoretical regression statistics of rifampicin N-oxide results

R squared	0.9913	Lower 95%	Upper 95%
Intercept	3.2059	-1.9903	8.4021
Slope	7775.7353	6435.8603	9115.6103

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Detection has a signal-to-noise ratio of 3:1).

A concentration of about 0.3 µg/ml rifampicin N-oxide solution (representing 0.04 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 3:1.

G.9.4.2 Limit of Quantitation

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Quantitation has a signal-to-noise ratio of 10:1).

A concentration of about 0.9 µg/ml rifampicin N-oxide solution (representing 0.12 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 10:1.

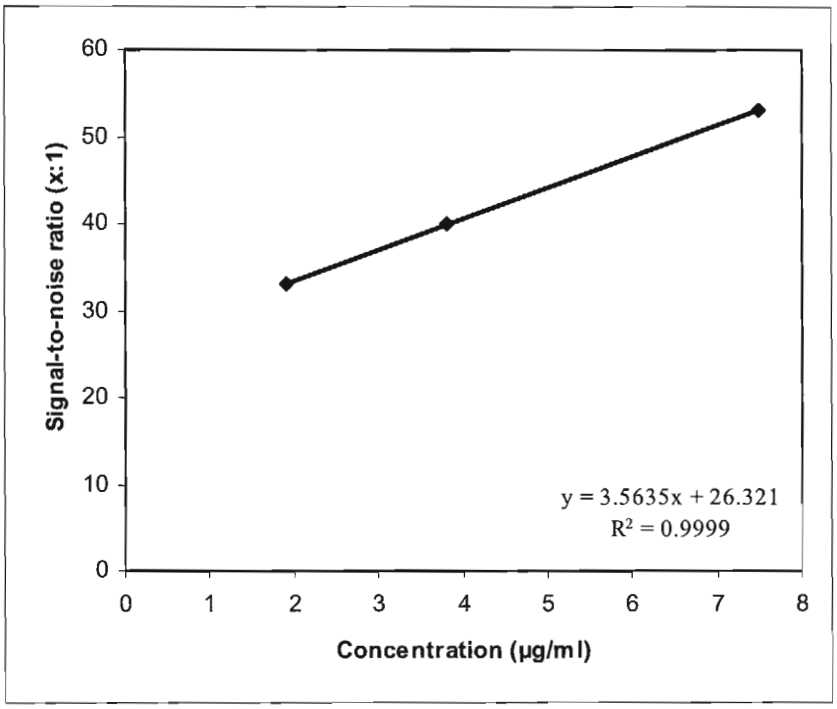


Figure G.5: Experimental linear regression graph for rifampicin N-oxide to determine the LOD and LOQ

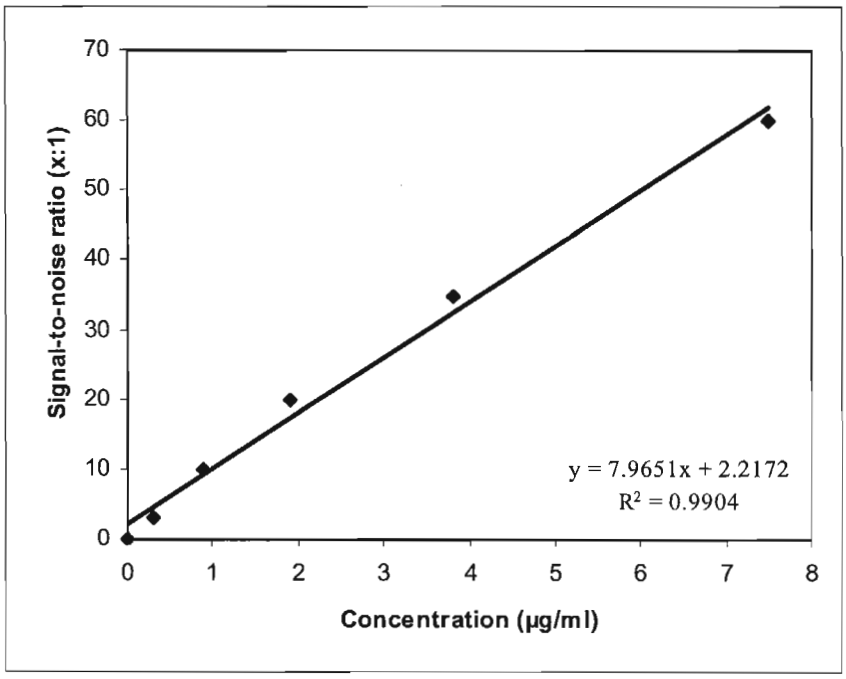


Figure G.6: Theoretical linear regression graph for rifampicin N-oxide to determine the LOD and LOQ

G.10. RIF-ISO HYDRAZONE

G.10.1 Linearity and range

Linearity and Range results for rif-iso hydrazone are given in Table G.11.

Table G.11: Results for rif-iso hydrazone to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
0	0	0	0
1.5	0.1	0.1	0.1
3.8	5.1	5.7	5.4
15.0	31.3	29.6	30.5
37.5	91.3	89.3	90.3
75.0	184.1	187.4	185.7

Regression statistics are given in Table G.12.

Table G.12: Regression statistics of rif-iso hydrazone results

R squared	0.9990	Lower 95%	Upper 95%
Intercept	-3.6152	-7.4374	0.2069
Slope	2512.6215	2402.8404	2622.4026

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range $0 - 75.0$ µg/ml ($0 - 10$ % of the expected rifampicin concentration) as shown in Figure G.7.

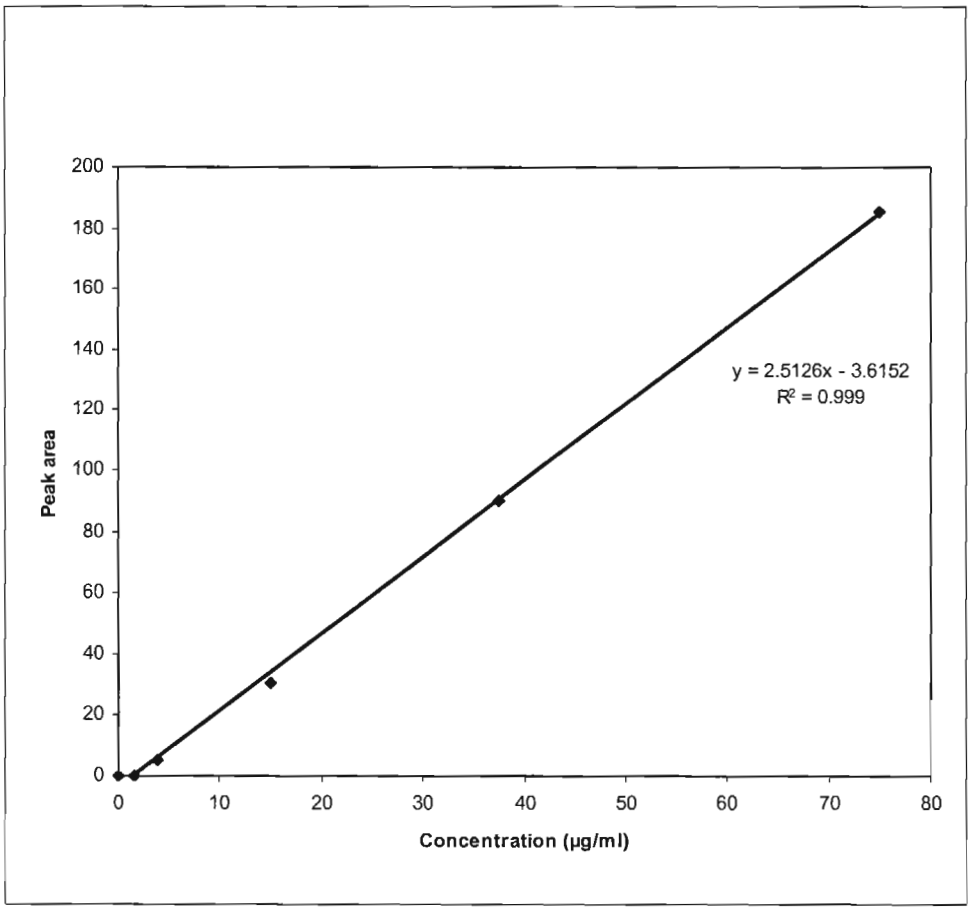


Figure G.7: Linear regression graph for rif-iso hydrazone to determine Linearity and Range

G.10.2 Precision

Precision results for a 75 µg/ml rif-iso hydrazone solution are given in Table G.13.

Table G.13: Results for Rif-iso hydrazone to determine Precision

Rif-iso hydrazone	Rifampicin	%
158.02	4861.55	3.3
152.85	4831.88	3.2
173.44	4890.12	3.5
159.87	4899.56	3.3
169.14	4981.54	3.4
158.61	4830.00	3.3
	Mean	3.3
	SD	0.1
	% RSD	3.7

Conclusion

(**Acceptance criterion:** The Percentage Relative Area must have an RSD of 5.0 % or less).

Precision was satisfactory with an RSD of 3.7 %.

G.10.3 Ruggedness

G.10.3.1 System Repeatability

A solution (representing 50 % of the related substance solution for rif-iso hydrazone) was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table G.14.

Table G.14: Results for rif-iso hydrazone to determine System Repeatability

	Area	Retention time (minutes)
	92.2	14.34
	92.3	14.35
	95.2	14.35
	93.1	14.36
	94.1	14.34
	92.9	14.35
Mean	93.3	14.35
SD	1.1	0.01
RSD %	1.1	0.08

Conclusion

(**Acceptance criterion:** The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.1 % for peak area and 0.1 % for retention time respectively.

G.10.4 Limit of Detection and Quantitation

G.10.4.1 Limit of Detection

Experimental signal-to-noise ratios for rif-iso hydrazone and the regression statistics thereof, are given in Tables G.15 and G.16, respectively. A linear regression graph is shown in Figure G.8.

Table G.15: Experimental signal-to-noise ratios for rif-iso hydrazone at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
1.5	14
3.8	22
15.0	76

Table G.16: Experimental regression statistics of rif-iso hydrazone results

R squared	0.9983	Lower 95%	Upper 95%
Intercept	5.7636	-16.0739	27.6011
Slope	4665.4738	2232.5185	7098.4291

Theoretical signal-to-noise ratios for rif-iso hydrazone and the regression statistics thereof, are given in Tables G.17 and G.18, respectively. A linear regression graph is shown in Figure G.9.

Table G.17: Theoretical signal-to-noise ratios for rif-iso hydrazone at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
0.3	3
1.0	10
1.5	14
3.8	22
15.0	76

Table G.18: Theoretical regression statistics of rif-iso hydrazone results

R squared	0.9957	Lower 95%	Upper 95%
Intercept	4.3203	0.2755	8.3650
Slope	4786.9778	4206.5170	5367.4387

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Detection has a signal-to-noise ratio of 3:1).

A concentration of about 0.3 µg/ml rif-iso hydrazone solution (representing 0.04 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 3:1.

G.10.4.2 LIMIT OF QUANTITATION

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Quantitation has a signal-to-noise ratio of 10:1).

A concentration of about 1.0 µg/ml rif-iso hydrazone solution (representing 0.13 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 10:1.

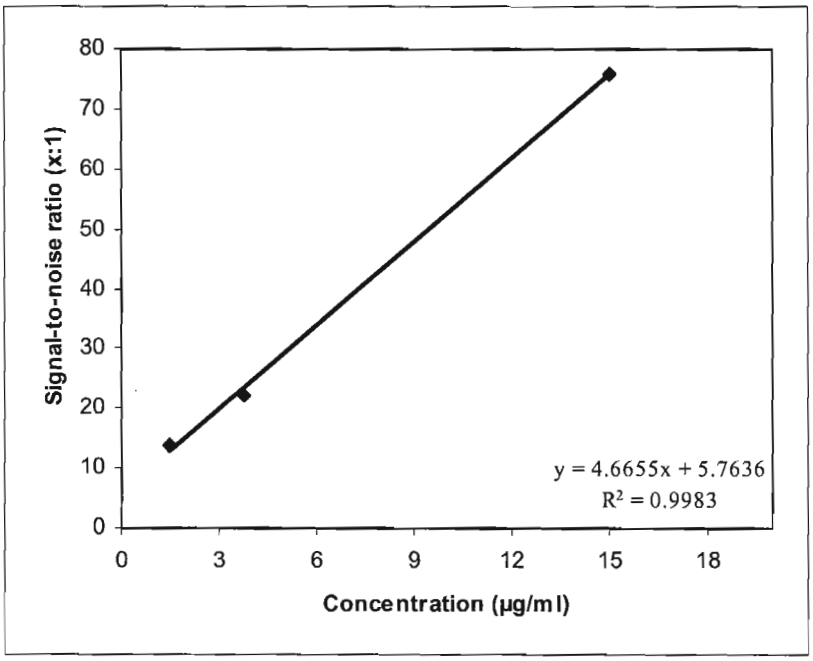


Figure G.8: Experimental linear regression graph for rif-iso hydrazone to determine the LOD and LOQ

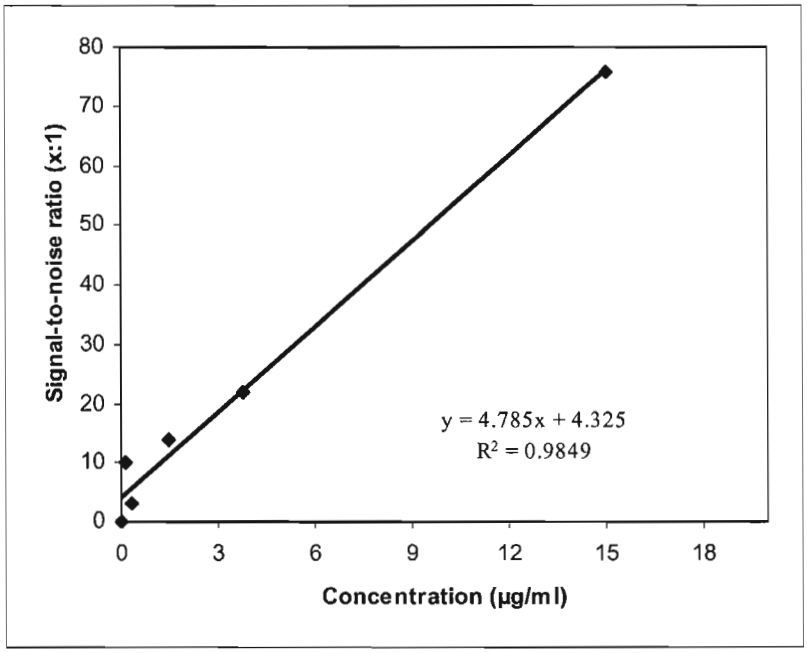


Figure G.9: Theoretical linear regression graph for rif-iso hydrazone to determine the LOD and LOQ

G.11. RIFAMPICIN-QUINONE

G.11.1 Linearity and Range

Linearity and Range results for rifampicin-quinone are given in Table G.19.

Table G.19: Results for rifampicin-quinone to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
0	0	0	0
1.9	18.4	19.9	0.25
3.8	29.4	29.7	0.50
7.5	64.2	63.9	1.00
18.8	171.6	185.7	2.50
37.5	365.8	359.2	5.00

Regression statistics are given in Table G.20.

Table G.20: Regression statistics of rifampicin-quinone results

R squared	0.9992	Lower 95%	Upper 95%
Intercept	-3.6654	-10.2642	2.9334
Slope	9725.4866	9348.0532	10102.9200

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 0 – 37.5 µg/ml (0 – 5.0 % of the expected rifampicin concentration) as shown in Figure G.10.

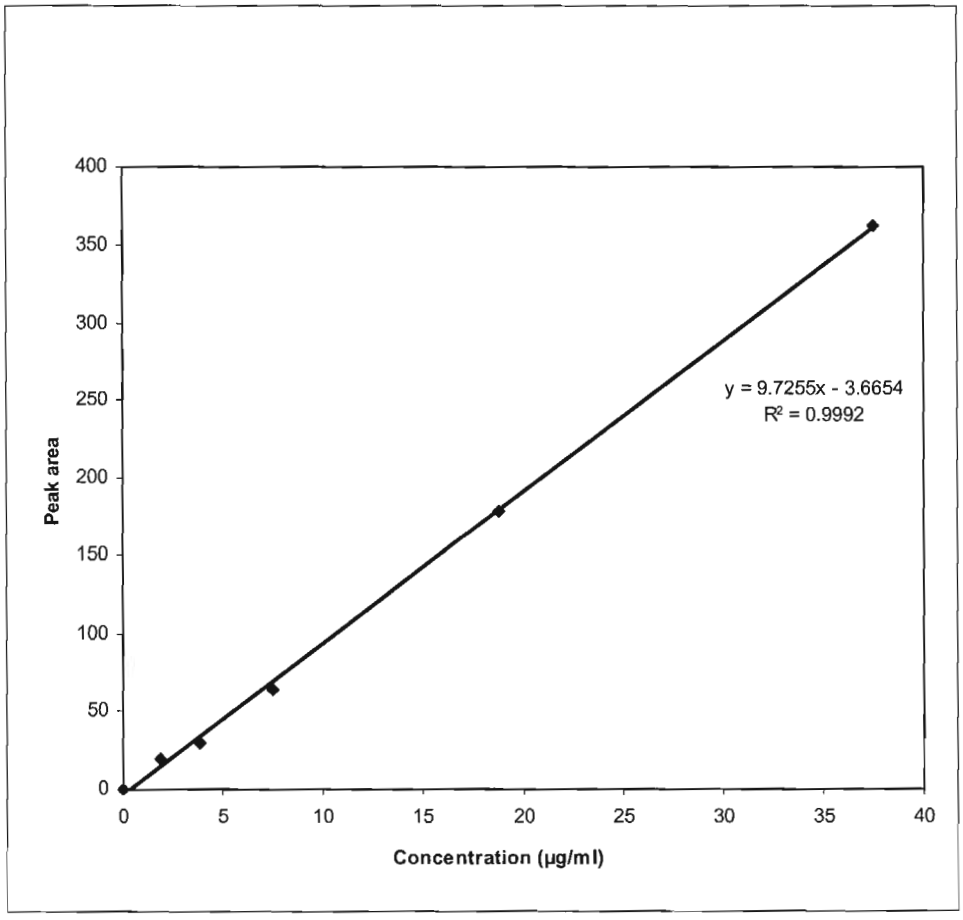


Figure G.10: Linear regression graph for rifampicin-quinone to determine Linearity and Range

G.11.2 Precision

Precision results for a 37.5 µg/ml rifampicin-quinone solution are given in Table G.21.

Table G.21: Results for rifampicin-quinone to determine Precision

Rifampicin-quinone	Rifampicin	%
180.26	4987.88	3.6
185.64	4984.75	3.7
190.76	5214.10	3.7
207.99	5162.95	4.0
189.73	4849.82	3.9
188.13	4866.90	3.9
	Mean	3.8
	SD	0.1
	% RSD	3.9

Conclusion

(**Acceptance criterion:** The Percentage Relative Area must have an RSD of 5.0 % or less).

Precision was satisfactory with an RSD of 3.9 %.

G.11.3 Ruggedness

G.11.3.1 System Repeatability

A solution (representing 50 % of the related substance solution for rifampicin-quinone) was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table G.22.

Table G.22: Results for rifampicin-quinone to determine System Repeatability

	Area	Retention time (minutes)
	163.3	18.80
	163.7	18.79
	168.2	18.67
	169.2	18.69
	167.1	18.75
	168.9	18.84
Mean	166.7	18.76
SD	2.4	0.06
RSD %	1.4	0.32

Conclusion

(**Acceptance criterion:** The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 1.4 % for peak area and 0.3 % for retention time respectively.

G.11.4 Limit of Detection and Quantitation

G.11.4.1 Limit of Detection

Experimental signal-to-noise ratios for rifampicin-quinone and the regression statistics thereof, are given in Tables G.23 and G.24, respectively. A linear regression graph is shown in Figure G.11.

Table G.23: Experimental signal-to-noise ratios for rifampicin-quinone at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
1.9	14
3.8	23
7.5	46

Table G.24: Experimental regression statistics of rifampicin-quinone results

R squared	0.9959	Lower 95%	Upper 95%
Intercept	2.2215	-21.1867	25.6298
Slope	5782.9840	1079.3247	10486.6432

Theoretical signal-to-noise ratios for rifampicin-quinone and the regression statistics thereof, are given in Tables G.25 and G.26, respectively. A linear regression graph is shown in Figure G.12.

Table G.25: Theoretical signal-to-noise ratios for rifampicin-quinone at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
0.5	3
1.0	10
1.9	14
3.8	23
7.5	46

Table G.26: Theoretical regression statistics of rifampicin-quinone results

R squared	0.9904	Lower 95%	Upper 95%
Intercept	2.0812	-2.0113	6.1738
Slope	5822.7143	4769.8779	6875.5508

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Detection has a signal-to-noise ratio of 3:1).

A concentration of about 0.5 µg/ml rifampicin-quinone (representing 0.07 % of the expected rifampicin concentration) solution will produce a signal-to-noise ratio of 3:1.

G.11.4.2 LIMIT OF QUANTITATION

Conclusion

(**Acceptance criteria:** The concentration determined as the Limit of Quantitation has a signal-to-noise ratio of 10:1).

A concentration of about 1.0 µg/ml rifampicin-quinone (representing 0.13 % of the expected rifampicin concentration) solution will produce a signal-to-noise ratio of 10:1.

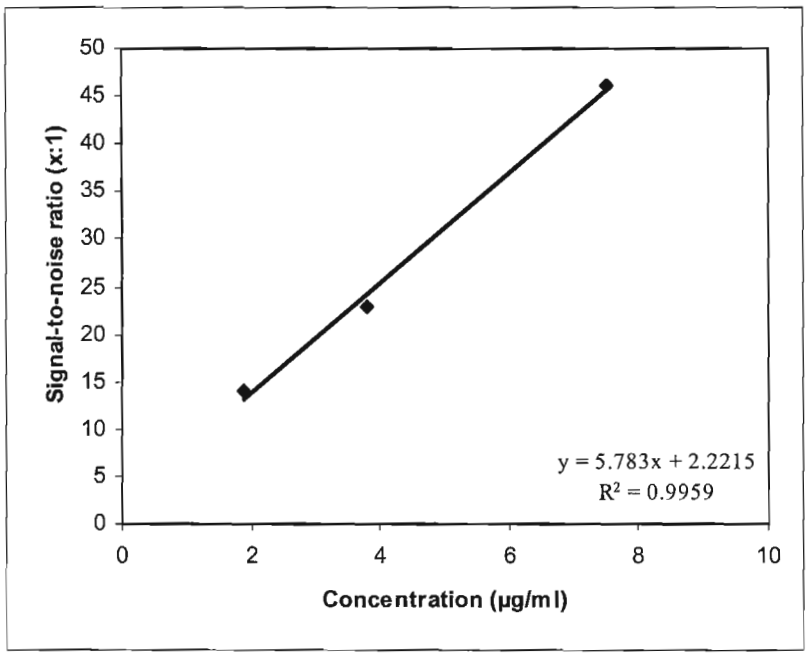


Figure G.11: Experimental linear regression graph for rifampicin-quinone to determine the LOD and LOQ

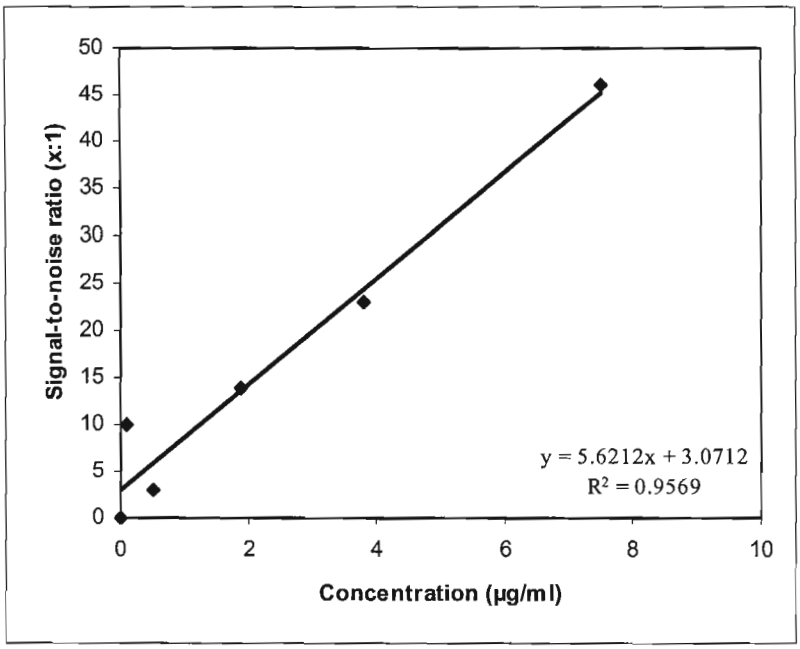


Figure G.12: Theoretical linear regression graph for rifampicin-quinone to determine the LOD and LOQ

G.12. 3-FORMYL RIFAMYCIN

G.12.1 Linearity and Range

Linearity and Range results for 3-formyl rifamycin are given in Table G.27.

Table G.27: Results for 3-formyl rifamycin to determine Linearity and Range

µg/ml	Area 1	Area 2	Mean
0	0	0	0
3.8	12.5	11.5	12.0
7.5	25.8	25.8	25.8
18.8	72.6	68.5	70.5
37.5	146.6	150.8	148.7

Regression statistics are given in Table G.28.

Table G.28: Regression statistics of 3-formyl rifamycin results

R squared	0.9989	Lower 95%	Upper 95%
Intercept	-2.6796	-7.2636	1.9044
Slope	4000.3801	3760.7952	4239.9650

Conclusion

(**Acceptance criteria:** The method is linear. R^2 is not less than 0.99).

The method is linear over the concentration range 0 – 37.5 µg/ml (0 – 5 % of the expected rifampicin concentration) as shown in Figure G.13.

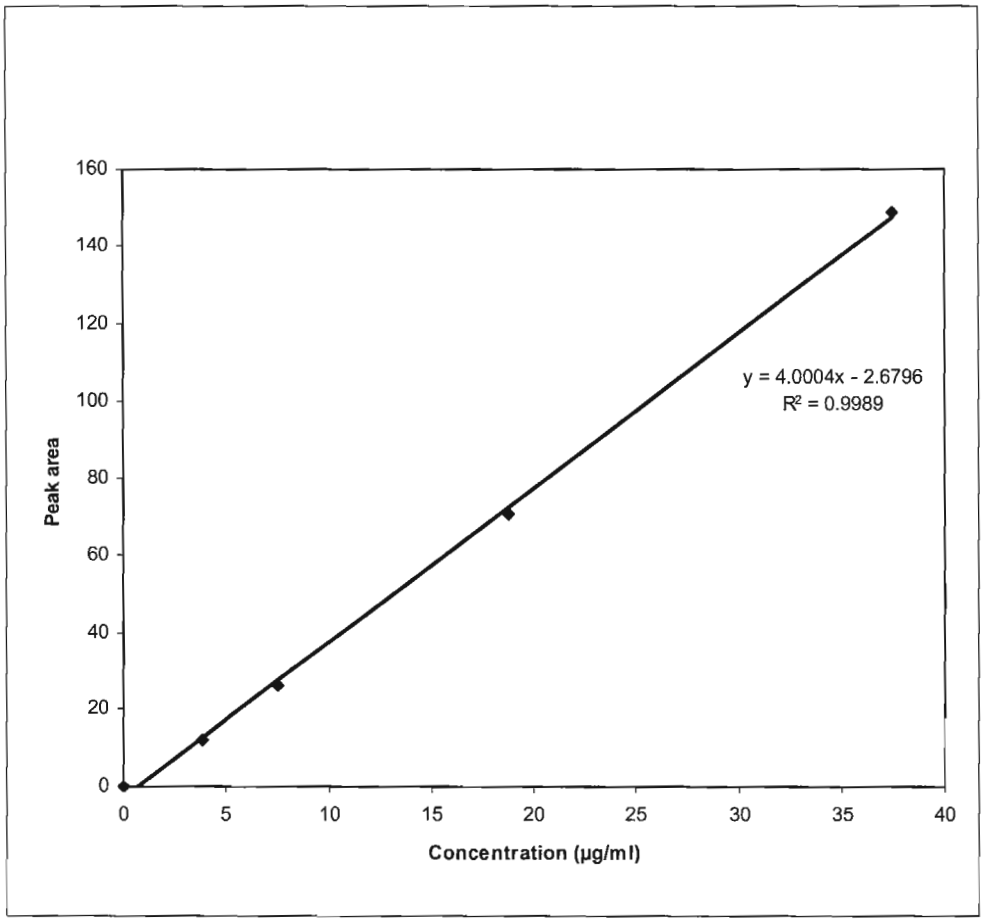


Figure G.13: Linear regression graph for 3-formyl rifamycin to determine Linearity and Range

G.12.2 Precision

Precision results for a 37.5 µg/ml 3-formyl rifamycin solution are given in Table G.29.

Table G.29: Results for 3-formyl rifamycin to determine Precision

3-Formyl rifamycin	Rifampicin	%
177.62	4867.65	3.6
179.89	4869.31	3.7
171.29	4873.65	3.5
175.05	4900.89	3.6
206.49	4830.34	4.3
196.21	4801.10	4.1
	Mean	3.8
	SD	0.1
	% RSD	1.8

Conclusion

(Acceptance criterion: The Percentage Relative Area must have an RSD of 5.0 % or less).

Precision was satisfactory with an RSD of 1.8 %.

G.12.3 Ruggedness

G.12.3.1 System Repeatability

A solution (representing 50 % of the related substance solution for 3-formyl rifamycin) was injected six times in order to test the repeatability of the peak area as well as the retention time. The results are presented in Table G.30.

Table G.30: Results for 3-formyl rifamycin to determine System Repeatability

	Area	Retention time (minutes)
	43.7	23.40
	40.3	23.57
	40.4	23.74
	42.1	23.83
	41.6	23.87
	42.5	23.57
Mean	41.8	23.66
SD	1.2	0.17
RSD %	2.9	0.70

Conclusion

(**Acceptance criterion:** The peak area should have an RSD of 5.0 % or less; and retention times should have an RSD of 2.0 % or less).

System performance proved well with RSD values of 2.9 % for peak area and 0.7 % for retention time respectively.

G.12.4 Limit of Detection and Quantitation

G.12.4.1 Limit of Detection

Experimental signal-to-noise ratios for 3-formyl rifamycin and the regression statistics thereof, are given in Tables G.31 and G.32, respectively. A linear regression graph is shown in Figure G.14.

Table G.31: Experimental signal-to-noise ratios for 3-formyl rifamycin at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
3.8	8
7.5	14
18.8	39

Table G.32: Regression statistics of 3-formyl rifamycin results

R squared	0.9969	Lower 95%	Upper 95%
Intercept	-0.7449	-18.3862	16.8963
Slope	2100.8243	617.1458	3584.5028

Theoretical signal-to-noise ratios for 3-formyl rifamycin and the regression statistics thereof, are given in Tables G.33 and G.34, respectively. A linear regression graph is shown in Figure G.15.

Table G.33: Theoretical signal-to-noise ratios for 3-formyl rifamycin at various concentrations

Concentration (µg/ml)	Signal-to-noise ratio (x:1)
1.0	3
5.0	10
3.8	8
7.5	14
18.8	39

Table G.34: Theoretical regression statistics of 3-formyl rifamycin results

R squared	0.9961	Lower 95%	Upper 95%
Intercept	0.0421	-2.1894	2.2736
Slope	2044.0345	1808.8486	2279.2203

Conclusion

(Acceptance criteria: The concentration determined as the Limit of Detection has a signal-to-noise ratio of 3:1).

A concentration of about 1.0 µg/ml 3-formyl rifamycin solution (representing 0.13 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 3:1.

G.12.4.2 Limit of Quantitation

Conclusion

(Acceptance criteria: The concentration determined as the Limit of Quantitation has a signal-to-noise ratio of 10:1).

A concentration of about 5.0 µg/ml 3-formyl rifamycin solution (representing 0.7 % of the expected rifampicin concentration) will produce a signal-to-noise ratio of 10:1.

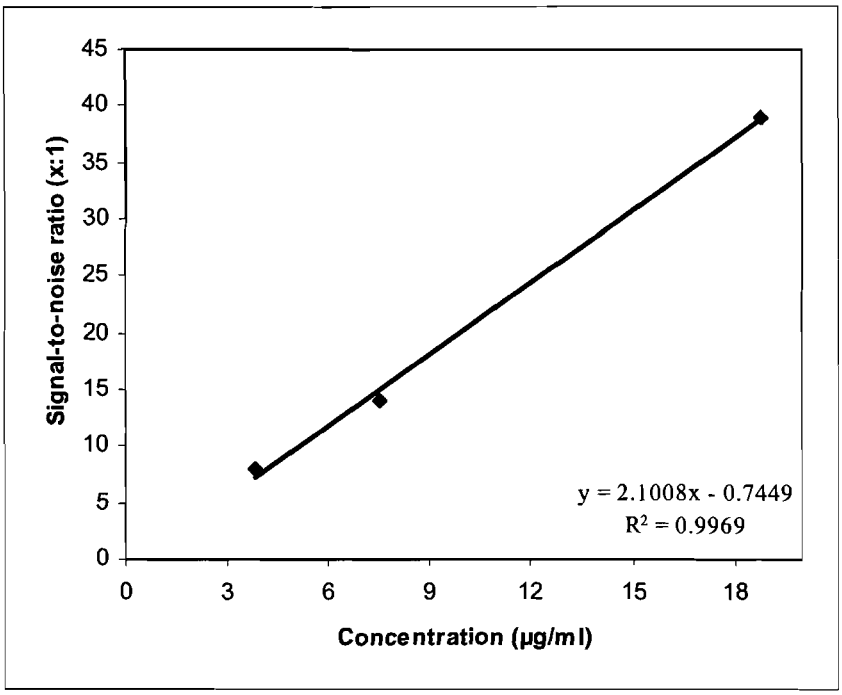


Figure G.14: Experimental linear regression graph for 3-formyl rifamycin to determine the LOD and LOQ

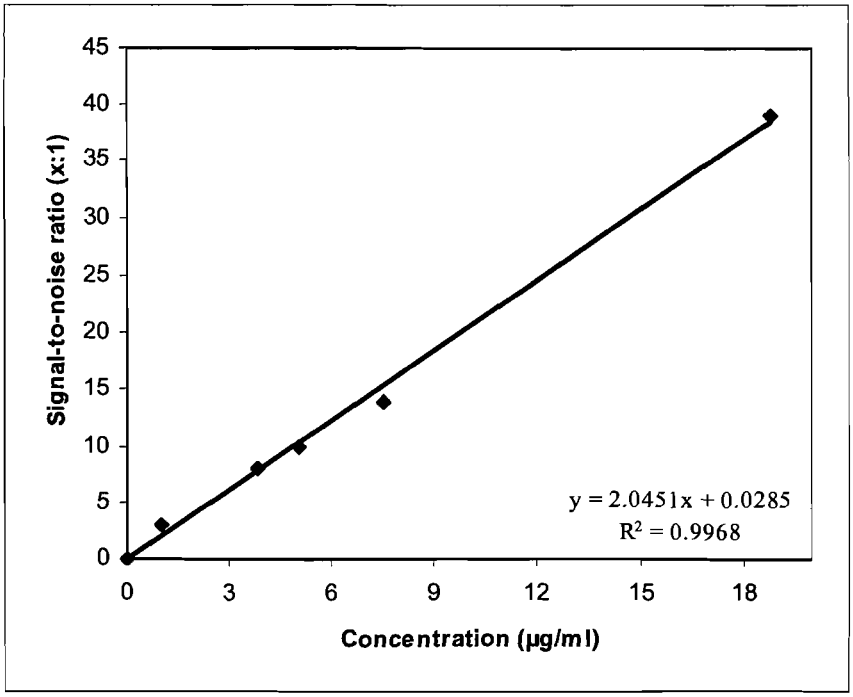


Figure G.15: Theoretical linear regression graph for 3-formyl rifamycin to determine the LOD and LOQ

G.13. CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Reference: USP (2006:2646)

Retention time (minutes):

Rifampicin N-oxide	± 7.3 minutes
Rif-iso hydrazone	± 14.4 minutes
Rifampicin-quinone	± 18.8 minutes
3-Formyl rifamycin	± 23.7 minutes
Rifampicin	± 28 minutes

Number of theoretical plates (N) plates/column (5 sigma method):

Rifampicin N-oxide	1 245
Rif-iso hydrazone	1 416
Rifampicin-quinone	2 340
3-Formyl rifamycin	11 276
Rifampicin	2 648

USP Tailing factor (T):

3-Formyl rifamycin	1.116
Rifampicin	1.321

Capacity factor (k'):

3-Formyl rifamycin	12.611
Rifampicin	14.470

Resolution between peaks (R):

3-Formyl rifamycin	-
Rifampicin	2.037

G.14. SYSTEM SUITABILITY PARAMETERS

* Inject the standard solution six times.
* Calculate the relative standard deviation of the peak areas obtained.
* Calculate the number of theoretical plates.
* Use the 5-sigma method to calculate the parameters in G.13.

The system is suitable to perform the analysis if the following criteria are met:

* RSD of 6 injections not more than 3.0 %.
* The column must have more than 1 800 theoretical plates for rifampicin.
* The resolution between the peaks of 3-formyl rifamycin and rifampicin must be at least 1.5.
* The USP tailing must be less than 1.5.

G.15. CONCLUSION

The method performed well and should be suitable to analyse the related substances in products for stability testing, quality control and batch release purposes.

APPENDIX H

Monograph on Rifampicin Capsules:

Final Text Approved for

The International Pharmacopoeia

Document QAS/04.113/FINAL
March 06

World Health Organization

RIFAMPICIN CAPSULES

Final text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Rifampicin capsules should be kept in a tightly closed container, protected from light.

Additional information. Strength in the current WHO Model list of essential medicines :150 mg, 300 mg.

Requirements

Comply with the monograph for "Capsules".

Definition Rifampicin capsules contain Rifampicin. They contain not less than **90.0%** and not more than **110.0%** of the amount of $C_{43}H_{58}N_4O_{12}$ stated on the label.

Identity tests

- Either test A alone or any two of tests B, C and D may be applied.

A. To a quantity of the contents of the capsules equivalent to about 20 mg of Rifampicin add 5 ml of dichloromethane R and shake. Filter and evaporate the filtrate to dryness. Carry out the examination with the residue as described under 1.7 Spectrophotometry in the infrared region. The infrared absorption spectrum is concordant with the spectrum obtained from rifampicin RS or with the *reference spectrum* of rifampicin.

B. To a quantity of the contents of the capsules equivalent to 1 mg of Rifampicin add 3 ml of water, shake and filter. To the filtrate add 3 drops of copper(II) sulfate (160 g/l) TS, shake and heat to boiling; a violet colour is produced.

C. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 85 volumes of dichloromethane R and 15 volumes of methanol R as the mobile phase. Apply separately to the plate 3 µl of each of the following 2 solutions in dichloromethane R. For solution (A) shake a quantity of the contents of the capsules equivalent to about 50 mg Rifampicin with 5 ml of dichloromethane R and filter. Solution (B) contains 10 mg of rifampicin RS per ml. After

removing the plate from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in daylight.
The principal spot obtained with solution A corresponds in position, appearance, and intensity to that obtained with solution B.

D. See Assay method A described below. The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

Related substances

Carry out the test as described under 1.14.4 High-performance liquid chromatography, preparing the solutions and using the conditions given below under Assay method A.

Inject 10 µl of solution (4). The test is not valid unless the resolution between the peaks is at least 1.5.

Inject 10 µl each of solutions (1), (3) and (5).

In the chromatogram obtained with solution (5) the following peaks are eluted at the following relative retention with reference to rifampicin (approximate retention time 25 minutes): rifampicin N-oxide about 0.3; rifampicin quinone about 0.7; 3-formylrifamycin about 0.9.

Note: The means of identifying the impurity peaks is subject to confirmation.

In the chromatogram obtained with solution (1), the area of any peak corresponding to rifampicin quinone is not greater than that the area of the principal peak in the chromatogram obtained with solution (3) (4.0%), the area of any peak corresponding to rifampicin N-oxide is not greater than 0.375 times the area of the principal peak in the chromatogram obtained with solution (3) (1.5%) and the area of any peak corresponding to 3-formylrifamycin is not greater than 0.125 times the area of the principal peak in the chromatogram obtained with solution (3) (0.5%). The area of any other peak is not greater than 0.25 times the area of the principal peak obtained with solution C (1.0%). The sum of the areas of all the peaks, other than the principal peak, is not greater than 1.5 times the area of the peak in the chromatogram obtained with solution (3) (6.0%). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with solution (3) (0.4%) and any peak with relative retention less than 0.23 with reference to rifampicin.

Assay

- Either method A or method B may be applied. For either method, prepare fresh solutions and perform the assay without delay. Low-actinic glassware is recommended.

A. Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1]. As the mobile phase, use a mixture of 6 volumes of methanol R and 4 volumes of phosphate buffer pH 7.0

[1] Luna® is suitable

(potassium dihydrogen phosphate R (0.01 mol/l), adjusted with sodium hydroxide (0.1 mol/l)VS).

Prepare the following solutions in methanol R. For solution (1) weigh and mix the contents of 20 capsules, shake a quantity equivalent to about 0.15 g Rifampicin, accurately weighed, in 100 ml methanol R, filter and dilute 5 ml to 10 ml with methanol R. For solution (2) use 0.75 mg rifampicin RS per ml. For solution (3) dilute a suitable volume of solution (1) to obtain a concentration equivalent to 30 µg Rifampicin per ml. Solution (4) contains 0.375 mg rifampicin RS per ml and 0.375 mg 3-formylrifamycin RS per ml. solution (5) contains ...mg of rifampicin impurity RS per ml. ***Note: see under test for Related substances above***

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 10 µl of solution (4). The assay is not valid unless the resolution between the peaks is at least 1.5.

Inject alternately 10 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$.

B Weigh and mix the contents of 20 capsules and transfer a quantity equivalent to about 0.10 g of Rifampicin, accurately weighed, to a 100-ml volumetric flask, add about 80 ml methanol R and shake. Dilute to volume with methanol R, mix, filter and discard the first 20 ml of the filtrate. Dilute 2 ml of the filtrate to 100 ml with phosphate buffer, pH 7.4, TS. Measure the absorbance of the resulting solution in a 1-cm layer at the maximum at about 475 nm, using as the blank phosphate buffer, pH 7.4, TS. Calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$, using the absorptivity value of 18.7 ($A^{1\%}_{1\,cm} = 187$).

Dissolution test. To be added.

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APPENDIX I

Monograph on Rifampicin Tablets:

Final Text Approved for

The International Pharmacopoeia

Document QAS/04.112/FINAL
March 06

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

RIFAMPICIN TABLETS

Text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Rifampicin tablets should be kept in a tightly closed container, protected from light.

Additional information. Strength in the current WHO Model list of essential medicines :150 mg, 300 mg. Rifampicin tablets are usually coated.

Requirements

Comply with the monograph for "Tablets".

Definition Rifampicin tablets contain Rifampicin. They contain not less than **90.0%** and not more than **110.0%** of the amount of $C_{43}H_{58}N_4O_{12}$ stated on the label.

Identity tests

- Either test A alone or any two of tests B, C and D may be applied.

A. To a quantity of the powdered tablets equivalent to about 20 mg of Rifampicin add 5 ml of dichloromethane R and shake. Filter and evaporate the filtrate to dryness. Carry out the examination with the residue as described under 1.7 Spectrophotometry in the infrared region. The infrared absorption spectrum is concordant with the spectrum obtained from rifampicin RS or with the *reference spectrum* of rifampicin.

B. To a quantity of the powdered tablets equivalent to 1 mg of Rifampicin add 3 ml of water, shake and filter. To the filtrate add 3 drops of copper(II) sulfate (160 g/l) TS, shake and heat to boiling; a violet colour is produced.

C. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 85 volumes of dichloromethane R and 15 volumes of methanol R as the mobile phase. Apply separately to the plate 3 µl of each of the following 2 solutions in dichloromethane R. For solution (A) shake a quantity of the powdered tablets equivalent to about 50 mg Rifampicin with 5 ml of dichloromethane R and filter. Solution (B) contains 10 mg of rifampicin RS per ml. After removing the plate

from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in daylight.
The principal spot obtained with solution A corresponds in position, appearance, and intensity to that obtained with solution B.

D. See Assay method A described below. The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

Related substances Carry out the test as described under 1.14.4 High-performance liquid chromatography, preparing the solutions and using the conditions given below under Assay method A.

Inject 10 µl of solution (4). The test is not valid unless the resolution between the peaks is at least 1.5.

Inject 10 µl each of solutions (1), (3) and (5).

In the chromatogram obtained with solution (5) the following peaks are eluted at the following relative retention with reference to rifampicin (approximate retention time 25 minutes.): rifampicin N-oxide about 0.3; rifampicin quinone about 0.7; 3-formylrifamycin about 0.9.
Note: The means of identifying the impurity peaks is subject to confirmation.

In the chromatogram obtained with solution (1), the area of any peak corresponding to rifampicin quinone is not greater than the area of the principal peak in the chromatogram obtained with solution (3) (4.0%), the area of any peak corresponding to rifampicin N-oxide is not greater than 0.375 times the area of the principal peak in the chromatogram obtained with solution (3) (1.5%) and the area of any peak corresponding to 3-formylrifamycin is not greater than 0.125 times the area of the principal peak obtained with solution (3) (0.5%). The area of any other peak is not greater than 0.25 times the area of the principal peak obtained with solution (3) (1.0%). The sum of the areas of all the peaks, other than the principal peak, is not greater than 1.5 times the area of the peak in the chromatogram obtained with solution (3) (6.0%). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with solution (3) (0.4%) and any peak with relative retention less than 0.23 with reference to rifampicin.

Assay

- Either method A or method B may be applied. For either method, prepare fresh solutions and perform the assay without delay. Low-actinic glassware is recommended.

A. Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1]. As the mobile phase, use a mixture of 6 volumes of methanol R and 4 volumes of phosphate buffer pH 7.0 (potassium dihydrogen phosphate R (0.01 mol/l), adjusted with sodium hydroxide (0.1 mol/l)VS).

[1] Luna® is suitable

Prepare the following solutions in methanol R. For solution (1) weigh and powder 20 tablets, shake a quantity of the powder equivalent to about 0.15 g Rifampicin, accurately weighed, in 100 ml methanol R, filter and dilute 5 ml to 10 ml with methanol R. Solution (2) contains 0.75 mg rifampicin RS per ml. For solution (3) dilute a suitable volume of solution (1) to obtain a concentration equivalent to 30 µg Rifampicin per ml. Solution (4) contains 0.375 mg rifampicin RS per ml and 0.375 mg 3-formylrifamycin RS per ml. Solution (5) contains ...mg of rifampicin impurity RS per ml. ***Note: see under test for Related substances above***

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 10 µl of solution (4). The assay is not valid unless the resolution between the peaks is at least 1.5.

Inject alternately 10 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$.

B. Weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to about 0.10 g of Rifampicin, accurately weighed, to a 100-ml volumetric flask, add about 80 ml methanol R and shake. Dilute to volume with methanol R, mix, filter and discard the first 20 ml of the filtrate. Dilute 2 ml of the filtrate to 100 ml with phosphate buffer, pH 7.4, TS. Measure the absorbance of the resulting solution in a 1-cm layer at the maximum at about 475 nm, using as the blank phosphate buffer, pH 7.4, TS. Calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$, using the absorptivity value of 18.7 ($A^{1\%}_{1\,cm} = 187$).

Dissolution test. To be added.

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APPENDIX J

Monograph on Rifampicin and Isoniazid Tablets:

Final Text Approved for

The International Pharmacopoeia

Document QAS/04.114/FINAL
March 06

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

RIFAMPICIN AND ISONIAZID TABLETS

Final text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Rifampicin and Isoniazid tablets should be kept in a tightly closed container, protected from light.

Additional information. Strengths in the current WHO Model list of essential medicines:
60 mg Rifampicin and 30 mg Isoniazid.
150 mg Rifampicin and 75 mg Isoniazid.
300 mg Rifampicin and 150 mg Isoniazid.
60 mg Rifampicin and 60 mg Isoniazid.
150 mg Rifampicin and 150 mg Isoniazid.
Rifampicin and Isoniazid tablets are usually film-coated.

Requirements

Comply with the monograph for "Tablets".

Definition Rifampicin and Isoniazid tablets contain Rifampicin and Isoniazid. They contain not less than **90.0%** and not more than **110.0%** of the amounts of rifampicin ($C_{43}H_{58}N_4O_{12}$) and isoniazid ($C_6H_7N_3O$) stated on the label.

Identity tests

- Either tests A and B or test C may be applied.

A. See Assay method A described below. The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

B. SeeAssay method B the test described below. The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

C. Carry out test C.1. or, where UV detection is not available, test C.2.

C.1.Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 100 volumes of methanol R and 1.5

volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and a proportional quantity (according to the ratio in the tablet) of rifampicin RS, per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in ultraviolet light (254 nm).

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

C.2. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and a proportional quantity (according to the ratio in the tablet) of rifampicin RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in daylight.

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

Rifampicin-related substances Carry out the test as described under 1.14.4 High-performance liquid chromatography, using the conditions given below under Assay method B.

Inject separately 10 µl each of solutions (1), (3) and (5).

In the chromatogram obtained with solution (5) the following peaks are eluted at the following relative retention with reference to rifampicin (retention time about 25 minutes.): rifampicin N-oxide about 0.3; 3-(isonicotinoylhydrazinomethyl)rifamycin [the "hydrazone" resulting from reaction between 3-formylrifamycin and isoniazid] about 0.5; rifampicin quinone about 0.7. The test is not valid unless the resolution between the peaks corresponding to rifampicin and rifampicin quinone is at least 4. ***Note: The means of identifying the impurity peaks is subject to confirmation.***

In the chromatogram obtained with solution (1), the area of any peak corresponding to the hydrazone impurity is not greater than the area of the principal peak in the chromatogram obtained with solution (3) (5.0%) , the area of any peak corresponding to rifampicin quinone is not more than 0.8 times the area of the principal peak in the chromatogram obtained with solution (3) (4.0%) and the area of any other peak is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with solution (3) (1.5%). The sum of the areas of all the peaks, other than the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with solution (3) (10.0%). Disregard any peak

with an area less than 0.02 times the area of the principal peak in the chromatogram obtained with solution (3) (0.1%) and any peak with a relative retention less than 0.23 with reference to rifampicin.

Assay

A. ***For isoniazid*** Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (15 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1]. As the mobile phase, use a solution prepared as follows: dissolve 50 g ammonium acetate R in 1000 ml of water and adjust to pH 5.0 with glacial acetic acid R. Mix 940 ml of this solution with 60 ml methanol R.

Prepare the following solutions in water. For solution (1) weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to about 30 mg Isoniazid, accurately weighed, to a 500 ml volumetric flask. Dissolve in about 400 ml water by shaking for about 15 minutes. [If foaming occurs, use 400 ml of a 4% solution of methanol R in place of the water.] Dilute to 500 ml with water. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtered solution. For solution (2) dissolve 30 mg isoniazid RS in 500 ml water.

Operate with a flow rate of 2.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 240 nm.

Note from Secretariat: EC agreed to delete as covered in general method text 1.14.4.

Inject alternately 20 µl each of solutions (1) and (2). The peak for isoniazid is eluted at a retention time of approximately 1.6 minutes.

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of isoniazid, $C_6H_7N_3O$.

B. ***For rifampicin*** Prepare fresh solutions and perform the assay without delay. Low-actinic glassware is recommended.

Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[2]. As the mobile phase, use a mixture of 6 volumes of methanol R and 4 volumes of phosphate buffer pH 7.0 (potassium dihydrogen phosphate R (0.01 mol/l), adjusted with sodium hydroxide (0.1 mol/l)VS).

Prepare the following solutions in methanol R. For solution (1) weigh and powder 20 tablets. Without delay shake a quantity of the powder equivalent to about 0.15 g Rifampicin, accurately weighed, in 100 ml methanol R, filter and dilute 5 ml to 10 ml

[1] Luna® is suitable
[2] Luna® is suitable

with methanol R. For solution (2) use 0.75 mg rifampicin RS per ml. For solution (3) dilute a suitable volume of solution (1) to obtain a concentration equivalent to 37.5 µg Rifampicin per ml. Solution (4) contains 0.375 mg rifampicin RS per ml and 0.375 mg rifampicin quinone RS per ml. Solution (5) contains ...mg of rifampicin impurity RS per ml. ***Note: see under test for Rifampicin-related substance above***

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 10 µl of solution (4). The assay is not valid unless the resolution between the peaks is at least 4.

Inject alternately 10 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Dissolution test. To be added for rifampicin.

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APPENDIX K

Monograph on Isoniazid and Ethambutol Hydrochloride Tablets:

Final Text Approved for The International Pharmacopoeia

Document QAS/04.094/FINAL
March 06

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

ISONIAZID AND ETHAMBUTOL HYDROCHLORIDE TABLETS

Final text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Isoniazid and Ethambutol hydrochloride tablets should be kept in a tightly closed container.

Additional information. Strength in the current WHO Model list of essential medicines: 150 mg Isoniazid and 400 mg Ethambutol hydrochloride. Isoniazid and Ethambutol tablets are usually film-coated.

Requirements

Comply with the monograph for "Tablets".

Definition Isoniazid and Ethambutol hydrochloride tablets contain Isoniazid and Ethambutol hydrochloride. They contain not less than **90.0%** and not more than **110.0%** of the amounts of isoniazid ($C_6H_7N_3O$) and ethambutol hydrochloride ($C_{10}H_{24}N_2O_2$, 2HCl) stated on the label.

Identity tests

- Either test A or test B may be applied.

A. See the Assay described below. The retention times of the two principal peaks in the chromatogram obtained with solution (1) correspond to those in the chromatogram obtained with solution (2).

B. Carry out test B.1, or, where UV detection is not available, test B.2.

B.1 Carry out the test as described under 1.14.1 Thin-layer chromatography , using silica gel R6 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and 2.67 mg ethambutol hydrochloride RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with

iodine vapours. and allow to stand for 20 minutes. Examine the chromatogram immediately in ultraviolet light (254 nm).
The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

B.2.Carry out the test as described under 1.14.1 Thin-layer chromatography , using silica gel R5 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and 2.67 mg ethambutol hydrochloride RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in daylight.
The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

Dissolution/Disintegration

- Either test A or test B may be applied

A. **Dissolution.** Carry out the test as described under 5.5 Dissolution test for solid oral dosage forms, using as the dissolution medium. 500 ml of dissolution buffer. pH 6.8, TS and rotating the paddle at 75 revolutions per minute. At 30 minutes withdraw a sample of 10 ml of the medium through an in-line filter. Allow the filtered sample to cool to room temperature and dilute 5 ml to 20 ml with water [solution (3)]. Determine the content of isoniazid, $C_6H_7N_3O$. and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2$. 2HCl as described below under Assay using solution (3) in place of solution (1).

For each of the six tablets tested, calculate the total amount of isoniazid, $C_6H_7N_3O$, and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2$. 2HCl in the medium from the results obtained. For both substances the average amount in solution is not less than 85% of the amount declared on the label. For either substance, if the amount of obtained for one of the six tablets is less than 80 %, repeat the test using a further six tablets: the average amount for all 12 tablets tested is not less than 85%.

B. **Disintegration.** Comply with 5.4 Disintegration test for tablets and capsules, operating the apparatus for 15 minutes. If the tablets do not comply, carry out test A above.

Assay Determine by 1.14.4 High-performance liquid chromatography , using a stainless steel column (15 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1]. As the mobile phase, use a solution prepared as follows: dissolve 50 g ammonium acetate R and 0.2 g copper(II) acetate R in 1000 ml of water and adjust to pH 5.0 with glacial acetic acid R. Mix 940 ml of this solution with 60 ml methanol R.

[1] Luna® is suitable

Prepare the following solutions in water. For solution (1) weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to about 100 mg Ethambutol hydrochloride, accurately weighed, to a 500 ml volumetric flask. Dissolve in about 400 ml water by shaking for about 15 minutes. [If foaming occurs, use 400 ml of a 4% solution of methanol R in place of the water.] Dilute to 500 ml with water. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtered solution. For solution (2) dissolve 100 mg ethambutol hydrochloride RS and 37.5 mg isoniazid RS in 500 ml water.

Operate with a flow rate of 2.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 270 nm.

Inject alternately 20 µl each of solutions (1) and (2). (The peak for isoniazid is eluted at a retention time of approximately 1.6 minutes, and that for ethambutol hydrochloride at a retention time of approximately 6 minutes.)

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of isoniazid, $C_6H_7N_3O$ and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2$, 2HCl.

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APPENDIX L

Monograph on Rifampicin, Isoniazid and Pyrazinamide Tablets:

Final Text Approved for The International Pharmacopoeia

Document QAS/04.115/FINAL
March 06

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE TABLETS

Final text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Rifampicin, Isoniazid and Pyrazinamide tablets should be kept in a tightly closed container, protected from light.

Additional information. Strengths in the current WHO Model list of essential medicines:
60 mg Rifampicin, 30 mg Isoniazid and 150 mg Pyrazinamide.
150 mg Rifampicin, 75 mg Isoniazid and 400 mg Pyrazinamide.
150 mg Rifampicin, 150 mg Isoniazid and 500 mg Pyrazinamide.
Rifampicin, Isoniazid and Pyrazinamide tablets are usually film-coated.

Requirements

Comply with the monograph for "Tablets".

Definition Rifampicin, Isoniazid and Pyrazinamide tablets contain Rifampicin, Isoniazid and Pyrazinamide They contain not less than **90.0%** and not more than **110.0%** of the amounts of rifampicin ($C_{43}H_{58}N_4O_{12}$), isoniazid ($C_6H_7N_3O$) and pyrazinamide ($C_5H_5N_3O$) stated on the label.

Identity tests

- Either tests A and B or test C may be applied.

A. See Assay method A described below. The retention times of the two principal peaks in the chromatogram obtained with solution (1) correspond to those of the principal peaks in the chromatogram obtained with solution (2).

B. See Assay method B described below. The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

C. Carry out test C.1. or, where UV detection is not available, test C.2.

C.1.Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and proportional quantities (according to the ratio in the tablet) of rifampicin RS and pyrazinamide RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air and examine the chromatogram in ultraviolet light (254 nm).

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

C.2. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and proportional quantities (according to the ratio in the tablet) of rifampicin RS and pyrazinamide RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in daylight.

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

Rifampicin-related substances Carry out the test as described under 1.14.4 High-performance liquid chromatography, using the conditions given below under Assay method B.

Inject alternately 10 µl each of solutions (1), (3) and (5).

In the chromatogram obtained with solution (5) the following impurity peaks are eluted at the following relative retention with reference to rifampicin (retention time about 25 minutes.): rifampicin N-oxide about 0.3; 3-(isonicotinoylhydrazinomethyl)rifamycin [the "hydrazone" resulting from reaction between 3-formylrifamycin and isoniazid] about 0.5; rifampicin quinone about 0.7.
Note: The means of identifying the impurity peaks is subject to confirmation.

In the chromatogram obtained with solution (1), the area of any peak corresponding to the hydrazone impurity is not greater than the area of the principal peak in the chromatogram obtained with solution (3) (5.0%), the area of any peak corresponding to rifampicin quinone is not greater than 0.8 times the area of the principal peak in the chromatogram obtained with solution (3) (4.0 %) and the area of any other peak is not greater than 0.3 times the area of the

principal peak in the chromatogram obtained with solution (3) (1.5%). The sum of the areas of all the peaks, other than the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with solution (3) (10.0%). Disregard any peak with an area less than 0.02 times the area of the principal peak in the chromatogram obtained with solution (3)(0.1 %) and any peak with a relative retention less than 0.23 with reference to rifampicin.

Assay

A. ***For isoniazid and pyrazinamide*** Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (15 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1]. As the mobile phase, use a solution prepared as follows: dissolve 50 g ammonium acetate R in 1000 ml of water and adjust to pH 5.0 with glacial acetic acid R. Mix 940 ml of this solution with 60 ml methanol R.

Prepare the following solutions in water. For solution (1) weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to about 30 mg Isoniazid, accurately weighed, to a 500 ml volumetric flask. Dissolve in about 400 ml water by shaking for about 15 minutes. [If foaming occurs, use 400 ml of a 4% solution of methanol R in place of the water.] Dilute to 500 ml with water. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtered solution. For solution (2) dissolve 30 mg isoniazid RS and a proportional quantity (according to the ratio in the tablet) of pyrazinamide RS in 500 ml water.

Operate with a flow rate of 2.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 240 nm.
Inject 20 µl of solution (2). The assay is not valid unless the resolution between the isoniazid and pyrazinamide peaks, eluting in this order, is at least 2.

Inject alternately 20 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of isoniazid, $C_6H_7N_3O$ and pyrazinamide, $C_5H_5N_3O$.

B. ***For rifampicin***

Prepare fresh solutions and perform the assay without delay. Low-actinic glassware is recommended.

Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[2]. As the mobile phase, use a mixture of 6 volumes of methanol R and 4 volumes of phosphate buffer pH 7.0 (potassium dihydrogen phosphate R (0.01 mol/l), adjusted with sodium hydroxide (0.1 mol/l)VS).

[1] Luna® is suitable
[2] Luna® is suitable

Prepare the following solutions in methanol R. For solution (1) weigh and powder 20 tablets. Without delay shake a quantity of the powder equivalent to about 0.15 g Rifampicin, accurately weighed, in 100 ml methanol R. filter and dilute 5 ml to 10 ml with methanol R. Solution (2) contains 0.75 mg rifampicin RS per ml. For solution (3) dilute a suitable volume of solution (1) to obtain a concentration equivalent to 37.5 µg Rifampicin per ml. Solution (4) contains 0.375 mg rifampicin RS per ml and 0.375 mg rifampicin quinone RS per ml. Solution (5) contains ...mg of rifampicin impurity RS per ml. ***Note: see under test for Rifampicin-related substance above***

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 10 µl of solution (4). The assay is not valid unless the resolution between the peaks is at least 4.

Inject separately 10 µl of solution (2) in six replicate injections in the chromatographic system. The assay is not valid unless the relative standard deviation for peak areas of rifampicin is less than 2.0%.

Inject alternately 10 µl each of solutions (1) and (2). Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Dissolution test. To be added for rifampicin.

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APPENDIX M

Monograph on Rifampicin, Isoniazid, Pyrazinamide and Ethambutol Hydrochloride Tablets:

Final Text Approved for The International Pharmacopoeia

Document QAS/04.097/FINAL
March 06

World Health Organization

RIFAMPICIN, ISONIAZID, PYRAZINAMIDE AND ETHAMBUTOL HYDROCHLORIDE TABLETS

Final text for addition to *The International Pharmacopoeia*

This monograph was adopted at the Fortieth WHO Expert Committee on Specifications for Pharmaceutical Preparations in October 2005 for addition to the 4th edition of the International Pharmacopoeia.

Category. Antituberculosis drugs.

Storage. Rifampicin, Isoniazid, Pyrazinamide and Ethambutol hydrochloride tablets should be kept in a tightly closed container, protected from light.

Additional information. Strength in the current WHO Model list of essential medicines : 150 mg Rifampicin, 75 mg Isoniazid, 400 mg Pyrazinamide and 275 mg Ethambutol hydrochloride. The tablets are coated.

Requirements

Comply with the monograph for "Tablets".

Definition Rifampicin, Isoniazid, Pyrazinamide and Ethambutol hydrochloride tablets contain Rifampicin, Isoniazid, Pyrazinamide and Ethambutol Hydrochloride. They contain not less than **90.0%** and not more than **110.0%** of the amounts of rifampicin ($C_{43}H_{58}N_4O_{12}$), isoniazid ($C_6H_7N_3O$), pyrazinamide ($C_5H_5N_3O$) and ethambutol hydrochloride ($C_{10}H_{24}N_2O_2$, 2HCl) stated on the label.

Manufacture The manufacturing process and the product packaging are designed and controlled so as to minimize the moisture content of the tablets. They ensure that, if tested, the tablets would comply with a loss on drying limit of not more than 30 mg/g when determined by drying freshly powdered tablets to constant mass under vacuum at 60°.

Identity tests

- Either tests A and B or test C may be applied.

A. See Assay method A described below. The retention times of the three principal peaks in the chromatogram obtained with solution (1) correspond to those of the principal peaks in the chromatogram obtained with solution (2).

B. See the test described below under "Assay method B". The retention time of the principal peak in the chromatogram obtained with solution (1) corresponds to that of the principal peak in the chromatogram obtained with solution (2).

C. Carry out test C.1. or, where UV detection is not available, test C.2.

C.1. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and proportional quantities (according to the ratio in the tablet) of rifampicin RS, pyrazinamide RS and ethambutol hydrochloride RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in ultraviolet light (254 nm).

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

C.2. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 100 volumes of methanol R and 1.5 volumes of strong ammonia solution R as the mobile phase. Apply separately to the plate 5 µl of each of the following two solutions in methanol R. For solution (A) shake a quantity of the powdered tablets equivalent to about 5 mg Isoniazid for 15 minutes with 5 ml of methanol R, filter, and use the filtrate. For solution (B) use 1 mg isoniazid RS and proportional quantities (according to the ratio in the tablet) of rifampicin RS, pyrazinamide RS and ethambutol hydrochloride RS per ml of methanol R. After removing the plate from the chromatographic chamber, allow it to dry in a current of air, place in a chamber with iodine vapours, and allow to stand for 20 minutes. Examine the chromatogram immediately in daylight.

The principal spots obtained with solution A correspond in position, appearance and intensity to those obtained with solution B.

Rifampicin-related substances Carry out the test as described under 1.14.4 High-performance liquid chromatography, preparing the solutions and using the conditions given below under Assay method B.

Inject 20 µl of solution (4). The test is not valid unless the resolution between the peaks is at least 4.

Inject 20 µl each of solutions (1), (3) and (5).

In the chromatogram obtained with solution (5) the following impurity peaks are eluted at the following relative retention with reference to rifampicin (retention time about 25 minutes): rifampicin N-oxide about 0.3; 3-(isonicotinoylhydrazinomethyl) rifamycin [the "hydrazone" resulting from reaction between 3-formylrifamycin and isoniazid] about 0.5; rifampicin quinone about 0.7.

Note: The means of identifying the impurity peaks is subject to confirmation.

In the chromatogram obtained with solution (1), the area of any peak corresponding to the hydrazone impurity is not greater than the area of the principal peak in the chromatogram obtained with solution (3) (5.0%). the area of any peak corresponding to rifampicin quinone is not more than 0.8 times the area of the principal peak in the chromatogram obtained with solution (3) (4.0%) and the area of any other peak is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with solution (3) (1.5%). The sum of the areas of all the peaks, other than the principal peak. is not greater than twice the area of the principal peak obtained with solution (3) (10.0% with reference to the content of rifampicin). Disregard any peak with an area less than 0.02 times the area of the principal peak in the chromatogram obtained with solution (3) (0.1%) and any peak with relative retention time less than 0.23 with reference to rifampicin.

Assay

A. ***For isoniazid, pyrazinamide and ethambutol hydrochloride*** Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (15 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups, (5 µm)[1]. As the mobile phase, use a solution prepared as follows: dissolve 50 g ammonium acetate R and 0.2 g copper(II) acetate R in 1000 ml of water and adjust to pH 5.0 with glacial acetic acid R. Mix 940 ml of this solution with 60 ml methanol R.

Prepare the following solutions in water. For solution (1) weigh and powder 20 tablets. Transfer a quantity of the powder equivalent to about 100 mg Ethambutol hydrochloride. accurately weighed, to a 500 ml volumetric flask. Dissolve in about 400 ml water by shaking for about 15 minutes. [If foaming occurs, use 400 ml of a 4% solution of methanol R in place of the water.] Dilute to 500 ml with water. Filter a portion of this solution through a 0.45 µm filter, discarding the first few ml of the filtered solution. For solution (2) dissolve 27.3 mg isoniazid RS, 145.5 mg pyrazinamide RS and 100 mg ethambutol hydrochloride RS
in 500 ml water.

Operate with a flow rate of 2.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 270 nm.

Inject 20 µl of solution (2). The assay is not valid unless the resolution between the isoniazid and pyrazinamide peaks is at least 2. (The peaks elute in the order, isoniazid, pyrazinamide and ethambutol hydrochloride.)

Inject alternately 20 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of isoniazid. $C_6H_7N_3O$. pyrazinamide, $C_5H_5N_3O$ and ethambutol hydrochloride, $C_{10}H_{24}N_2O_2$, 2HCl.

B. ***For rifampicin***

Prepare fresh solutions and perform the assay without delay. Low-actinine glassware is recommended.

[1] Luna® is suitable

Determine by 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with particles of silica gel, the surface of which has been modified with chemically bonded octadecylsilyl groups, (5 µm)[2]. As the mobile phase, use a mixture of 6 volumes of methanol R and 4 volumes of phosphate buffer pH 7.0 (potassium dihydrogen phosphate R (0.01 mol/l), adjusted with sodium hydroxide (0.1 mol/l)VS).

Prepare the following solutions in methanol R. For solution (1) weigh and powder 20 tablets. Without delay, shake a quantity of the powder equivalent to about 150 mg Rifampicin in 100 ml methanol R, filter and dilute 5 ml to 10 ml with methanol R. Solution (2) contains 0.75 mg rifampicin RS per ml. For solution (3) dilute a suitable volume of solution (1) to obtain a concentration equivalent to 37.5 µg Rifampicin per ml. Solution (4) contains 0.375 mg rifampicin RS per ml and 0.375 mg rifampicin quinone RS per ml. Solution (5) contains …mg of rifampicin impurity RS per ml. ***Note: see under test for Rifampicin-related substance above***

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of about 254 nm.

Inject 20 µl of solution (4). The assay is not valid unless the resolution between the peaks is at least 4.

Inject alternately 20 µl each of solutions (1) and (2). Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of rifampicin, $C_{43}H_{58}N_4O_{12}$.

Dissolution test. ***To be added for rifampicin.***

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[2] Luna® is suitable

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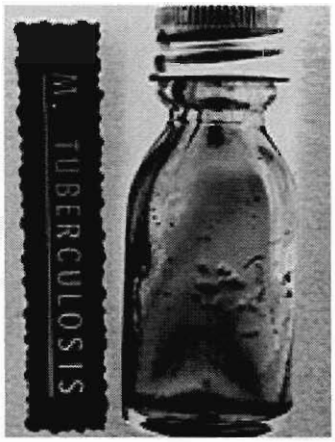
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