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A DETAIL STUDY ON ALBENDAZOLE AND RIFAMPCIN VIA SPECTROPHOTOMETRIC AND HPLC METHODS

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CHAPTER 1

INTRODUCTION

1.1 ANTHELMINTICS

Anthelmintics are substances used to cure infections with parasitic worms. They include flat worms (e.g., flukes, tapeworms) and round worms (e.g., nematodes). They are of huge importance for human tropical medicine and for veterinary medicine, which in therapeutics is an anthelmintic drug having a wide spectrum of activity. The World Health Organisation estimates that staggering 2 billion people harbour parasitic worm infections.

These also infect livestock and crops, affecting food production with a resultant economic impact. Also of importance is the infection of domestic pets. Indeed, the companion animal market is a major economic consideration for animal health companies undertaking drug discovery programs.

Epidemiology

Helminths are distributed globally and infection is most common and serious in poor countries. The distribution of helminth diseases is determined by climate, hygiene, diet and exposure to vectors.

Infection

The transmission of helminth diseases varies with the type of worms; it may involve ingestion of eggs or larvae, penetration by larvae, bite of vectors, or ingestion of stages in the meat of intermediate hosts. Worms are often long-lived.

Pathogenesis

Most infections are asymptomatic, pathologic manifestations depend on the size, activity and metabolism of the worms. Immune and inflammatory responses also cause pathology. Most patients are asymptomatic with the initial infection. Some patients will complain of abdominal pain, diarrohea, and fever as the worms mature in the

small intestine and penetrate through the intestinal wall. In severe (sometimes fatal) cases, larvae may invade heart muscle and brain tissue.

Host defences Antibody and cell-mediated responses are important for inflammation. Non-specific defence mechanisms limit susceptibility. Parasites survive defences through many evasion strategies.

Diagnosis

Diagnosis of helminth diseases in humans usually requires a medical history and physical examination, a laboratory analysis of stools, and sometimes other tests.

Prevention

It requires frequent washing of hands, frequent cleaning of bathrooms and kitchens and thorough cooking of the foods they infest mainly beef, pork, sausage and bear meat. Water supplies should be chlorinated, if possible.

Treatment

In most cases involves the use of highly effective anti-worm drugs known as vermifuges that kill the worms., clove, tansy tea, garlic, pineapple, honey, papaya and chive juice.

Characters of ideal anthelmintics

1) Orally active

2) Effective in single dose

3) Inexpensive

4) Wide safety margin between toxicity to worm and toxicity to host.

Classification of anthelmintics

Albendazole– effective for threadworms, roundworms, whipworms, tapeworms, hookworms. Mebendazole – effective for pinworms, roundworms and hookworms.

Diethylcarbamazine– effective for Wuchereria bancrofti, Brugia malayi, Brugia timori, tropical pulmonary eosinophilia, loiasis.

Pyrantel pamoate – effective for most nematode infections.

Thiabendazole – effective for roundworms, hookworms.

Fenbendazole – effective for gastrointestinal parasites.

Triclabendazole – effective for liver flukes.

Flubendazole – effective for most intestinal parasites.

Abamectin – effective for most common intestinal worms, except tapeworms, for which praziquantel is commonly used in conjunction with abamectin.

Niclosamide – effective for tapeworms.

Ivermectin– effective for most common intestinal worms (except tapeworms).

Praziquantel – effective for cestodes, some trematodes.

Octadepsipeptides (e.g. Emodepside)– effective against a variety of gastrointestinal helminths.

Aminoacetonitrile derivatives (e.g. Monepantel): effective against a variety of gastrointestinal helminths including those resistant to the other drug classes.

Based on the mode of action, Albendazole anthelmintic drugs have been chosen for the present study.

Albendazole: appears to cause cytoplasmic microtubular degeneration, which in turn impairs vital cellular processes and leads to parasite death. There is some evidence that the drug also inhibits helminth-specific ATP generation by fumarate reductase.

Albendazole has a broad spectrum of activity against intestinal nematodes and cestode, as well as the liver flukes. It is also effective in treating cerebral and spinal neurocysticercosis.

1.2 ANTI-TUBERCULOSIS AGENTS

Anti-Tuberculosis agents are drugs that are used to cure tuberculosis. Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis and characterized by the development of cellular allergies, specific granulomas in various organs and tissues and polymorphic clinical picture.

Mycobacterium tuberculosis or Koch's bacillus usually affects lungs, lymphatic system, bones, joints, genito-urinary organs, skin, eyes, and nervous system. Left untreated, the disease progresses and ends fatally. Tuberculosis is transmitted through inhalation of aerosolized droplets. TB mainly attacks the lungs, but can also affect other parts of the body.

TB is highly contagious during the active stage of the disease and can infect an individual through inhalation of as few as 10 Mycobacterium tuberculosis (MTB) bacteria. After inhalation, these bacteria are mainly captured by the alveolar macrophages, but they can evade the host immune system and remain in the dormant stage for a long period of time, at which point they can reactivate to a virulent form under immune-compromised conditions of the host.

This is possible because M. tuberculosis can persist in slow growing as well as in fast growing stages which makes treatment challenging. Almost all of the antibiotics that can be used to treat TB work when the bacteria are actively dividing.

In the intensive phase of TB treatment, the antibiotics mainly kill rapidly growing bacteria, which causes rapid sputum conversion, and the eradication of clinical symptoms. However, in order to kill the persistent or slow growing strains of MTB, the continuation phase of the treatment is essential.

TB can be treated effectively by using first line drugs isoniazid (INH), rifampin (RIF), pyrazinamide, ethambutol and streptomycin. However, this first line therapy often fails to cure TB for several reasons. Relapse and the spread of the disease contribute to the emergence of drug-resistant bacteria.

The emergence of multidrug resistant TB (MDR-TB), i.e. which is resistant to at least INH and RIF, is of great concern, because it needs the use of second-line drugs that are difficult to procure and are much more toxic and expensive than first line drugs.

Therefore, the detection and treatment of drug susceptible or single drug-resistant TB is an important strategy for preventing the emergence of MDR-TB.M. tuberculosis strains with extensively drug resistant-TB (XDR-TB), that is resistant to either INH or RIF (like MDR tuberculosis), any fluoroquinolone, and at least one of three second line antituberculosis injectable drugs i.e., capreomycin, kanamycin, and amikacin have also been reported.

Signs and symptoms

About 5–10% of those without HIV, infected with tuberculosis, develop active disease during their lifetimes. In contrast, 30% of that co infected with HIV develops active disease. General signs and symptoms are fever, chills, night sweats, appetite loss, weight loss, fatigue and finger clubbing may also occur.

Prevention

Tuberculosis prevention and control efforts primarily rely on the vaccination of infants with the Bacillus Calmette-Guérin (BCG) and the detection and appropriate treatment of active cases. The World Health Organization has achieved some success with improved treatment regimens, and a small decrease in case numbers.

Classification of Anti-Tuberculosis

First line drugs Isoniazid, rifampin, ethambutol, streptomycin and pyrazinamide.

Second line drugs Ethionamide, cycloserine, tetracycline, para-aminosalicylic acid, amikacin and fluoroquinolones.

Classification of Anti-tuberculosis drugs of the International Union against Tuberculosis and Lung Disease

I Group (preparations of high efficiency) Isoniazid, rifampicin

II Group (moderate efficiency) Cycloserine, ethionamide, streptomycin, kanamycin, viomycin, ethambutol, protionamide, pyrazinamide

III Group (low efficiency) Aminosalicylic acid, thioacetazone

Isoniazid (INH) is one of the most effective and specific anti-tuberculosis drugs, which was introduced in 1952. M. tuberculosis is highly susceptible to INH (MIC 0.02–0.2 μg/ml).

INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. INH enters the mycobacterial cell by passive diffusion. The most significant adverse reactions associated with INH administration are hepatotoxicity and neurotoxicity.

Rifampicin (RIF) was introduced in 1972 as an anti-tuberculosis drug and has excellent sterilizing activity. RIF acts by binding to the β-subunit of RNA polymerase (rpoB), the enzyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity and thereby killing the organism.

An important characteristic of RIF is that it is active against actively growing and slowly metabolizing (non-growing) bacilli. RIF produces relatively few adverse reactions. It may cause gastrointestinal upset. Hepatotoxicity occurs less frequently than with INH administration.

Ethionamide (ETN) is a derivative of isonicotinic acid and has been used as an anti-tuberculosis agent since 1956. ETN and the similar drug prothionamide act as pro-drugs, like INH . Once delivered into the bacterial cell, ETN undergoes several changes. Its sulfo group is oxidized by flavin monooxygenase, and the drug is then converted to 2-ethyl-4-aminopyridine. The intermediate products formed before 2-ethyl-4-aminopyridine seems to be toxic to mycobacteria, but their structures are unknown.

1.3 PHARMACEUTICALS

The drug is any material intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease. It is also called medicine, medication or medicament and can be defined as any chemical substance intended for use in the medical diagnosis, cure, treatment, or prevention of disease. A drug is any substance when taken into the body, alters the function of the body either physically and/or psychologically.

Pharmaceutical drugs may be used for a limited span, or on a regular basis for chronic disorders. There are many classes of drugs which will have different function on the body. In the ancient times, a large part of medicinal products used were natural products or herbs mostly derived from plants but in subsequent centuries it was agreed that such products must be pure for effective utilization as pharmaceutical substances.

Pharmaceutical analysis provides information on the identity, purity, content and stability of raw materials, excipients, active pharmaceutical ingredients (APIs) and also the secondary pharmaceutical product(s) i.e., the dosage forms having either single or multi-component formulated products. The quality of the drug product may deviate from the standard required but in carrying out an analysis one can confirm that whether the quality of the product is of the required standard or not.

The process of drug development involves a sequence of activities which are carried out together. Once a molecule is synthesized that has desirable biological activity, the process of creating a pharmaceutical drug product from this molecule begins. As toxicology and efficacy studies are undertaken, methods for manufacture of the active molecule and for its delivery in therapeutic doses are sought.

Critical to the latter effort is finding a form of the active molecule which exhibits appropriate physical properties. The form ultimately selected, called the active pharmaceutical ingredient (API), or drug substance or bulk material, must be stable and bio available enough to be formulated into a drug product, such as a tablet/capsule or syrup/suspension. This formulation must be effective at delivering the active molecule to the targeted bio system.

1.4 PHARMACEUTICAL ANALYSIS

Pharmaceutical industry requires increasing regulators of high quality and safety standards worldwide. To ensure that these standards are met, reliable analysis tools and methods are constantly required and developed by analytical scientists. Pharmaceutical analysis, therefore, plays a prominent role in advancing the concepts and theories of analytical chemistry, as well as providing important information on practical aspects of drug design, quality control, and quality assurance of industrial manufacturing.

The methods of pharmaceutical analysis are traditionally and commonly applied to the chemical analysis of drug molecules. However, in the last two decades, modern pharmaceutical analysis has evolved enormously, capitalizing on combination techniques, high-throughput technologies, chemometrics, and most recently miniaturization and nanotechnology.

The combination of several techniques allows the modern pharmaceutical analyst to exploit the virtues of each technique and, in turn, to improve the overall quality of analysis. Indeed, modern analytical techniques and methods offer the possibility of increasing the amount of information received from individual analysis, with reduced cost, analysis time, and sample volumes.

The purpose of analytical method

An analytical method describes the steps necessary to perform an analysis. The use of analytical method during the development and manufacturing provides the following information:

i) Potency, which can relate directly to the requirement of a known drug
ii) Impurities, which will relate with the safety of the drug.
iii) Key characteristic form such as crystallinity, drug release, etc.
iv) iv) Degradation products, to confirm the method is stability-indicating.

1.5 METHOD DEVELOPMENT

Pharmaceutical analysis plays an important role in quality assurance as well as quality control of bulk drugs and pharmaceutical dosage forms. Rapid increase in pharmaceutical industries and production of drug in various parts of the world has brought a rise in demand for new analytical techniques in the pharmaceutical industries.

As a consequence, analytical method development became the key role of any pharmaceutical drug development program. Effective method development confirms that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development.

Recent development in analytical methods has been resulted from the advancement of analytical instruments. The improvement of the analytical method development and analytical instruments have reduced the analysis time, increased precision and accuracy and reduced costs of analysis.

As a consequence, most of pharmaceutical companies are investing huge amount of money for the establishment of advanced analytical laboratories. Analytical techniques are developed and validated for active pharmaceutical ingredients, additives, bulk products, degradation products and related substances, residual solvents, etc. As a result, it has become an integral part of the requirements of the regulatory organization.

Analytical method development finally results in official test methods. These methods are used in quality control laboratories to ensure the identity, purity, safety, efficacy and performance of drug products. Regulatory authorities are placing greater emphasis on analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods.

1.6 METHODS OF PHARMACEUTICAL ANALYSIS

In recent years, pharmaceutical analysis is done by techniques, which include HPLC-UV, HPLC-NMR, HPLC-NMR-MS, GC-MS, LC-MS/MS, UPLC, FI-spectrofluorimetry, phosphorimetry, supercritical fluid chromatography (SFC) UV, voltammetry, etc. It is obvious that these techniques make possible a great many things that could not be accomplished previously using conventional methods.

They also make possible, in some cases, faster analysis than was possible with the chemical methods. Although modern analytical chemistry is dominated by sophisticated instrumentation, the basis of analytical chemistry and some of the principles used in modern instruments are from traditional techniques many of which are still used today.

There are many reasons for the persistence of the chemical types of analysis, can be summarized as follows: There are many chemical situations which are better handled by chemical, rather than instrumental methods. The broad spectrum of reactions available gives the analyst quite versatility. For e.g., we find that the analysis of complex systems relies heavily on conventional methods, since specific reactions are generally available for classes of organic compounds of pharmaceutical importance.

In addition, the area of trace analysis relies heavily on chemical methods to develop specific colors for the materials in question.

Another advantage of the conventional methods, particularly titrimetry, can be stated as follows: Most instrumental analyses are dependent on calibration curves which require pure samples of the compounds in question.

Titrimetry does not require such calibrations. Titration methods are still widely used in the analysis for the assay of pure drug. Its share in the European Pharmacopoeia is almost 70%, and more than 40% in United States Pharmacopoeia (USP) for the determination of low molecular weight organic compounds. Hence, this approach is the most practical when the analytical laboratory is faced with problem of pure samples. The cost of equipment of conventional analysis is generally quite low.

Furthermore, majority of the methods currently in use in pharmaceutical analysis, like HPLC, LC-MS, TLC, HPTLC, GC, GC-MS, capillary electrophoresis, etc., are purely physical methods. The use of purely physical methods in quantitative analysis in recent years has given rise to a concern among the academicians that there is a negligence of basic chemistry in analytical chemistry.

CHAPTER 2

LITERATURE REVIEW

Table 2.1: Literature review table

S.NO	AUTHOR	JOURNAL	YEAR
1.	E.A. Swinyard,	Remington's Pharmaceutical Sciences	1990
2.	L.M. Rollo	The Pharmacological Basis of Therapeutics	1991
3.	K.N. Rao, D. Subrahmanyam	Ind. J. Med. Res.	1992
4.	D.J. Vadodaria,et.al;	Indian J. Pharm	1995
5.	D. Bhaskar,et.al;	Int. J. Pharm. Bio. Sci.	1996
6.	R.G. Santosh, et.al;	Asian J. Pharm.	2000
7.	D. Bhoopathy et.al;	Asian J. Res. Chem.	2002
8.	J.M. Reddy et.al;	Indian J. Pharm. Sci.	2006
9.	M.I. Walash, et.al;	J. Assoc. Off. Anal. Chem.	2009
10.	. W. Jezzy, et.al;	J. Pharm. Biomed. Anal.	2010
11.	A. Wadhwa et.al;	Indian J. Chem. Sec.	2013
12.	M.G. Pateria, et.al;	J. Ind. Chem. Soc.	2014
13.	R. Saxena et.al;	J. Ind. Chem. Soc.	2015
14.	S.N. Nema, et.al;	J. Ind. Chem. Soc.	2015
15.	K.N. Prashanth, et.al;	Acta Pol. Pharm. Drug Res.	2018
16.	A.M.A. Sameer, et.al;	Int. J. Anal. Chem.	2020
17.	M.S. Raghu, et.al;	Chem. Soc. Ethiop.	2021

18.	K. Basavaiah, et.al;	Thai J. Pharm. Sci.	2021
19.	M.X. Cijo, et.al;	Chem. Ind. Chem. Eng. Q.	2021
20.	M.S. Raghu, et.al;	J. Assoc. Arab Univ. Basic Appl. Sci.	2022
21.	A.S. Douglas, et.al;	'Principles of Instrumental Analysis',	2022
22.	. K.B. Vinay, et.al;	Thai J. Pharm. Sci.	2022
23.	M.S. Raghu, et.al;	Proc. Natl. Acad. Sci. India, Sect. A. Phys. Sci.	2023
24.	K.B. Vinay, et.al;	Drug Test. Analysis,	2024
25.	A.A. Gouda,et.al;	J. Anal. Methods Chem.	2025

CHAPTER 3

AIM AND OBJECTIVES

3.1 The cure for disease caused by helminths is now possible with orally administrated anthelmintic drugs and tuberculosis by vaccination with Bacillus Calmette-Guérin (BCG) and prolonged oral drugs. There are several classes of anthelmintic and anti-tubercular drugs. Drawing each drug from each of anthelmintics and anti-tuberculosis drugs, the following two drugs were selected for the present study**.**

1)Albendazole (ALB)

2) Rifampicin (RIF)

An extensive survey of the analytical methods reported for the determination of the eight drugs in pharmaceuticals indicated that there existed a lot of scope for more and new methods for the determination.

The aim of the present work was to develop various sensitive and selective analytical methods, employing easily available chemicals and cost- effective techniques such as titrimetric (including aqueous and non-aqueous), UV/visible spectrophotometry and chromatography, for the assay of selected drugs.

3.2 OBJECTIVE OF THE PRESENT STUDY

1. To develop simple, sensitive and economical methods for the determination of aforementioned drugs in pharmaceuticals using titrimetric, HPLC to optimize the working conditions to maximize the performance characteristics.
2. To validate the developed methods for the accuracy and precision, sensitivity, selectivity, robustness and ruggedness, according to the current ICH guidelines. To ascertain accuracy further by recovery test via standard addition method.

3. To compare the developed methods with the existing methods with respect to performance characteristics.

3.3 METHOD VALIDATION

Analytical methods used in pharmaceutical analysis must be sufficiently accurate, specific, sensitive and precise to conform to the regulatory requirements as set out in the relevant ICH guidelines [36], which are applied by the licensing authorities and by pharmacopoeias. The ICH guideline is regarded as the basis and philosophical background to analytical validation.

The objective of validation of an analytical method is to demonstrate that it is suitable for its intended purpose. Following the ICH guideline, the developed methods were validated for linearity, sensitivity, precision, accuracy, robustness, ruggedness and selectivity.

CHAPTER 4

MATERIALS AND METHODS

4.1 DRUG PROFILE

Albendazole (ALB) is an anthelmintic drug, chemically known as methyl 5-propylthio 2-benzimidazole carbamate (Fig. 2.0.1) [1]. It has the molecular formula C12H15N3O2S and has the molecular weight 265.33 g mol-1.

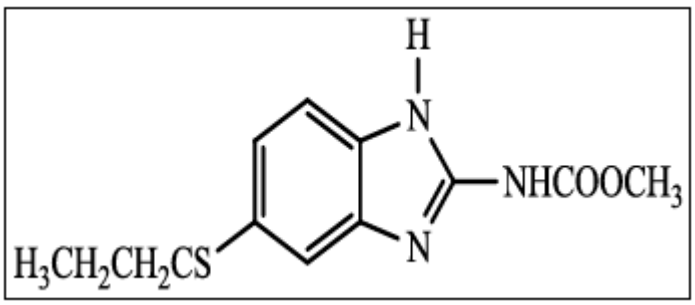

Figure 4.1: Chemical structure of albendazole.

4.2 PHYSICOCHEMICAL PROPERTIES

1) ALB is a white to yellowish, fluffy, odourless crystalline powder

2) It melts at 208-210 oC.

3) It is practically insoluble in water, soluble in dimethyl sulphide, alcohol, dichloromethane, chloroform, and glacial acetic acid.

4) ALB is a benzimidazole anthelmintic drug widely used in veterinary and human medicine for the treatment of infections caused by helminates.

5) In higher doses it is also used in the treatment of echinococcosis.

6) ALB is official in the United States Pharmacopoeia (USP) and British Pharmacopoeia (BP), which recommend non-aqueous titrimetric assay of the drug where the end point is located either visually using crystal violet indicator or potentiometrically using modified glass-SCE system.

4.3 LITERATURE SURVEY OF ANALYTICAL METHODS FOR ALBENDAZOLE Diverse analytical methods are found in the literature for the determination of ALB in pharmaceuticals.

Titrimetric methods

In addition to the official methods, a few titrimetric methods are available in the literature. Two groups of workers employed the non-aqueous titrimetric for the quantification of ALB in pharmaceuticals using acetous perchloric acid and sodium methylate in DMF medium [8] as titrants. Basavaiah and Prameela employed chloramine-T as an oxidimetric agent for the titrimetric assay of ALB. The same authors reported titrimetric and kinetic methods using bromate bromide mixture and methyl orange as reagents. The same group of workers has reported the application of N-Bromo succinimide (NBS) N chlorosuccinimide (NCS) and sodium periodate as oxidimetric titrants for the assay of ALB in pharmaceuticals.

4.4 Chromatographic methods

High performance liquid chromatographic (HPLC) technique has been employed for the determination of ALB in formulations either alone [29-32] or in combination with some other drugs [33]. But none of them is stability-indicating. A lone ultra-performance liquid chromatographic (UPLC) method has earlier been used for the assay of ALB in Turkey blood plasma [34], and is not stability-indicating despite many fold advantages of UPLC.

4.5 VALIDATED TITRIMETRIC AND SPECTROPHOTOMETRIC ASSAY OF ALBENDAZOLE USING CERIUM(IV), P-DIMETHYLAMINOBENZALDEHYDE AND O-DIANISIDINE AS REAGENTS

4.5.1 EXPERIMENTAL Apparatus

A Systronics model 166 digital spectrophotometer (Systronics Ltd., Ahmedabad, Gujarat, India) with 1-cm path length matched quartz cells was used to record the absorbance values.

4.5.2 Reagents and materials

All chemicals used were of analytical reagent grade. Double distilled water was used throughout the investigation.

Cerium (IV)sulphate (0.01M)

An approximately 0.01M solution was prepared by dissolving about 5.054 g of the chemical Ce(SO4)2.4H2O (LOBA Chemie Pvt. Ltd., Mumbai, India) in 0.5M sulphuric acid with the aid of heat, and diluted to 250 mL with the same acid, and standardized using standard ferrous ammonium sulphate solution; iron(II) [65]. It was used for titrimetric work and diluted to obtain working concentrations of 300 µg mL-1 Ce(IV) with 4M perchloric acid (method B) and 100 µg mL-1 with 0.5M H2SO4 (method C).

Perchloric acid (4M)

Perchloric acid (4M) was prepared by diluting appropriate volume of commercially available acid (70%; Merck Ltd., Mumbai, India) with water.

Sulphuric acid [H2SO4] (5M)

A 5M H2SO4 was prepared by appropriate dilution of concentrated acid (98%; Sp. gr. 1.84, Merck Ltd., Mumbai, India) with water, used in both methods B and C.

Ferrous ammonium sulphate [iron(II)] solution (0.01M)

About 3.92 g of [NH4]2[Fe][SO4]2·6H2O (Loba Chemie Ltd., Mumbai, India) was dissolved in 10 mL of 0.5M H2SO4 in a 1000 mL volumetric flask and volume was brought up to the mark with water, and standardized with a solution of pure potassium dichromate.

Ferroin indicator

Prepared by dissolving 0.742 g of 1,10-phenanthroline monohydrate (Qualigens Fine Chemicals, Mumbai, India, assay 100%) in 50 mL of 0.025M ferrous sulphate (Merck Ltd., Mumbai, India) solution (0.348 g of ferrous sulphate heptahydrate in 50 mL water).

p-Dimethylaminobenzaldehyde [DMAB] (0.5%)

An accurately weighed 0.25 g of DMAB (Merck Ltd., Mumbai, India) was transferred to a 50 mL volumetric flask, dissolved in 4M perchloric acid and the volume was made up to the mark with the same solvent.

o-Dianisidine [ODA] (0.05%)

An accurately weighed 25 mg of reagent (Merck Ltd., Mumbai, India) was transferred to a 50 mL volumetric flask, dissolved in ethanol and the volume was made up to the mark with the same solvent.

Acetic acid (3:2) solution

Acetic acid:water (3:2) mixture was prepared by mixing 300 mL of glacial acetic acid (Merck Ltd., Mumbai, India) with 200 mL water.

Standard ALB solution

Pharmaceutical grade ALB (99.7% pure) was procured from Cipla India, Ltd., Mumbai, as a gift and used as received. A stock standard solution equivalent to 1 mg mL-1 ALB was prepared by dissolving 100 mg of pure drug in 3:2 acetic acid in a 100 mL calibrated flask. This solution was used in the titrimetric work and a working concentration of 20 µg mL-1 was obtained by step wise dilution with same acid and used in method C. For method B, 200 µg mL-1 ALB solution was prepared separately by dissolving accurately weighed 20 mg of pure drug in 4M perchloric acid and diluting to volume with the same solvent in a 100 mL standard flask. Alworm-400 (Medopharm Ltd., Malur, India), Bandy-400 (Mankind Pharma. Ltd., New Delhi, India) tablets and Zentel suspension (GSK Pharma. Ltd., Bangalore, India) were purchased from local commercial sources.

4.5.3 ASSAY PROCEDURES

Procedure for bulk drug

Titrimetric (method A)

A 10 mL aliquot of the ALB solution containing 1-10 mg of ALB was transferred into a 100 mL conical flask. To this, 10 mL of 0.01M Ce(IV) was added using a pipette, the contents were mixed well and the flask set aside for 5 min. Finally, the unreacted oxidant was titrated with 0.01M iron (II) solution using one drop of ferroin indicator. Simultaneously, a blank titration was performed, and the amount of the drug in the measured aliquot was calculated from the amount of Ce (IV) reacted.

The amount of ALB in the aliquot was calculated using the formula,

$$\text{Amount (mg)} = \frac{V.Mw.C}{n}$$

V = volume of cerium (IV) reacted, mL

Mw = molecular weight of drug, g mol-1

C = concentration of cerium (IV), mol L-1

n = number of moles of cerium (IV) reacting with each mole of ALB.

Spectrophotometry using DMAB (method B)

Different aliquots (0.0, 0.1, 0.25, 0.5, 1.0, …… 1.6 mL) of 200 µg mL-1 standard ALB solution in 4M perchloric acid were transferred into a series of 10 mL calibrated flasks by means of a micro burette and the total volume in all the flasks was adjusted to 1.6 mL by adding 4M perchloric acid. To each flask, 4 mL of 5M H_2SO_4 and 1 mL of 300 µg mL-1 Ce(IV) solution were added, and the content was mixed well. After a standing time of 5 min, added 1 mL DMAB to each flask and the volume was made up to mark with 4M perchloric acid. After 5 min, the absorbance of the each coloured product was measured at 460 nm against a water blank.

Spectrophotometry using ODA (method C)

Varying aliquots (0.0, 0.25, 0.5, 1.0, 2.0, ……4.0 mL) of 20 µg mL-1 ALB solution were transferred into a series of 10 mL volumetric flasks using a micro burette and the total volume was brought to 4 mL by adding water. To each flask, were added 2 mL of 5M H_2SO_4 and 1 mL of 100 µg mL-1 Ce(IV) solution; and the content was mixed well, kept aside for 5 min. Finally, 1 mL of 0.05% ODA was added to each flask, the volume was made up to mark with water, and mixed well. The absorbance of each solution was measured at 470 nm against a water blank after 5 min.

A standard graph was prepared by plotting absorbance against concentration, and the unknown concentration was computed from the regression equation derived using Beer's law data.

4.5.4 Procedure for the analysis of tablets

Twenty tablets were weighed and finely powdered. An accurately weighed quantity of the tablet powder equivalent to 100 mg ALB was transferred into a 100 mL calibrated flask, and about 60 mL 3:2 acetic acid added. The content of the flask was shaken for 20 min, finally the volume completed to the mark with same acid. The content was mixed well, and filtered through a Whatman No. 42 filter paper. First 10 mL portion of the filtrate was discarded, and a suitable aliquot of the filtrate (1 mg mL-1 ALB) was then subjected to titrimetric analysis (method A). This tablet extract was diluted stepwise to get 20 µg mL-1 ALB with water, used in spectrophotometry (method C), by following the procedure described earlier. Tablet extract equivalent to 200 µg mL-1 ALB was prepared separately using 4M perchloric acid and subjected to analysis in spectrophotometric method B by following the general procedure.

4.5.5 Procedure for suspension

The content of a full bottle of Zentel suspension (10 mL) containing 400 mg ALB was quantitatively transferred to a 250 mL separating funnel, the bottle was rinsed with 4×10 mL of chloroform and the washings were transferred to the separating funnel followed by the addition of another 50 mL each of chloroform and water. The

funnel was shaken for 5 min. and the organic layer was transferred to a 400 mL beaker after drying over anhydrous Na2SO4. The content was again extracted with 4×10 mL portions of chloroform, dried and combined with the chloroform layer in the beaker. The solvent was evaporated to dryness on a water bath, and the residue dissolved in 3:2 acetic acid and the solution was transferred quantitatively to a 100 mL volumetric flask, diluted to the mark with the same acetic acid and mixed well. The resulting solution (4 mg mL-1 ALB) was diluted to 1 mg mL-1 solution with 3:2 acetic acid and used for titrimetric assay. The above was diluted stepwise with appropriate solvents to working concentrations of 200 and 20 µg mL-1 for assay by spectrophotometric methods.

4.5.6 Procedure for the analysis of placebo blank and synthetic mixture

A placebo blank containing starch (20 mg), acacia (25 mg), hydroxyl cellulose (20 mg), sodium citrate (30 mg), talc (20 mg), magnesium stearate (25 mg) and sodium alginate (20 mg) was prepared by combining all components to form a homogeneous mixture. A 50 mg of the placebo blank was accurately weighed and its solution was prepared as described under 'tablets', and then subjected to analysis by following the general procedures. A synthetic mixture was prepared by adding an accurately weighed 100 mg of ALB to about 100 mg the placebo mentioned above. The extraction procedure used for tablets was applied to prepare 1 mg mL-1 ALB solution. This was diluted to get 200 and 20 µg mL-1 ALB solutions, respectively for spectrophotometry. Three different volumes of the resulting synthetic mixture solution (equivalent 3, 6 and 9 mg ALB in method A; 10, 20 and 30 µg mL-1 in method B; 2, 4 and 6 µg mL-1 in method C) were subjected to analysis as described previously.

4.5.7 RESULTS AND DISCUSSION

ALB is reported to undergo oxidation with several oxidizing reagents such as CAT, in situ bromine, NBS, NCS, and sodium periodate. The present work is based on the oxidation of the sulphur atom of the propyl thio group of the ALB molecule by a measured excess of Ce (IV) in acid medium, and the surplus oxidant was determined by either titrimetric or spectrophotometry. The probable reaction scheme is given below;

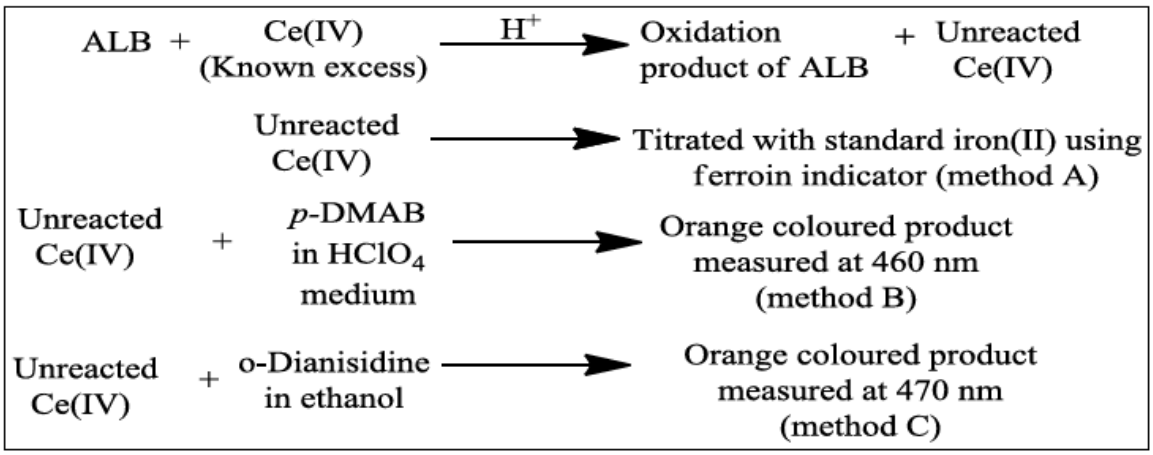

Figure 4.2: Schematic representation of reaction

The reaction between ALB and Ce (IV) was found to occur in 1:2 (drug: oxidant) stoichiometric ratio and all the calculations are based on this stoichiometric ratio in titrimetric. Using 0.01M Ce (IV), 1-10 mg of ALB was conveniently determined.

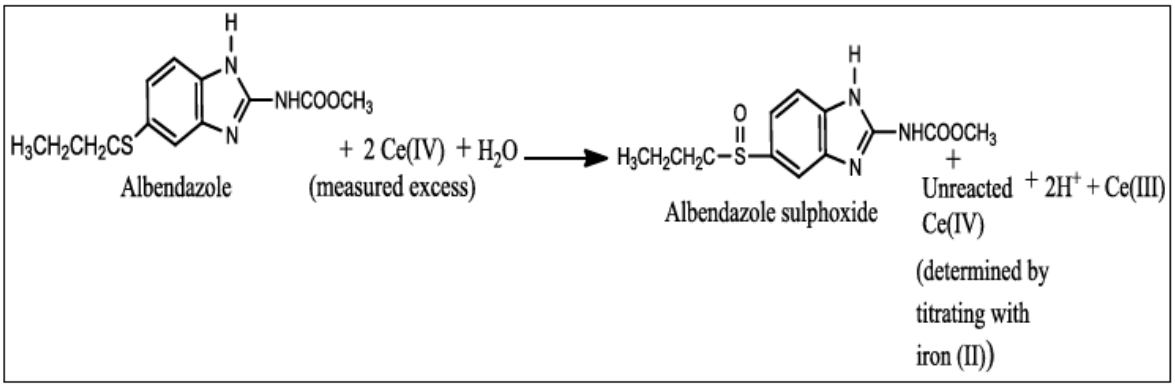

Figure 4.3: Schematic representation of reaction

In spectrophotometry (method B), the unreacted Ce(IV) was treated with DMAB in HClO4 medium to yield formic acid and p-dimethylaminophenol, which upon further; oxidation gave the corresponding quinoimine derivative, measured at 460 nm and in method C, the surplus oxidant was treated with ODA in acid medium to form the orange red coloured oxidation product, which was measured at 470 nm .

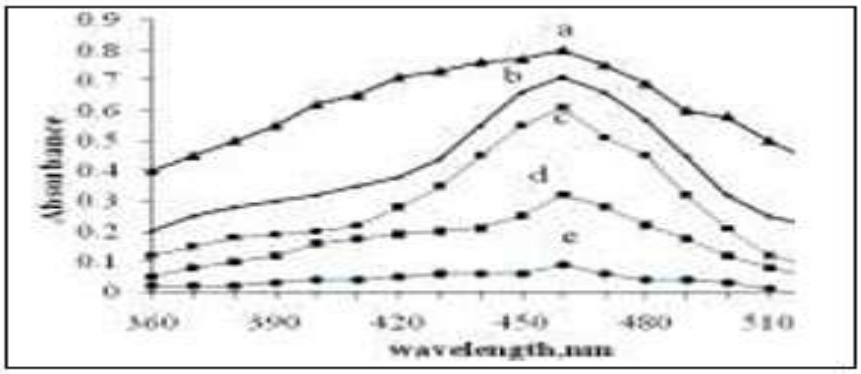

Figure 4.4: Absorption spectra of colored species after treating with: a. 0.0, b. 5.0, c. 10.0, d. 20.0, e. 30 µg mL-1 ALB, in method B.

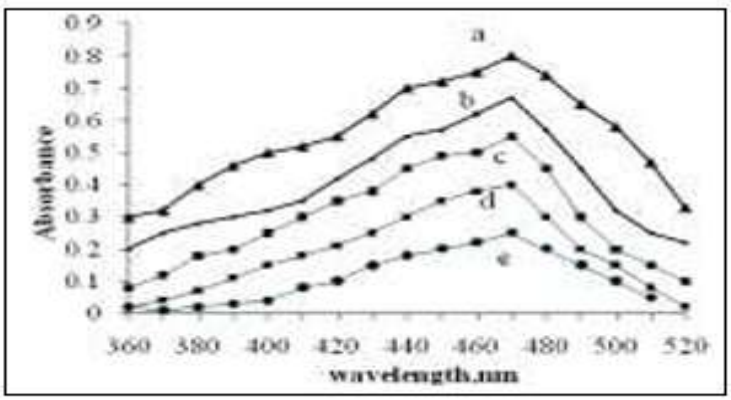

Figure 4.5: Absorption spectra of colored species after treating with: a. 0.0, b. 1.0, c. 2.0, d. 4.0, e. 6.0 µg mL-1 ALB, in method C.

4.5.8 METHOD VALIDATION

The methods were validated for linearity, selectivity, precision, accuracy, robustness and ruggedness.

Linear range and sensitivity

Over the range investigated (1-10 mg), a fixed stoichiometry of 1:2 [ALB: Ce (IV)] was obtained in titrimetric which served as the basis for calculations. In spectrophotometry, the calibration graphs were found to be linear from 2.0–32.0 µg mL-1 and 0.5-8.0 µg mL-1 ALB in method B and method C, respectively, in the inverse manner.

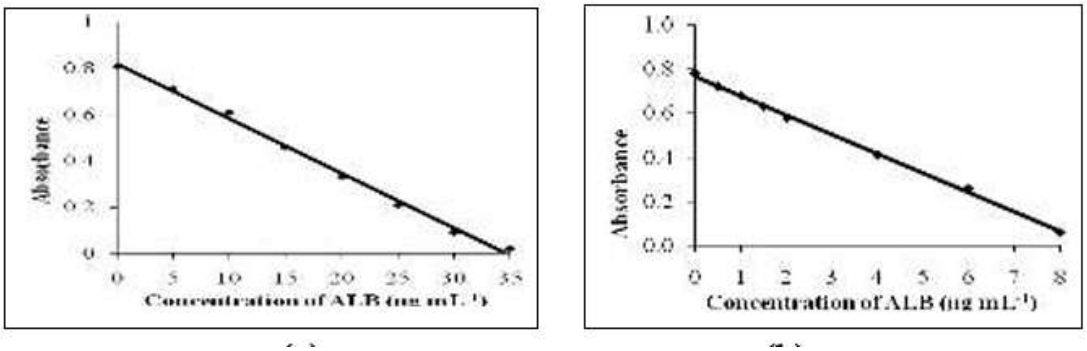

Figure 4.6: Calibration plot: (a) method B and (b) method C.

The calibration graphs are given by the equation Y = a + bX
where, Y = absorbance, a = intercept, b = slope, and X = concentration in µg mL-1 obtained by the method of least squares. Sensitivity parameters such as molar absorptivity and Sandell sensitivity values and the limits of detection (LOD) and quantification (LOQ) were calculated according to the ICH guidelines [68] using the formulae:

$$LOD = \frac{3.3 \times \sigma}{S} \qquad LOQ = \frac{10 \times \sigma}{S}$$

where, 'σ' is the standard deviation of the absorbances of seven blanks prepared by mixing all reactants and Ce (IV) and 'S' is the slope of the calibration curve. These are summarized in Table:

Table 4.1 : Sensitivity and regression parameters.

S. No	Parameter	Method B	Method C
1.	λmax, nm	460	470
2.	Linear range, µg mL-1	2.0–32.0	0.5–8.0
3.	Molar absorptivity(ε), L mol-1 cm-1	5.98×103	2.4×104
4.	Limit of detection (LOD), µg mL-1	1.58	0.64
5.	Limit of quantification (LOQ), µg mL-1	4.79	1.93
6.	Regression equation, Y Intercept	0.8271	0.7580
7.	Slope	-0.0240	-0.0829
8.	Standard deviation of a (Sa)	1.90×10-2	1.40×10-2
9.	Standard deviation of b (Sb)	8.52×10-4	3.352×10-3
10.	Regression coefficient (r)	-0.9968	-0.9993

4.5.9 RAPID QUANTITATIVE ASSAY OF ALBENDAZOLE IN BULK DRUG AND PHARMACEUTICALS BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

INTRODUCTION

Ultra performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 µm particles of stationary phase.

The upper limit of pressure used for the mobile phase flow is 15,000 psi, which is twice that of the pressure limit of conventional HPLC. Owing to its speed and sensitivity, this technique has gained considerable attention in recent years in different fields of pharmaceutical and biomedical analysis. Stability testing and stress testing (forced degradation studies) are critical components development strategy.

The studies help us to understand the mechanism of a drug's decomposition, which further helps in obtaining information on physical and chemical factors that result in instability. These factors are then controlled in order to stabilize the drug or drug composition, resulting in increased shelf-life or improved efficacy. Stress testing is defined as the stability testing of drug substances and drug products under conditions exceeding those of used for accelerated testing.

These studies are undertaken to elucidate the intrinsic stability of the drug substance. According to International Conference on Harmonisation (ICH) guidelines, Q1A(R2), the stability testing of drug substances should be carried out under different stress conditions (hydrolysis, oxidation, photolysis, and thermolysis) to assess the stability indicating ability of analytical methods used for the analysis of stability of sample. The standard conditions for photo stability testing are described in ICH guidelines Q1B.

The guidelines explicitly require conduct of forced degradation studies under a variety of conditions like pH, light, oxidation, dry heat, etc. and separation of drug and degradation products. A review on the development of validated stability-indicating assay methods (SIAMs) for drug substances and products is available.

Inspite of many-fold advantages of UPLC, the technique was not applied to the determination of ALB in pharmaceuticals, though, ALB in Turkey blood was determined by the technique. This prompted the author to develop an UPLC method, which is stability-indicating, for the determination of ALB.

4.5.10 EXPERIMENTAL

Materials and reagents: HPLC grade acetonitrile and methanol were purchased from Merck Ltd., Mumbai, India. Orthophosphoric acid and triethylamine were from Qualigens Fine Chem., Mumbai, India. Water purified by the Milli-Q system (Millipore, Milford, Massachusetts, USA) was used throughout the investigation. Sodium hydroxide solution (NaOH, 5M) was prepared by dissolving required amount of pellets in water. Hydrochloric acid (HCl, 5M) was prepared by appropriate dilution of concentrated acid (Sp. gr. 1.18) with water. A 5% solution of H2O2 was prepared by diluting required volume of the commercially available 30% reagent with water.

Chromatographic conditions and equipment's

UPLC was performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler and tunable UV (TUV) detector. The output signal was monitored and processed using Empower-2 software. The chromatographic column used was Agilent Zorbax Extend C18 (50×4.6 mm; 1.8 um particle size). Isocratic elution process was adopted throughout the analysis.

Mobile phase preparation

Two mL of orthophosphoric acid was diluted to 1000 mL with water and pH adjusted to 7.0 using triethylamine. A 50 mL portion of this resulting buffer was mixed with 50 mL acetonitrile, shaken well and filtered using 0.22 µm nylon membrane filter. Methanol: water (50:50 v/v) was used as diluent in all preparations of the sample.

Instrumental parameters

The isocratic flow rate of mobile phase was maintained at 0.5 mL min-1. The column temperature was adjusted to 30 °C. The injection volume was 4 µL. Eluted sample was monitored at 295 nm and the run time was 5 min. The retention time of the analyte was about 2.219 min.

Preparation of standard solution

To prepare a stock standard solution of ALB (100 µg mL-1), 10 mg of pure drug was accurately weighed into a 100 mL volumetric flask and 50 mL methanol was added. The content was sonicated for complete dissolution of ALB, made up to the mark with water and filtered through a 0.22 µm nylon membrane filter.

4.5.11 ASSAY PROCEDURES

Procedure for bulk drug Preparation of calibration curve

Working solutions containing 0.24-60 µg mL-1 ALB were prepared by serial dilution of aliquots of the stock solution. Four µL aliquots were injected (triplicates) and eluted with the mobile phase under the stated chromatographic conditions. A plot of average peak area versus the concentration of ALB in µg mL-1 was prepared. The concentration of unknown was computed from the regression equation derived using mean peak area concentration data.

Procedure for formulations

Portion of the powdered tablet or suspension equivalent to 10 mg of ALB was transferred in to a 100 mL volumetric flask and 50 mL of methanol was added. The content was sonicated for 20 min to achieve complete dissolution, and the content was then diluted to volume with water to yield a concentration of 100 µg mL-1 and filtered through 0.22 µm nylon membrane filter. The solution obtained was injected in to the UPLC column after appropriate dilution with diluent.

Procedure for selectivity study

A placebo blank composition used was the same as described above and its solution was prepared as described under "procedure for tablets", by taking about 10 mg, and then subjected to analysis. A synthetic mixture was

prepared by homogeneous mixing of 10 mg of pure ALB with 10 mg of the above-mentioned placebo blank. Its solution was prepared as described under 'procedure for tablets and a suitable aliquot was analysed, after appropriate dilution.

Sample preparation for forced degradation

Two mL of 100 µg mL-1 standard ALB was transferred into four different 10 mL volumetric flasks. Two mL of 5M HCl, 5M NaOH, 5% H2O2 or water was added separately, and the flasks were heated for 3 hour on a water bath maintained at 80 °C. Then the solutions were cooled and neutralized by adding base (acid hydrolysis) or acid (base hydrolysis), the volume in each flask was brought to the mark with diluent, and the appropriate volume (4 µL) was injected for analysis. Solid state thermal degradation was carried out by exposing pure drug to dry heat at 105 °C for 24 h. For photolytic degradation studies, pure drug in solid state was exposed to 1200K flux hours in a photo stability chamber. The sample after exposure to heat and light was used to prepare 100 µg mL-1 solutions in diluent and the chromatograms were recorded as usual.

4.5.12 RESULTS AND DISCUSSION

Method development Optimization of chromatographic conditions: Preliminary trials using different compositions of mobile phases consisting of water, acetonitrile, and methanol, and also different ratios of these solutions, did not give good peak shape. When a solution containing 2 mL of H3PO4 in one litre of water, pH adjusted to 7.0 was used as buffer, peak shape of ALB was improved.

To optimize the separation, the f low rate was altered between 0.25- and 0.75-mL min−1. The best separation flow rate was kept constant at 0.5 mL min−1 and the peak response was monitored at wavelength of 295 nm. Different trials were made with stationary phase C18, phenyl and C8 for good peak shape and retention. Finally good peak shape and retention were found with stationary phase Agilent Zorbax Extend C18 (50 × 4.6 mm; 1.8 um particle size) column. The column temperatures varied between 25 and 50 °C and the column temperature was chosen as 30 °C. Finally, a buffer of pH 7.0 and acetonitrile (50:50 v/v) resulted in a good peak shape with a minimum background noise.

Linearity

Seven-point calibration curve was obtained in the concentration range 0.24-60 µg mL-1 for ALB. The correlation coefficient and intercept were found to be 0.9998 and -15412, respectively. The linearity data is given below:

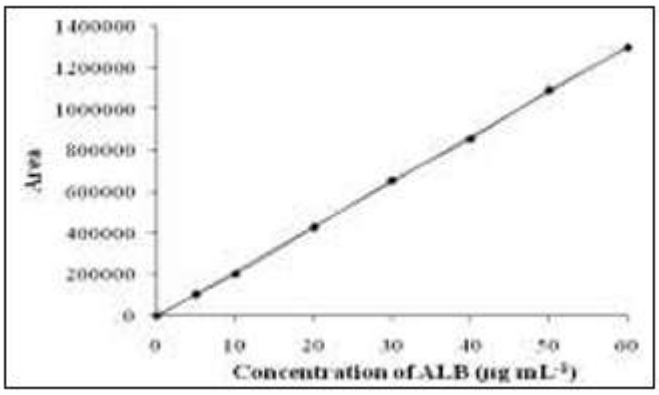

Figure 4.7: Calibration curve.

Table 4.2: Sensitivity and regression parameters.

S. No	Parameter	Values
1.	λmax, nm	
2.	Linear range, µg mL-1	0.24-60
3.	Molar absorptivity(ε), L mol-1 cm-1	
4.	Limit of detection (LOD), µg mL-1	0.01
5.	Limit of quantification (LOQ), µg mL-1	0.04
6.	Regression equation, Y Intercept	-15412
7.	Slope (b)	21921.1
8.	Standard deviation of a (Sa)	129.2
9.	Standard deviation of b (Sb)	282.7
10.	Regression coefficient (r)	0.9998

4.5.13 METHOD VALIDATION

Linearity

To evaluate the linearity of the method, solutions of seven concentrations (0.24, 5.0, 10.0, 20.0, 30.0, 40.0 and 60 µg mL-1) were prepared and 4 µL injected in triplicates. The calibration curve was plotted for peak area of ALB against the corresponding concentration using linear regression analysis. The intercept, slope and correlation coefficient were calculated using the formula:

$Y = a+bX$

where, Y is the mean peak area, X concentration in µg mL-1, a intercept, b slope.

Accuracy and precision

The precision of an analytical method expresses the closeness of agreement (degree of scatter) among a series of measurements obtained from multiple sampling of the same homogeneous sample under the stated optimum conditions. Triplicate injections, of three different concentrations (15, 30, 45 µg mL-1) were given on the same day and the values of relative standard deviation (%RSD) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision. The mean %found ALB in the range from 98.33 to 101.8. The results of accuracy are given in Table below:

Table 4.3 : Results of accuracy study (n=5).

S.NO	ALB injected, µg mL-1	%ALB found	% REa
1.	15	98.33	1.07
2.	30	98.44	1.53
3.	45	99.45	1.69

The peak area based and retention time based %RSD values were < 1.03 indicating excellent precision of the method. The results are given in Table.

Table 4.4: Results of precision study.

S.NO	Mean area ±SD	% RSD a	Mean retention time ±SD	% RSD b
1.	340642 ±1409	0.41	2.238 ±0.020	0.67
2.	654421 ±1932	0.29	2.240 ±0.022	1.03
3.	997349	0.16	2.239 ±0.019	0.38

a Relative standard deviation based on peak area; b Relative standard deviation based on retention time.

Robustness

To determine the robustness of the method, the experimental conditions were deliberately changed. The column oven temperature (28, 30 & 32 ºC), mobile phase composition (45:55, 50:50, 55:45; acetonitrile: buffer v/v), flow rate of the mobile phase (0.45, 0.50 & 0.55 mL min-1) and detection wavelength (293, 295 & 297 nm) were the varied parameters. In each case the %RSD values were calculated for the obtained peak area. The number of theoretical plates and tailing factors were compared with those obtained under the optimized conditions. The system suitability parameters were found to be USP plate count more than 6000, USP tailing less than 1.11, %RSD for system precision less than 2% and signal-to-noise ratio more than 10. The selectivity of the ALB peak was also not affected by varied conditions, which confirms robustness of the method. The results are given in Table

Table 4.5 : Method robustness.

S.NO	ALB injected, µg mL-1	Mean area ±SD	% RSD a	Mean retention time ±SD	% RSD b
1.	15	340642	0.41	2.238	0.89
2.	30	654421	0.29	2.240	0.98
3.	45	997349	0.16	2.239	0.85

a Relative standard deviation based on peak area; b Relative standard deviation based on retention time.

Ruggedness

The study was performed by three analysts and three columns for three different concentrations of ALB (triplicate injections). The area, retention time, theoretical plates and tailing factor obtained for fixed concentration of drug was compared with that of the optimized one. The intra-day and inter-day relative standard deviation values were evaluated for each parameter. The results are summarized in Table

Table 4.6 : Method ruggedness.

S.NO	Variables	Mean peak area± SD*	% RSD	Mean Rt± SD*	% RSD	Mean theoretical plates ± SD	% RSD	Mean tailing factor ±SD	% RSD
1.	Analyst (n=3)	447196 ±1409	0.32	2.239 ±0.02	0.89	6616± 119.63	1.81	1.11± 0.004	0.36
2.	Column (n=3)	444454 ±1418	0.32	2.236 ±0.03	1.34	6588± 120.33	1.83	1.05± 0.002	0.46

4.5.14 Solution stability and mobile phase stability:

Stability of the ALB solution was evaluated by injecting the sample solution into the chromatographic system at different time interval. The peak area was recorded in the time interval 0, 12 and 24 hrs and the RSD values were calculated. Freshly prepared solution was injected at the same time intervals for mobile phase stability (0, 12 and 24 hrs) and RSD values of the peak areas were calculated. At the specified time interval, %RSD for the peak area obtained from drug solution stability and mobile phase stability were within 1%. This showed no significant change in the elution of the peak and its system suitability criteria (%RSD, tailing factor, theoretical plates). The results also confirmed that the standard solution of drug and mobile phase were stable at least for 24 hours during the assay performance. The results are compiled in Table

Table 4.7 : Solution stability.

S.NO	Peak Area	Mean peak area± SD*	% RSD	RT	Mean Rt ± SD*	% RSD	Mean theoretical plates ± SD	% RSD	Mean tailing factor ± SD*	% RSD
1.	443236	444559 ± 1342	0.30	2.24	2.237 ± 0.01	0.45	6588 ± 62.42	0.95	1.09 ± 0.005	0.46
2.	443265			2.54						
3.	443258			2.36						

Table 4.8 : Mobile phase stability.

S.NO	Peak Area	Mean peak area± SD*	% RSD	RT	Mean Rt ± SD*	% RSD	Mean theoretical plates ± SD	% RSD	Mean tailing factor ± SD*	% RSD
1.	443936	444693 ±1072	0.24	2.24	2.24 ±0.01	0.45	6588 ±59.23	0.90	1.08 ±0.005	0.46
2.	445919			2.25						
3.	444223			2.31						

Selectivity

Selectivity of the method was evaluated by injecting the mobile phase, placebo blank, pure drug solution, and tablet extract. No peaks were observed for mobile phase and placebo blank and no extra peaks were observed for tablet extracts and synthetic mixture.

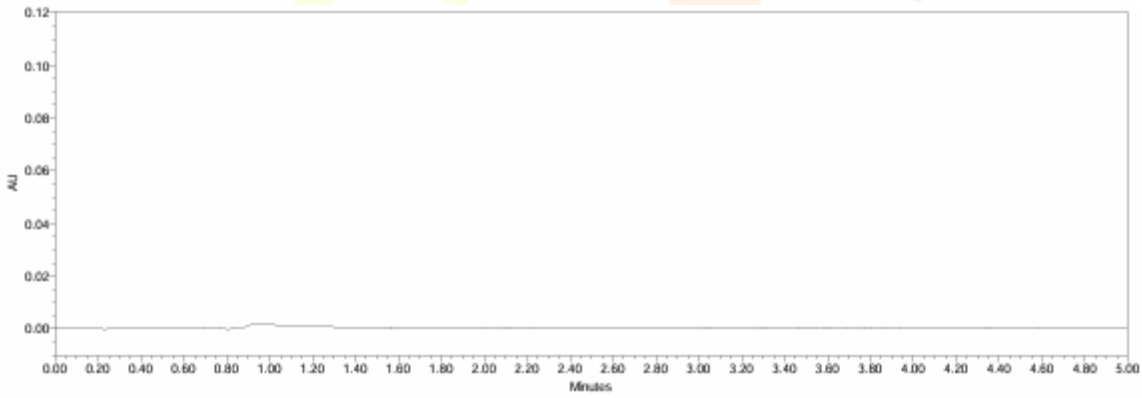

Figure 4.8: Chromatogram of placebo blank.

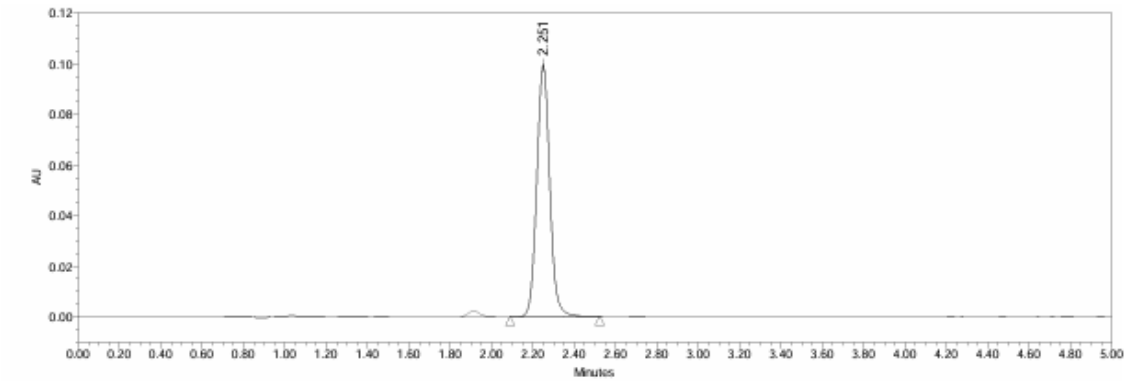

Figure 4.9: Chromatogram of 20 µg mL-1 ALB synthetic mixture.

4.5.15 Application to formulations

A 100-µg mL-1 solution of tablets or suspension was prepared as per 'procedure for formulations' and injected in triplicate to the UPLC system after suitable dilution. The mean peak area of the tablets extract was found to be equivalent to the pure drug and the results were compared with those of an official method [6]. The accuracy and precision of the proposed method was further evaluated by applying Student's t- test and Variance ratio F- test, respectively. The t- and F- values at 95% confidence level did not exceed the tabulated values and this further confirmed that there was significant difference between the official and proposed methods.

Table 4.9: Results of analysis of formulations by the proposed method and statistical comparison of the results with the official method.

S.No	Formulation brand name	Nominal amount, mg	% ALB found ± SD		t- value	F- value
			Official method	Proposed method		
1.	Alworm-400	400	99.8±0.68	99.54±0.52	0.67	1.71
2.	Bandy-400	400	100.6±0.72	99.95± 0.86	1.30	1.43
3.	Zentel suspension	400	101.8±0.71	102.3± 0.89	0.98	1.57

Accuracy by recovery study

A standard addition procedure was followed to further evaluate the accuracy of the method. The solutions were prepared by spiking pure drug into a pre-analysed tablet powder or suspension at three different levels and injected to chromatographic column. The recovery of the known amount of added analyte was computed. The percentage recovery of ALB from tablet ranged from 99.60-101.4%. The results given in Table reveal good accuracy of the proposed method.

Table 4.10: Results of recovery study by standard addition method.

S.No	Formulation studied	ALB in tablet, µg mL-1	Pure ALB added, µg mL-1	Total found, µg mL-1	Pure ALB recovered % ±SD*
1.	Alworm-400	19.96	10.0	29.83	99.60±1.68
2.	Bandy-400	19.06	20.0	40.48	101.3±0.86
3.	Zentel suspension	19.23	30.0	50.26	100.6±1.58

4.5.16 SUMMARY AND CONCLUSIONS

Assessment of methods

The reported titrimetric and chromatographic methods for ALB surveyed. The reported titrimetric methods use unstable oxidant, require scruposely anhydrous medium, large quantities of organic solvent and are applicable over semi micro ranges.

The UPLC method in particular, is the highly sensitive with an LOD 0.01 mL-1.

The proposed UPLC method is sensitive, stability indicating, has wide linear dynamic range and shorter analysis run time.

4.6 UV/VISIBLE SPECTROPHOTOMETRIC AND CHROMATOGRAPHIC ASSAY OF RIFAMPICIN

4.6.1 DRUG PROFILE AND LITERATURE SURVEY

DRUG PROFILE

Rifampicin (RIF), chemically known as 3-[[4-methyl-1-piperazinyl) imino]-methyl]-rifamycin SV (Fig. 6.0.1), is potentially hepatoxic and an established first-line anti-tuberculosis agent derived from rifamycin SV, and its use in other serious infections, such as HIV, is increasing [1]. It is metabolized in the liver mainly by deacetylation and is excreted with its metabolites in bile. This requires careful monitoring of its serum concentration, when used by patients with liver disease, to optimize the dose [2]. Its molecular formula is C43H58N4O12 and has a molecular weight of 823 g mol-1.

RIF has the following structure

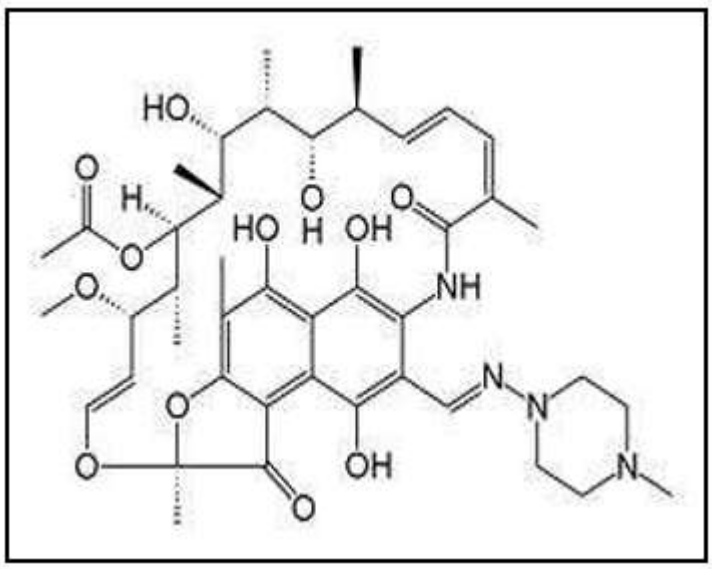

Figure 4.10: Chemical structure of rifampicin

4.6.2 PHYSICOCHEMICAL PROPERTIES OF RIFAMPCIN

RIF is soluble in dimethylsulfoxide (~100 mg mL-1), dimethylformamide, methanol (16 mg mL-1, 25 oC), chloroform (349 mg mL-1, 25 °C), ethyl acetate (108 mg mL-1, 25 °C), and acetone (14 mg mL-1, 25 °C).

RIF is slightly soluble in water at 25 °C: 2.5 mg mL-1, pH 7.3; 1.3 mg mL-1, pH 4.3; and in 95% ethanol (~10 mg mL-1).

RIF is soluble at 37 °C: in 0.1N HCl, 200 mg mL-1 and in phosphate buffer pH 7.4, 9.9 mg mL-1.

RIF should be stable for at least two years when stored desiccated at -20 °C and protected from light.

RIF is stable as a solid at temperatures up to 70 °C.

The drug is official in Indian Pharmacopoeia (IP) which suggests a microbiological assay method.

The European Pharmacopoeia describes a UV-spectrophotometric method in which the drug equivalent to 100 µg mL-1

RIF was prepared in methanol and 5 mL of this extract was diluted to 10 mL with phosphate buffer of pH 7.4, and absorbance measured at 475 nm versus buffer.

4.6.3 LITERATURE SURVEY OF ANALYTICAL METHODS FOR RIFAMPICIN

UV-spectrophotometric methods

To the best of author's knowledge, there is only one report on the application of direct UV-spectrophotometry for the determination of RIF in formulations in which absorbance of an aqueous solution was measured at 340 nm. However, this method is not stability indicating.

UV-spectrophotometry with its variants constitutes an evident alternative to the already existing methods. Multivariation calibration based on partial least square methods were presented for the simultaneous determination of RIF, isoniazid (INH) and pyrazinamide (PYR) , and RIF, INH, PYR and ethambutol.

Bennetton et al.described a first derivative UV-spectrophotometric method for RIF and INH whereas the same method was employed by Rote and Sharma for the determination of RIF in combination with INH and PYR.

A method based on the convolution of the double divisor ratio spectra was employed by Rasha and Hadir for resolving and assaying a ternary mixture containing RIF, INH and PYR. Three methods based on the measurement of graphical absorbance ratio, derivative ratio and additivity of absorbances were developed for the simultaneous determination of RIF and INH.

Visible spectrophotometric methods Few visible spectrophotometric methods are available in the literature for RIF. Sastry et al. have described two methods by measuring the absorbance of complexes formed by RIF with VO2 + or Th (IV) in the presence of PrOH at 490 or 525 nm, respectively. Cu (II) was found to form a colored complex with RIF in methanolic medium paving the way for the assay of drug.

The drug was determined by Sadegi and Karimi through complex formation with iron (III) and charge-transfer complexation with three π-electron acceptors in acetonitrile medium. Sastry et al. [16] used the route of ternary complex formation reactions involving RIF & ZrOCl2, La (NO3)2, NiCl2, or CeCl3 in the presence of pyridine in methanolic medium. Ion-pair extraction assay, using either alizarin violet 3B or alizarin brilliant violet R and measuring the absorbance of the colored complex at 560 nm, has been reported by Reddy et al. Complex formation with Cu (II) and CT complexation reaction with halogenated quinones were used by Shereen et al. for the assay of RIF.

Halogenated quinones such as chloranil and chloranilic acid were also used as chromogenic agents through CT complexation reaction for RIF. Shukla et al. developed a method by reacting RIF with ammonioum metavanadate in acid medium. In an indirect method reported by Barsoum et al., the drug was reacted with a known excess of N-bromosuccinimde and the unreacted oxidant was treated with KI and the resulting triiodide ion I3 _ was measured. Two spectrophotometric methods based on reaction of RIF with 2,6-dichloroquinone chlorimide and K2Cr2O7 are also found in the literature. In a method reported by Diwakar et al. , the drug was reacted with p-N, N dimethylphenylene diamine and chloramine-T, and the resulting chromogen was extracted into butyl alcohol and absorbance measurement at 520 nm.

HPLC methods

To the best of author's knowledge, there is only one report dealing with the HPLC assay of RIF in pharmaceutical formulations when present alone [24]. In this method, the assay was carried out under isocratic conditions with an octadecyl silane column and an aqueous mobile phase containing methanol and 0.02M Na2HPO4 pH 4.5 adjusted to H3PO4 (75:25 v/v).

The detection was done at 254 nm. But this method is not stability-indicating. There are a few reports that describe HPLC methods for the analysis of whole blood and plasma concentrations of antituberculosis agent in combination with isoniazid and pyrazinamide, and their metabolites. In combined dosage forms with isoniazid and pyrazinamide or pyrazinamade, RIF has been determined simultaneously using HPLC with UV-detection.

1.6.4 STABILITY-INDICATING UV-SPECTROPHOTOMETRIC ASSAY OF RIFAMPICIN

1.6.5 INTRODUCTION

UV-spectrophotometry, because of its simplicity, sensitivity, accuracy and precision and ease of handling, is one of the most widely used techniques in pharmaceutical analysis, because many such substances absorb radiation in the UV region (200-400 nm). Because of the increasing usefulness of UV spectrophotometry as a means of assaying pharmaceutical substances described in the pharmacopeias, many pharmaceutical compounds have been successfully assayed by this technique.

4.6.6 EXPERIMENTAL

Instrument

Shimadzu Pharmaspec 1700 UV/visible spectrophotometer provided with 10-mm matched quartz cells was used to record native absorbances.

Reagents and chemicals

Solvents such as chloroform (99.4% alcohol stabilized and spectroscopic grade), sodium hydroxide, potassium hydrogenorthophosphate, hydrochloric acid, orthophosphoric acid, hydrogen peroxide were purchased from Merck Ltd. Mumbai, India. Double distilled water was used throughout the investigation.

Sodium hydroxide solution (NaOH, 5M) was prepared by dissolving required amount of pellets in water.

Hydrochloric acid (HCl, 5M) was prepared by appropriate dilution of concentrated acid (Sp. gr. 1.18) with water. This solution was diluted to 0.1M and standardized.

Orthophosphoric acid (H3PO4 0.1M) was prepared by appropriate dilution of concentrated acid (Sp. gr. 1.57) with water, and standardized.

A 3% solution of H2O2 was prepared by diluting required volume of the commercially available 30% reagent with water.

A phosphate buffer of pH 7.4 was prepared by mixing 50 mL of 0.1M potassium hydrogen phosphate and 25 mL of 0.1M NaOH, and diluted to 100 mL with water; its pH adjusted using pH meter.

Pure drug sample and dosage form

Pharmaceutical grade RIF (99.9% purity) was a gift from Lupin Limited, Tarapur, Maharashtra, India, and was used as received. Capsules in two strengths R-Cin 300 and R-Cin 450 RIF (Lupin Limited, Chikaltana, Aurangabad, India) were purchased from local commercial stores.

Preparation of standard drug solutions

Two standard solutions of 100 µg mL-1 RIF both were prepared by dissolving 10 mg of pure RIF separately in 0.1M HCl (method A) and 0.1M H3PO4 (method B), and diluted separately to 100 mL with respective solvent in calibrated flasks.

4.6.7 PROCEDURES FOR CALIBRATION CURVE

Procedure for bulk drug

HCl method

Into a series of 10 mL calibrated flasks, aliquots of standard drug solution (0.15 to 3.0 mL of 100 µg mL-1) equivalent to 1.5-30 µg mL-1 RIF were accurately transferred and the volume was made up to the mark with 0.1M HCl. The absorbance of each solution was then measured at 263 nm against 0.1M HCl.

H3PO4 method

Aliquots (0.0, 0.15, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL) of RIF standard solution (100 µg mL-1) were accurately measured into a series of 10 mL graduated flasks and the volume was made up to the mark with 0.1M H3PO4 and the absorbance of each solution was measured at 259 nm against 0.1M H3PO4.

Preparation of calibration graph and computation of the unknown concentration are similar to those described in previous chapters.

4.6.8 Procedure for capsules

Contents of twenty capsules were pooled and pulverized. The amount of capsule powder equivalent to 10 mg RIF was quantitatively transferred into two separate 100 mL volumetric flasks. The content was shaken well with about 50 mL of 0.1M HCl or 0.1M H3PO4 separately for 20 minutes and the content was diluted to the mark with the respective solvent. It was filtered using Whatman No 42 filter paper. First 10 mL portion of the filtrate was discarded and 2 mL portion of the subsequent portion was subjected to analysis following the general procedures.

4.6.9 Procedure for placebo and synthetic mixture

A placebo blank of the composition : urea (10 mg), sodium oxalate (15 mg), camphor (10 mg), glucose (10 mg), lactose (20 mg), sucrose (15 mg) and ascorbic acid (10 mg) was made and its solution prepared as described in ‘procedure for capsules’, and then subjected to analysis, by taking 2 mL aliquot.

To assess the role of the inactive ingredients on the assay of RIF, a synthetic mixture was separately prepared by adding 10 mg of RIF to the 10 mg placebo. The drug was extracted and solution prepared as described under ‘procedure for capsules’. The solutions were analyzed for RIF using the recommended procedures.

4.6.10 RESULTS AND DISCUSSION

In HCl method, 0.1M HCl was used as the solvent and the absorbance measured at 263 nm where as in H3PO4 method, 0.1M H3PO4 was used as a solvent with the measurement being made at 259 nm analytical wavelength.

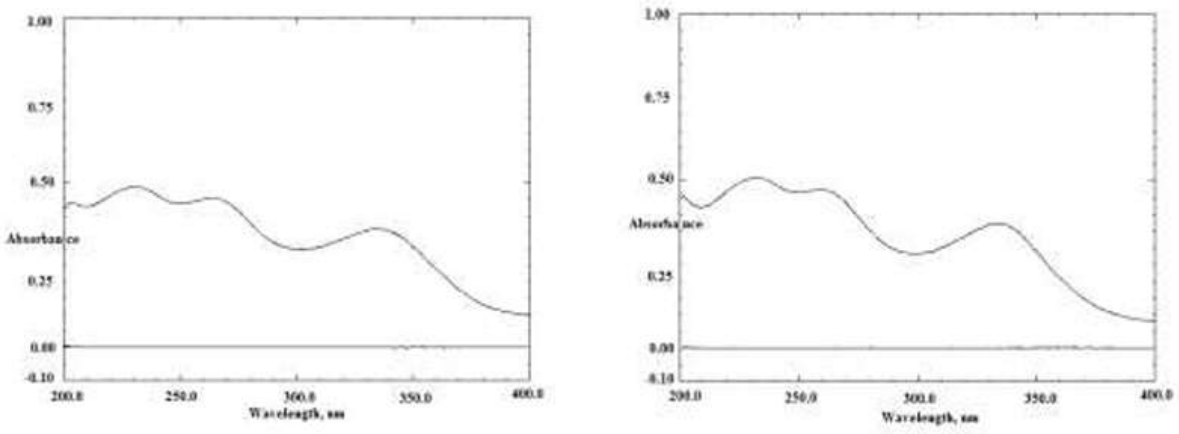

Figure 4.11: UV absorption spectra of: (a) RIF (15 µg mL-1) in 0.1M HCl and (b) RIF (15 µg mL-1) in 0.1M H3PO4.

4.6.11 METHOD VALIDATION

Linearity and sensitivity: A linear relationship was found between the absorbance at 263 nm in HCl method and 259 nm in H3PO4 method, and the concentration of RIF in the range 1.5–30 µg mL-1 in both methods as shown in Figure. The correlation coefficients (r) were 0.9995 and 0.9997 indicating good linearity. The representative linear equations were Y = 0.0008 + 0.0303X and Y = 0.0046 + 0.0316X calculated by the respective least squares method, for HCl method and H3PO4 method, respectively. The uncertainties with the y-axis (Sy), intercept (Sa) and slope (Sb) were also calculated for both methods. These results are presented in Table Measures of sensitivity are also compiled in the same Table and speak of high sensitivity of the methods.

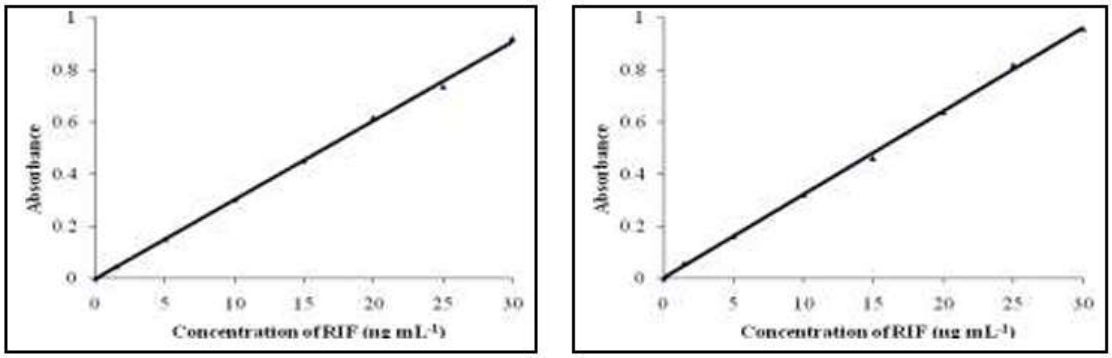

Figure 4.12: Calibration plot for: (a) HCl method and (b) H3PO4 method.

Table 4.11: Parameters

S. No	Parameter	HCl method	H3PO4 method
1.	λmax, nm	263	259
2.	Linear range, µg mL-1	1.5–30	1.5–30
3.	Molar absorptivity (ε), L mol-1cm-1	2.49×104	2.71×104
4.	Limit of detection (LOD), µg mL-1	0.19	0.14
5.	Limit of quantification (LOQ), µg mL-1	0.57	0.44
6.	Regression equation, Y, Intercept (a)	0.0008	0.0046
7.	Slope (b)	0.0303	0.0316
8.	Standard deviation of a (Sa)	0.72×10-4	0.15×10-4
9.	Standard deviation of b (Sb)	3.89×10-3	3.89×10-3
10.	Regression coefficient (r)	0.9995	0.9997

Precision and accuracy

The results of analysis of standard solution are given in below Table . As evident, %RSD values of the data obtained were all below 2%The %RE values were < 2.05% indicating high accuracy of the methods.

Table 4.12: Results of intra-day and inter-day accuracy and precision study.

S. No	RIF taken (µg mL-1)	RIF founda (µg mL-1)	RSDb %	REc %
HCl	10	10.12	1.02	1.20
H3PO4	15	15.78	1.68	1.42

a Mean value of 7 determinations; b Relative standard deviation (%); c Relative error (%).

Selectivity

The absorbance of the placebo solution in each case was almost equal to the absorbance of the blank which revealed no interference. The absorbance resulting from 20 µg mL-1 (in both methods) was nearly the same as those obtained for pure RIF solutions of identical concentrations. The percent recoveries of RIF were 98.94±2.1 and 102.1±1.5 for HCl method and H3PO4 method, respectively.

4.6.12 Application to capsules

Commercial RIF capsules were analysed using the proposed methods and also by a reference method. Capsule extract equivalent to 100 µg mL-1 RIF was prepared in methanol and 5 mL of this extract was diluted to 10 mL with phosphate buffer of pH 7.4, and absorbance measured at 475 nm vs buffer. The results obtained were compared statistically and are presented in Table which reveal close similarity in accuracy and precision between the proposed and reference methods.

Table 4.13: Results of analysis of tablets by the proposed methods and statistical comparison of the results with the reference method.

S. No	**Capsules brand name**	**Nominal amount (mg/tablet)**	**Reference method**	**Proposed methods**	
				HCL METHOD	**H3PO4 METHOD**
1.	R-Cin 300	300	98.27±1.29	99.15±0.72	99.69±1.12
2.	R-Cin 500	450	101.4±0.91	99.35±1.45	102.1±1.72

CHAPTER 5

CONCLUSION

The quality control laboratories in pharmaceutical companies require analytical methods that should predict accurate and precise data of the drug content. The aim of the thesis work was to develop and validate simple, rapid, accurate, precise, robust and rugged analytical methods for the assay of four each of anthelmintics and anti-tubercular drugs, by adopting simple and cost- effective techniques like, UV/visible spectrophotometry and advanced techniques such as high-performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC).

The thesis contains description of the analyses of anthelmintic drugs namely albendazole (ALB) anti-tuberculosis agents namely rifampicin (RIF), using UV/visible spectrophotometry, HPLC and UPLC methods.

The proposed methods well-earned their applicability for assaying the respective drug in developing and under developed countries. The UV/visible spectrophotometric methods are more economical because they utilized cheaper chemicals and low-cost instrument, they don't require longer analysis time and they rely decisively on the selectivity of the reaction chemistry adopted.

The calibration of each and every method is only by using pure drug. The proposed UPLC and HPLC methods are highly sensitive, stability-indicating, have wide linear dynamic ranges and shorter analysis run time. The developed methods were applied to the determination of active drug in bulk form, tablet/capsule preparations, suspension/syrup, and a few methods in spiked human urine. No interferences were observed by inactive ingredients present in tablets/capsules or biochemical substances present in urine. These reassure the excellent selectivity of the proposed methods.

UV visible spectrophotometric, and one UPLC methods were developed for the assay of albendazole (ALB) in bulk drug, its pharmaceuticals, and validated according to ICH guidelines.

The proposed UPLC method is sensitive, has wide linear dynamic range and shorter analysis run time. This is the first stability indicating UPLC method for ALB. The reported UPLC method is not stability indicating. The assessment of the developed methods was made at the end of the chapter.

UV-spectrophotometric, HPLC and UPLC methods, were developed and validated for rifampicin (RIF). The proposed methods use 0.1M HCl, 0.1M H3PO4, FC reagent, potassium ferricyanide, 1,10-phenanthroline, 2,2'-bipyridyl as analytical reagents, which an ordinary analytical laboratory can afford, and the procedures do not involve any critical reaction conditions or tedious sample preparation.

The proposed methods are rapid, simple and highly sensitive. The developed methods do not require an internal standard unlike the reported HPLC method and less solvent consumption. The methods are applicable over wide linear dynamic concentration ranges.

Developed methods were validated as per the current requirements of the International Conference on Harmonisation Guidelines (ICH) which is the ultimate for validations all over the world. Method selectivity and accuracy were further confirmed by standard addition procedure.

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