

**Chemometrically-Assisted Spectrophotometric  
Determination of Certain Anti-Tubercular  
Combinations**

**By**

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## **List of Abbreviations**

CLS	Classical least square
<sup>1</sup> D	First derivative zero crossing
DOTS	Directly observed therapy short course
<sup>1</sup> D ratio	First derivative ratio
EDL	Essential drug list
INH	Isoniazid
PCR	Principal Component régression
PZA	Pyrazinamide
RIF	Rifampicin

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## **Abstract**

The UV absorption spectra of the rifampicin, isoniazid and pyrazinamide in the range of 200-400 nm, showed a considerable degree of spectral overlap. It has been reported that rifampicin and isoniazid are known to interact with each other in solid formulation milieu to yield isonicotinyl hydrazone. The use of chemometrics assisted spectrophotometric method is presented for the simultaneous determination of isoniazid and rifampicin tablets used in the treatment of tuberculosis. Resolution of binary mixtures of the drugs has been accomplished by using first derivative ratio, first derivative zero crossing. The multivariate methods, classical least squares (CLS) regression and principal components regression (PCR) has also been used for the determination of binary and ternary (in presence of pyrazinamide) mixtures. The amount of isoniazid and rifampicin in binary mixture were determined by measuring the first derivative zero-crossing wave lengths at 230 and 250.5 nm (zero-crossing wave length of rifampicin) and 320.5 and 360.5 nm (zero-crossing wave length of isoniazid) respectively. By using rifampicin as a divisor the amount of isoniazid in the same binary mixture were determined by measuring the first derivative ratio amplitudes at 222.5, 247.5, and 282.5 nm. Also by using isoniazid as divisor the amount of rifampicin were determined by measuring the first derivative ratio amplitudes at 322.5 and 327.5 nm. Although the components show an important degree of overlap, good recoveries were obtained with both the laboratory prepared mixtures and commercial tablets. No interference has been observed from the tablet excipients. The stability study indicated that the rate of degradation of rifampicin in the presence of isoniazid follows first order kinetics and the decreased amount is about 23.2% (RIF-INH in mixtures) and 30.9% (RIF-INH in formulations) through 150 minute at 60 °C.

*Keywords:* Chemometrics; Spectrophotometry; Rifampicin; Isoniazid; Pyrazinamide; Stability

## **1. Introduction**

### **1.1. Tuberculosis**

Tuberculosis (TB) is the world's second most common cause of death from infectious disease, next to human immunodeficiency virus (HIV). TB has become an increasing health problem since emergence of HIV; TB/HIV co-infection adds further complications in treating the disease. It is also a major concern for industrialized nations because of emergence of drug resistance, alcohol / drug abuse, growth of immigrants and other factors (Dye et al., 1999).

#### **1.1.1. Extent of the problem**

It is estimated that one-third of the global population, i.e., "two billion people", is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) with approximately eight million new cases and two million deaths each year (WHO, 2006). Only one in ten people infected by *M. tuberculosis* actually develop the disease, as the healthy immune system keeps the infection dormant. This infection can reactivate years; even decade later if the immune system is weak. Transmission is accelerated by confined housing conditions, and the activation of the infection is facilitated by poor nutrition and general ill health. Tuberculosis is a disease that hits the poor hardest (Dye et al., 1999).

*M. tuberculosis* is a highly contagious, airborne, slow-growing, aerobic, rod-shaped acid-fast bacillus, which can withstand weak disinfectants and can survive in dry state for weeks but can grow only within a host organism. *M. tuberculosis* has a cell wall but lacks a phospholipid outer membrane and classified as a gram-positive. However, if a gram-positive stain is performed *M. tuberculosis* either stains very weakly gram-positive or does not retain dye due to the high lipid and mycolic acid content of its cell wall. *M. tuberculosis* divides extremely slowly (every 15 to 20 hours) compared to other bacteria, which tend to have division times measured in minutes. *M. tuberculosis* is identified microscopically by its staining characteristics, it retains certain stains after being treated with acidic solution, and is thus classified as an "acid-fast bacillus". In the most common staining technique, the Ziehl-Neelsen stain, acid-fast bacilli are stained bright red, which stands out clearly against a blue background. The reason for the acid-fast staining is because of its lipid rich cell wall.

The exact method by which the stain is retained is unclear but it is thought that some of the stain becomes trapped within the cell and some forms a complex with mycolic acid. Mycolic acid allows the bacteria to survive within acidic milieu like in macrophages. It also provides the organism with a resistant barrier to many common drugs (Williams and Lemke, 2002).

*M. tuberculosis* usually affects the lungs (80 to 85% of the cases). It is characterized by a persistent cough, shortness of breath and chest pain. Other symptoms include weight loss, fever and night sweats. If not treated, each person with active pulmonary TB on the average will infect 10 to 15 other people every year. The mycobacteria can also infect almost any part of the body, such as the lymph nodes, the spine or bones (extra pulmonary TB). Pulmonary TB is the most frequent site of involvement and extra pulmonary tuberculosis is less frequent but it is equally vital to diagnose and treat the disease rapidly, as all forms are deadly if left untreated (Williams and Lemke, 2002).

There are 22 TB high burden countries in the world, which constitute 80% of global tuberculosis cases. Five Asian countries (India, China, Indonesia, Bangladesh and Pakistan) account for more than half of all incidence cases. Out of the 22 TB high burden countries, ten are countries with the highest rate per capita; nine are in Africa, south of the Sahara. Particular reference to Africa; the increase in TB incidence is strongly associated with the prevalence of HIV infection: rates of HIV infection among TB patients are correspondingly high, exceeding 60% in South Africa, Botswana, Zambia and Zimbabwe (Stop TB partnership, 2006).

Ethiopia ranks eighth among the world's top 22 TB high-burden countries. According to the World Health Organization Global TB Report 2006, the country had more than 267,000 TB cases in 2004, with an estimated incidence rate of 353 cases per 100,000 people. The number of TB cases is likely to increase. Ethiopia's HIV/AIDS epidemic expands, as nearly one-fourth of adult TB cases are HIV-positive (WHO, 2006). Tuberculosis often is the first co-infection that appears in an HIV-infected person. The consequence of this interaction of HIV/AIDS and *M. tuberculosis* in Ethiopia is a cause for large increase in the number of tuberculosis cases. This suggests that tuberculosis with HIV/AIDS co-infection aggravates the likelihood of spreading the disease in the country (Converse, 2000).

### 1.1.2. Development of TB treatment

Tuberculosis was first described in 1882 by Robert Koch. Following Sanatorium, Collapse therapy and the development of Bacillus Calmette-Guérin vaccine, the era of chemotherapy for TB dawned with the discovery of streptomycin in 1943 by Selman Waksman. After the discovery of streptomycin, p-aminosalicylic acid, isoniazid, pyrazinamide, ethambutol and rifampicin were introduced as anti-TB agents. The first clinical study started when streptomycin became available for therapy and was followed by the finding that a combined regimen with p-aminosalicylic acid reduced the emergence of bacterial resistance, compared to that seen when either of the two was given alone. Studies showed that combining isoniazid with p-aminosalicylic acid and streptomycin was more effective than any two drug combination in preventing the emergence of resistance early in the course of treatment when the viable bacterial population was high (Panchagnula *et al.*, 2004).

The three important properties of anti-TB drugs are antibacterial activity, capacity to inhibit the development of resistance and capacity to kill intracellular persisting organisms. A significant breakthrough came in 1956 with the comparative study at the Tuberculosis Chemotherapy Center, Madras, India (presently known as Tuberculosis Research Center, Chennai, India), which provided scientific justification for the domiciliary treatment of TB, and hence made effective chemotherapy available for even the poorest developing countries. Currently combination of isoniazid, rifampicin, pyrazinamide and ethambutal in the form of fixed dose combination is widely circulated providing effective TB treatment (Panchagnula *et al.*, 2004). The development of chemotherapy for tuberculosis and year of introduction of the drugs are summarized in Table 1.

Table 1: Development of chemotherapy for tuberculosis ([Panchagnula et al., 2004](#))

Treatment mode	Specifications	Year of introduction
Separate formulations	Streptomycin	1944
	p-aminosalicylic acid (PAS)	1946
	Isoniazid (INH)	1951
	Pyrazinamide (PZA)	1954
	Ethambutol (ETH)	1962
	Rifampicin (RIF)	1970
Combination drugs (SCC)	Streptomycin + PAS	1948
	INH + PAS	1952-1955
	INH + Streptomycin	1952-1955
DOT	Directly observed treatment	1958 onwards
	2-drug (Thiacetazone + INH)	Early 1980s
Fixed dose combinations (FDCs)	(ETH + INH)	
	(RIF + INH)	
DOTS	3 drug RIF + INH + PZA	1950s-1970s
	Directly observed treatment, Short course	Early 1990s
	4 drug FDC	1993 (available in market)
Pediatric FDC	RIF + INH + ETH + PZA	1999 (in WHO EDL)
	RIF + INH	1999 (in WHO EDL)
	RIF + INH + PZA	1999 (in WHO EDL)

### 1.1.3. Directly observed therapy short course (DOTS)

The lack of organization of services to ensure widespread detection and cure of patients has resulted in the current burden of TB and the emergence of drug resistance. However, there is proven cost effective management package known as directly observed treatment short course. This was effectively launched in 1991. The DOTS strategy, the most significant advance in tuberculosis intervention in the past decades, was not the direct outcome of basic research but of a carefully planned and executed program of operational research which ensures effective delivery of health services to TB patients ([Small, 1999](#)).

DOTS is one of the most tangible health interventions available, contributing to social and economic development. It is actually a package of the minimal core essential for national tuberculosis control, and includes political commitment, sputum smear based diagnosis, careful tracking of each patient, uninterrupted drug supply and direct observation of pill consumption. Treatment with properly implemented DOTS has a success rate exceeding 95% and prevents the emergence of further multi-drug resistant strains of tuberculosis. More than 1.2 million people received DOTS care since WHO adopted it in 1992 as a strategy to combat tuberculosis (Small, 1999).

Ethiopia adopted the DOTS strategy in 1992 and began to implement it in a few zones of Oromiya (Press Release for World TB Day, 2003). DOTS is a major plan in the WHO global TB eradication program. By 1998, 119 countries had adopted the strategy and 21% of all estimated smear positive cases were reported under DOTS. The targets are to achieve 85% treatment success and 70% smear-positive case detections world wide (WHO, 2006).

#### **1.1.4. Drug resistance to tuberculosis**

Organisms are said to be drug-resistant when drugs meant to neutralize them have reduced effect. When an organism is resistant to more than one drug, it is said to be multi-drug resistant. The most prominent example of this is antibiotic resistance. To counter drugs, bacteria and viral pathogens have evolved sophisticated mechanisms to inactivate these compounds (e.g., by pumping out compounds mutating residues required for the compound to bind, etc.), and they do so at a rate that far exceeds the pace of new development of drugs. Examples include *M. tuberculosis* among bacterium and HIV-1 among viruses (Tomasz, 1994).

The successful management of tuberculosis in the 21<sup>st</sup> century will be reliant upon the synergy provided by a balanced portfolio that includes application of existing interventions to maintain our grip on the disease while we simultaneously search for new knowledge that will evolve into better tools for confronting the epidemic. Balancing these efforts will ensure success (Small, 1999).

Drug resistant TB was observed since the early days of the introduction of chemotherapy. The first molecular study of drug resistance mechanisms in *M. tuberculosis* was published by Zhang

and colleagues in 1992 on the mechanism of action of isoniazid resistance in *M. tuberculosis* (Zhang *et al.*, 1992). Finding of the first survey of drug resistance was released in 1998, which included data from 35 countries. Conclusion of third world congress on TB in 1992 was the existence of little information on the global magnitude of multi-drug resistance-TB, defined as resistance to at least rifampicin and isoniazid (Isman, 1993).

Drug supply management of anti-TB drugs is complicated since individual drugs must frequently be ordered from different sources and there is a risk of out of stock situations in which patients may be treated with too few drugs or even only one drug in isolation. Subsequently, erratic drug intake may result in the selection of drug-resistant strains of the TB bacillus (Blomberg, *et al.*, 2001b).

## **1.2. Anti -tubercular drugs**

Tuberculosis is out of control in many parts of the world and it is now the world's second most common cause of death from infectious disease. Against this worrisome disease the first line drugs are isoniazid, rifampicin, pyrazinamide and ethambutol. Some second-line drugs are capreomycin, cycloserine, streptomycin, clarithromycin and ciprofloxacin. These second-line drugs may be used for infections with tubercle bacilli, likely to be resistant to first-line drugs or when the first-line drugs have to be abandoned because of the unwanted reactions. To decrease the possibility of the emergence of resistant organisms, combined drug therapy is employed involving the following: for first initial phase of about 2 months consisting of the three drugs isoniazid, rifampicin and pyrazinamide in the form of fixed dose combinations: (plus ethambutol if the organism is suspected to be resistant). A second, continuation phase of four months duration, consists of two drugs; isoniazid and ethambutol. A longer term treatment is needed for patients with meningitis, bone/joint involvement or drug-resistant infection (Williams and Lemke, 2002).

### 1.2.1. Rifampicin

Rifampicin (3-[4-methylpiperazinyliminomethyl]-rifamycin SV) is a semisynthetic derivative of rifamycin SV, an antibiotic isolated from *Amycolatopsis mediterranei* (formerly known as *Streptomyces mediterranei*). This molecule is a member of the ansamycins class of natural products. Members of this class are characterized as molecules with an aliphatic chain forming bridge between two non-adjacent positions of an aromatic moiety as shown in (Figure 1). It is orally active, highly effective against a variety of gram-positive and gram-negative organisms and it is used alone or in combination with other drugs, such as isoniazid and pyrazinamide in treatment of tuberculosis, leprosy and other infectious diseases specially those resulting from HIV/AIDS (Williams and Lemke, 2002).

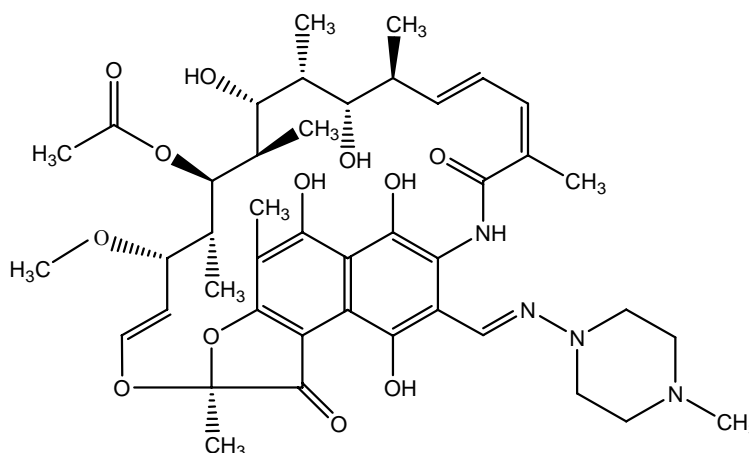


Figure 1: Structural formula of rifampicin

#### 1.2.1.1. Chemistry of rifampicin

Rifampicin is a complex macrocyclic, red-orange crystalline compound, which has zwitter ionic properties. The presence of phenolic groups results in acidic properties ( $pK_a = 1.7$ ), where as the piperazine moiety gives basic properties ( $pK_a = 7.9$ ). The mechanism of action suggests an inhibition of mycobacterial mRNA synthesis by binding to beta subunit of DNA-dependent RNA polymerase in a broad range of microbial pathogens (Calvori *et al.*, 1965). It has bactericidal action and a potent sterilizing effect against tubercle bacilli in both intracellular and extracellular locations. It is active against DNA-dependent RNA polymerase from both gram-positive and

gram-negative bacteria, but because of poor penetration of the cell wall of gram-negative organisms by rifampicin, the drug has less value in infections caused by such organisms (Williams and Lemke, 2002).

### 1.2.2. Isoniazid

Isoniazid (Pyridine-4-carboxylic acid hydrazide) is a synthetic antibacterial agent with bactericidal action against *M. tuberculosis*. It is the hydrazide of isonicotinic acid (Figure 2). It was the cornerstone in tuberculosis chemotherapy for almost half a century since its discovery as a potent antituberculous drug in 1952. It is a beneficial agent, effective against intracellular and extra cellular bacilli and is generally considered the primary drug for treatment of *M. tuberculosis* (Williams and Lemke, 2002).

It is bactericidal against replicating organisms but appears to be only bacteriostatic against non-replicating organisms. After treatment with isoniazid, *M. tuberculosis* organism loses its acid fastness, which may be interpreted as indicating that the drug interferes with cell wall development (Williams and Lemke, 2002).

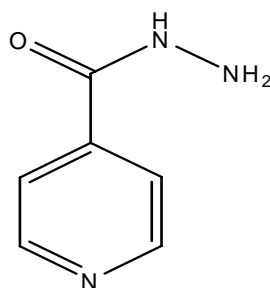


Figure 2: Structural formula of isoniazid

#### 1.2.2.1. Chemistry of isoniazid

Isoniazid is a prodrug and its antitubercular function requires *in vivo* activation by an enzyme. It is highly bactericidal against replicating tubercle bacilli and its antibacterial activity is limited to mycobacteria. It's major use is for chemotherapy of tuberculosis (in combination with rifampicin, pyrazinamide and/or ethambutol), tuberculosis prophylaxis, occasionally to prevent transmission

to close contacts at high risk of disease, progression of infection to primary complexity in recently infected asymptomatic individuals, and recurrence of infection in immuno deficient individuals. The mechanism of action of isoniazid is poorly understood but may involve inhibition of mycolic acid biosynthesis which is a long fatty acid-containing constituent of cell wall, by affecting the enzyme mycolase synthase which is unique for mycobacteria (Benfang *et al.*, 2000)

### 1.2.3. Pyrazinamide

Pyrazinamide (pyrazine carboxamide) is a synthetic analogue or bioisoster of nicotinamide (also called vitamin B3), which is a weak bactericidal against *M. tuberculosis* (Figure 3). The discovery of pyrazinamide in the late 1940s as a powerful drug against tuberculosis was based on the serendipitous observation that nicotinamide curiously had activity against tubercle bacilli in animal models. Subsequent synthesis of analogues of nicotinamide led to the identification of pyrazinamide as the most active derivative against *M. tuberculosis* (Rang *et al.*, 2003).

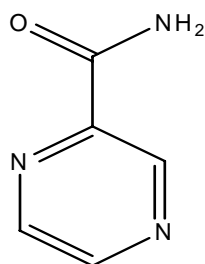


Figure 3: Structural formula of Pyrazinamide

#### 1.2.3.1. Chemistry of pyrazinamide

Pyrazinamide is an important component of the current 6-month short-course TB chemotherapy. It plays a unique role in shortening the therapy from a period of 9 to 12 months down to 6 months, because pyrazinamide kills a population of semi-dormant tubercle bacilli residing in macrophages. It is inactive at neutral pH but it has potent sterilizing activity, particularly in the relatively acidic intracellular environment of macrophages at pH = 5.5, since after phagocytosis the organisms are contained in phagolysosomes in which the pH is low, which are not killed by other TB drugs (Rang *et al.*, 2003).

Unlike other TB drugs, pyrazinamide despite its remarkable activity *in vivo* has no activity against tubercle bacilli *in vitro* in normal culture medium except under acidic-pH conditions (Zhang *et al.*, 1999).

The mechanism of action of pyrazinamide is poorly understood, but some findings suggest that pyrazinamide may be active totally or in part as a prodrug. Its activity depends on its conversion to pyrazinoic acid; susceptible organisms produce amidase enzyme, which is responsible for conversion to pyrazinoic acid, which may lower the pH in the immediate surrounding of the *M. tuberculosis* to an extent that the organism is unable to grow. Resistant strains of *M. tuberculosis* do not produce this amidase enzyme suggesting that the acid form of the drug is the active form (Williams and Lemke, 2002). It is highly effective during the first two months of treatment while acute inflammatory changes persist and its use has enabled treatment regimens to be shortened and the risk of relapse to be reduced.

#### 1.2.4. Analysis of the drugs

Several analytical methods are available for the individual determination of the above compounds in bulk and pharmaceutical preparations such as voltammetry (Alonso Lomillo *et al.*, 2002; Ghoneim *et al.*, 2003; Gao *et al.*, 2006), amperometry (Alonso Lomillo *et al.*, 2003; Alonso Lomillo *et al.*, 2005; Quintino and Angnes, 2006), titrimetry (El-Brashy and El-Ashuy, 1992), gas chromatography (Khuhawar and Zardari, 2006), HPLC (Gowda *et al.*, 2002; Khuhawar *et al.*, 2005), spectrophotometry (Galal *et al.*, 1992; Safavi *et al.*, 2004; Salem *et al.*, 2005; Sadeghi and Karimi 2006), spectro-fluorimetry (Lapa, *et al.*, 2000), chemiluminescence (Song *et al.*, 2001; Xi *et al.*, 2004), kinetic determination (Espinosa-Mansilla, *et al.*, 1998; Kulkarni, *et al.*, 2004) and flow injection (Li *et al.*, 1999; Zhang and Li, 2001; Zhang *et al.*, 2001, Safavi *et al.*, 2003; Safavi *et al.*, 2004).

The available method for the simultaneous determination of rifampicin and isoniazid is HPLC which has advantages in terms of analysis of complex mixtures. But the method has some disadvantages like costs of chemicals i.e. using internal standards and tetra butyl ammonium hydroxide as ion pairing agent in the mobile phase which shortens the column life, complex and tedious procedures for sample extraction and requirement of running the reference standards every time for assay of sample in quality control laboratories (Shah *et al.*, 1992).

Chemometric-assisted spectrophotometric technique has so many advantages one of it is reduction of interferences by using the full spectrum in the case of multivariate calibration. It does not require running the reference standards every time when the assay of sample because once absorbance matrix for the standards stored on the computer it serves for long period of time (Mahalanabis *et al.*, 1989).

Other analytical methods available are voltammetry (Hammam *et al.*, 2004), visible spectrophotometry for rifampicin at pH 7.4; which may not good for determination of rifampicin in the presence of degradation product i.e. isonicotinyl hydrazone both have similar absorption maxima in colorimetric region and first-derivative ultraviolet spectrophotometry for isoniazid (Benetton *et al.*, 1998), spectrophotometric methods based on graphical absorbance ratio at selected wavelength, derivative spectroscopy, additivities of absorbance (Kakde *et al.*, 2002) and Chemiluminescence with artificial neural network (Li *et al.*, 2005).

The analytical techniques which are developed for the simultaneous assay of rifampicin, isoniazid and pyrazinamide in pharmaceutical preparations are HPTLC (Argekar *et al.*, 1996), HPLC (Calleri *et al.*, 2002; Mohan *et al.*, 2003), micellar electrokinetic capillary chromatography (Acedo-Valenzuela *et al.*, 2002), normal and first derivative UV spectrophotometry (Rote and Sharma, 1996; Rote and Sharma, 1997), chemometrics-assisted spectrophotometry based on PLS regression (Goicoechea and Olivieri, 1999; Espinosa-Mansilla *et al.*, 2001), PCR and PLS regression (Madan *et al.*, 2005) and recently rifampicin, isoniazid and pyrazinamide have been determined by high performance liquid chromatography with artificial neural network (Glass *et al.*, 2007).

### 1.3. Anti-tubercular fixed dose combinations

Fixed dose combinations have a long history of development starting with combination therapy. Combination therapy refers to treatment with two or more active drugs administered at one time in their individual formulations. Combination therapy has proven successful in the treatment of cancer, infectious diseases, hypertension and neurological disorders. The use of combination therapy in a standardized regimen is the fundamental strategy of WHO and International Union against Tuberculosis and Lung Disease for the treatment of TB. However, increasing the number of drugs to be taken increases the problem of patient compliance. One of the best ways of ensuring compliance with multi-drug regimens is to combine the requisite drugs physically into one preparation, a fixed dose combination product (Laing *et al*, 1999).

Fixed dose combinations are available for treatment of diseases in a number of classes such as cardiovascular, infectious, gastro intestinal, etc. At the beginning of 1970s, fixed dose combinations accounted for over half of the pharmaceutical products and for 40% of the best-selling drugs in the United State of America. Currently treatment of TB involves administration of a combination of two or more first-line anti-TB drugs namely, rifampicin, isoniazid, and pyrazinamide in a fixed proportion in a single dosage form for the initial 2 months, followed by rifampicin and isoniazid for 4 months. The rationale for using fixed dose combinations for TB is based on the fact that the combination is more effective than each of the drugs alone, on account of the known resistance to antitubercular drugs (Laing *et al*, 1999).

The development of fixed dose combinations as a single formulation was facilitated by the fact that the component drugs were generic and their safety and efficacy had been proven. That is they are highly effective, minimally toxic, orally administered, and well tolerated. Since 1998, WHO and international union against tuberculosis and lung disease have recommended replacing single-drug regimens with fixed-dose combinations for TB treatment (Blomberg *et al*, 2001a). The potential advantages associated with the use of fixed dose combinations are: safety and efficacy, simplified treatment, better management of DOTS, simplified drug supply management, shipping and distribution, and reduced risk of emergence of drug-resistance strains (Blomberg *et al*, 2001b).

Despite these advantages, the problem of drug toxicity during treatment with these agents still exists, especially in patients with HIV infection, and there has been a global increase in the prevalence of drug-resistant tuberculosis (Panchagnula *et al.*, 2004).

In Ethiopia two-drug, three-drug, and four-drug fixed dose combination products containing isoniazid, rifampicin, pyrazinamide, and ethambutol are used as anti-TB products. Reports show that patients treated with three-drug formulation had more rifampicin and isoniazid resistant isolates than those treated with individual drugs (Wolde Mariam, 2003). An integral part of the strategy to fight the disease is the use of quality anti-TB fixed dose combinations as recommended by WHO and international union against tuberculosis and lung disease. The major challenge in using fixed dose combination tablets is to ensure that only combined tablets of good quality are used. Although fixed dose combination products provide many advantages, combinations of the drugs may lead to problems associated with quality of the products. The problems associated with quality of fixed dose combination products and identification of the quality assessment parameters were and still is the main focus, and there are several reports in literature which have raised concern about the quality of these products. Moreover, fixed dose products with lower than the required strength of rifampicin than single-drug formulations were found to be in wide circulation (Kenyon *et al.*, 1999; Laserson *et al.*, 2001).

The two major problems associated with quality of fixed dose combinations are:-

- (i) Loss of bioavailability of rifampicin upon administration (Shishoo *et al.*, 2002).
- (ii) Instability of drugs within the formulation environment (Singh and Mohan, 2003; Bhutani, 2004).

In both cases, the problem has been ascribed to the interaction of rifampicin with isoniazid to form isonicotinyl hydrazone [(3-(isonicotinylhydrazinomethyl) rifamycin] (Figure 4) which is the key degradation product (Mariappan *et al.*, 2005).

When rifampicin is present along with isoniazid in fixed dose formulations the two drugs interact with each other (Jindal *et al.*, 1994; Bhutani *et al.*, 2005a), resulting in conversion of rifampicin to 3-formylrifamycin, which further reacts with isoniazid to form isonicotinyl hydrazone (Sankar *et al.*, 2003; Singh *et al.*, 2000 a; Singh *et al.*, 2000b).

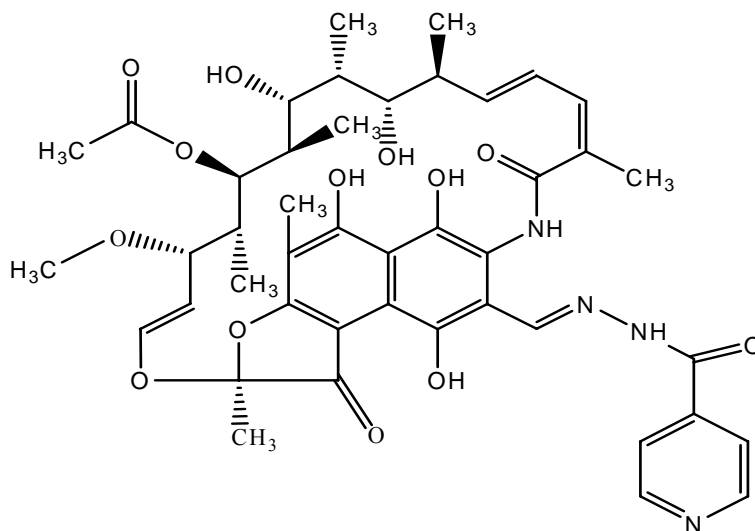


Figure 4: Structural formula of isonicotinyl hydrazone

A significant amount of rifampicin is converted to isonicotinyl hydrazone in the presence of isoniazid *in situ* under stomach acid pH conditions and also in the formulation environment.

There is a speculation for the formation of isonicotinyl hydrazone which may be formed by direct interaction of the imino group of rifampicin with the hydrazine group of isoniazid without formation of 3-formylrifamycin (Dekker and Lotter, 2003).

The formation of isonicotinyl hydrazone was also found when fixed dose combination products were exposed to accelerated stability test conditions of temperature and humidity (Singh and Mohan, 2003). Isonicotinyl hydrazone was of particular interest as it lacks both *in vitro* and *in vivo* anti-tubercular activity against *M. tuberculosis* (Mariappan, *et al.*, 2005). Therefore, it is being considered for control in pharmacopoeial monographs (WHO, 2004).

It was observed that three-four drug combinations containing rifampicin and isoniazid along with pyrazinamide and / or ethambutol hydrochloride showed far more chemical instability than two drug fixed dose combinations containing rifampicin and isoniazid (Bhutani *et al.*, 2004; Bhutani *et al.*, 2005a). The main stability concern with anti-TB fixed dose combination products is the interaction between rifampicin and isoniazid; the reaction may be catalyzed by pyrazinamide and ethambutol (Bhutani *et al.*, 2005b).

Isonicotinyl hydrazone interferes in the microbiological analysis of rifampicin (Mariappan *et al.*, 2004a) and also in the conventionally used colorimetric method of analysis of rifampicin at 475 nm (Ellard, 1999).

It was reported in literature that rifampicin was over estimated to an extent of 33%, when it was present along with isonicotinyl hydrazone (Mariappan, *et al.*, 2004b). Corresponding over estimation of rifampicin was also observed on dissolution analysis of samples of marketed fixed dose combination product and when the product was exposed to accelerated stability test condition of 40°C and 75% relative humidity. Based on this observation, recently a derivative spectroscopic method is developed, which can specifically analyze rifampicin in the presence of isonicotinyl hydrazone from fixed dose combination products (Mariappan *et al.*, 2006).

Rifampicin and isoniazid show closely overlapping UV absorption bands in the range from 200-400 nm. Hence it is not possible to analyze rifampicin and isoniazid combinations with normal UV-spectrophotometry. In this study, chemometrics-assisted spectrophotometric methods are investigated for the simultaneous determination of the above two drugs in some synthetic mixtures and commercial dosage forms that are found in Ethiopian market.

## **2. Objectives of the study**

### **2.1. General objective**

Finding a simple, selective and accurate method for determination of the individual components present in the studied dosage forms and measuring accurately the rate of formation of isonicotinyl hydrazone product that may be produced between the interaction of rifampicin and isoniazid.

### **2.2. Specific objectives**

- ✘ Providing simple, rapid and precise spectrophotometric method for determination of individual anti-TB components present in the studied anti-TB combinations.
- ✘ Providing accurate and precise chemometrics assisted method for determination of each anti-TB components in the presence of each other.
- ✘ Providing an accurate stability-indicating assay for the combination product, this may be formed due to the interaction between rifampicin and isoniazid.
- ✘ Analyzing commercial dosage forms having different production dates as an application of the developed procedures.

### **3. Experimental**

#### **3.1. Equipments**

Spectrophotometric measurements were carried out on a double beam UV-visible Spectrophotometer (Varian Cary100, Varian, Inc., Walnut Creek, California, USA) connected to a PC AMD-K6 microcomputer with Cary UV software, version 9. The absorption spectra of test and reference solutions were recorded over the range 200-400 nm. The subsequent statistical manipulation was performed by transferring the spectral data to Microsoft excel 2003 program and processing them with the standard curve fit package and matrix calculations.

#### **3.2. Chemicals and solvents**

The reference standards for rifampicin, isoniazid and pyrazinamide were gift samples from (Sanofi-Aventis, Pretoria, SA). Analytical grade hydrochloric acid (HCl) (Reagent Chemicals Service Ltd., England), methanol and chloroform (BDH chemicals Ltd., England) were used during this experiment.

#### **3.3. Pharmaceutical preparation**

The following film coated anti-tubercular tablets were obtained from Kazanchis health center and subjected to analysis by the proposed procedures: AkuriT<sup>®</sup>, Lupin, Ltd., Aurangabad, India, (Batch No 50016, Manufacturing date: December 2005, Expiry date: November 2007, containing 150 mg rifampicin, 75 mg isoniazid per tablet) and AkuriT-Z<sup>®</sup>, Lupin.Ltd., Aurangabad, India, (Batch No 52002, Manufacturing date: December 2005, Expiry date: November 2007, containing 150 mg rifampicin, 75 mg isoniazid , 400 mg pyrazinamide per tablet).

### **3.4. Preparation of standards**

In to 200 ml volumetric flask an accurately weighed 50 mg of rifampicin, isoniazid or pyrazinamide was transferred into 200 ml volumetric flask and dissolved in about 150 ml of 0.1 M hydrochloric acid with continuous shaking and then adjusted to volume with 0.1 M hydrochloric acid to give a final concentration of 250 µg/ml of the drugs.

The prepared stock solutions of rifampicin, isoniazid, and pyrazinamide were diluted quantitatively with 0.1 M HCl to obtain the appropriate dilutions for each drug according to its calibration range. The absorbance measurements were carried out in the range of 200-400 nm (digitized every 1.0 nm, 200 points per spectrum, with scan speed of 600 nm/min) using 1.00 cm quartz cells against a reagent blank (0.1 M HCl) and stored in a computer for subsequent data analysis.

### **3.5. Preparation of sample solution of rifampicin and isoniazid from the pharmaceutical formulations**

Ten tablets were accurately weighed and powdered; an amount of the powder equivalent to the average weight of one tablet was taken in a 100 ml beaker, 20 ml of 3:2 mixture of chloroform: methanol was added and the flask was shaken for 10 min. The supernatant solution was decanted in 100 ml volumetric flask and the residue was washed three times with 20 ml of the same solvent mixture. The volume was completed to the mark with chloroform:methanol mixture after combining the extract and all the washings. Aliquots of 2.67 and 1.34 ml of this solution were transferred to 100 ml volumetric flasks and diluted with 0.1 M HCl up to the mark. Absorbance measurements were also carried out in the range of 200-400 nm (digitized every 1.0 nm, 200 points per spectrum, with scan speed of 600 nm/min) using 1.00 cm quartz cells against a reagent blank (0.1 M HCl) and stored in a computer for subsequent data analysis.

### **3.6. Preparation of sample solution of rifampicin, isoniazid and pyrazinamide from the pharmaceutical formulations**

The same procedures were used as described in section 3.5 except for an aliquot of 3.33 ml of this solution were transferred to 100 ml volumetric flask and diluted with 0.1 M HCl up to the mark. The absorbance measurements were carried out in the range of 200-400 nm (digitized every 1.0 nm, 200 points per spectrum, with scan speed of 600 nm/min) using 1.00 cm quartz cells against a reagent blank (0.1 M HCl) and stored in a computer for subsequent data analysis.

### **3.7. Interactions of rifampicin and isoniazid**

Stability studies of mixture of rifampicin and isoniazid were carried out using synthetic drug mixtures in proportions equivalent to that generally encountered in dosage forms (150 mg of rifampicin and 75 mg of isoniazid) in addition to the commercial tablets. From the prepared synthetic binary mixtures and commercial tablets, 1.34 ml proportions were diluted into 100 ml with 0.1 M HCl to obtain working solutions which contains 20 and 10  $\mu\text{g/ml}$  of rifampicin and isoniazid, respectively. All the flasks were kept in a thermostatically controlled oven ( $\pm 0.1^\circ\text{C}$ )  $60^\circ\text{C}$  for the time given below. Samples for measurements were taken at 0, 30, 60, 90, 120, and 150 minutes. The absorption measurements were carried out in the range of 200-500 nm after cooling of the flasks to the room temperature.

### **3.8. Data analysis**

Data was processed on Intel Pentium IV, PC-compatible computer equipped with the essential software Microsoft excel 2003 for first derivative (Zero-crossing and ratio), CLS and MVSP version 3.13 (1985-2003) and VISTA 6.4. 3436-EWU (2001) was used for PCR calculations.

## **4. Results and discussion**

### **4.1. Preliminary studies**

The experiments were done in triplicate in all cases in three different days following the same procedure, using the same laboratory equipment and by the same analyst (to confirm both repeatability and reproducibility). Mean of the triplicate analysis for each day and all the three days was taken in this analysis.

The absorbance spectrum of pure, synthetic mixtures and dosage forms of rifampicin, isoniazid and pyrazinamide was recorded in a wavelength range of 200-400 nm in the linear calibration domain of rifampicin (10-50  $\mu\text{g/ml}$ ), isoniazid (5-40  $\mu\text{g/ml}$ ) and pyrazinamide (2-20  $\mu\text{g/ml}$ ). First derivative zero crossing, first derivative of the ratio spectra, CLS, and PCR techniques are used for the determination of pure drugs (calibration step), laboratory prepared synthetic mixtures (testing step) and the dosage forms (prediction step). From the results of this analysis various graphs are sketched using software such as and Harvard graphics 2.0.

This preliminary study may help for arriving at a final conclusion about the data structure and the proper selection of proposed analytical methods.

#### **4.1.1. Overlay plots for the given spectra**

This will make clear the presence or absence of gross outliers and clusters in the given responses. [Figures \(5-7\)](#) show the overlay plots for the studied pure compounds of isoniazid, rifampicin, and pyrazinamide, respectively at different concentration levels.

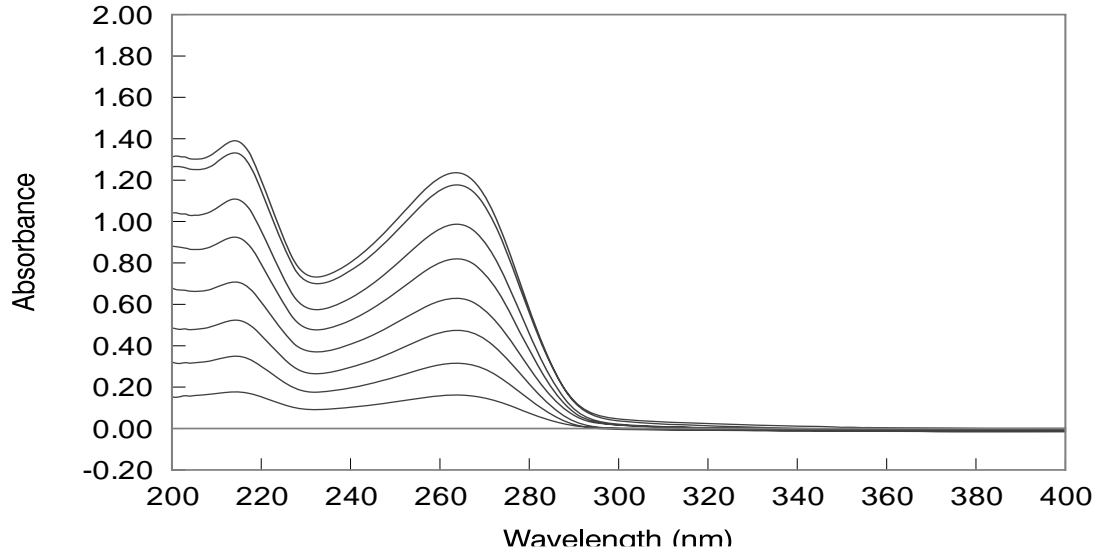


Figure 5: Overlay absorption curves of isoniazid in the calibration range (5-40  $\mu\text{g/ml}$ )

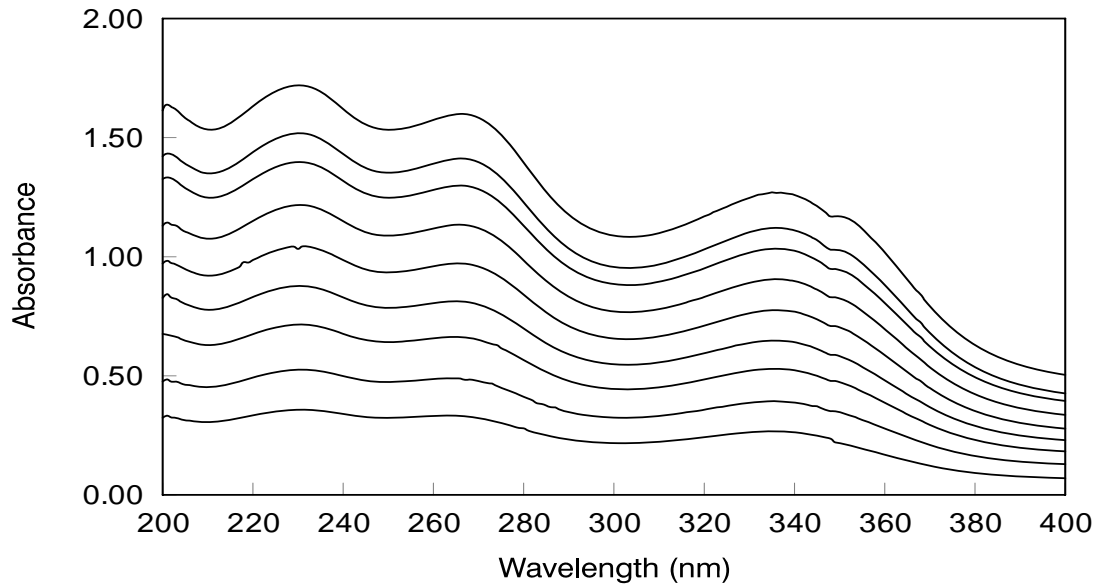


Figure 6: Overlay absorption curves of rifampicin in the calibration range (10-50  $\mu\text{g/ml}$ )

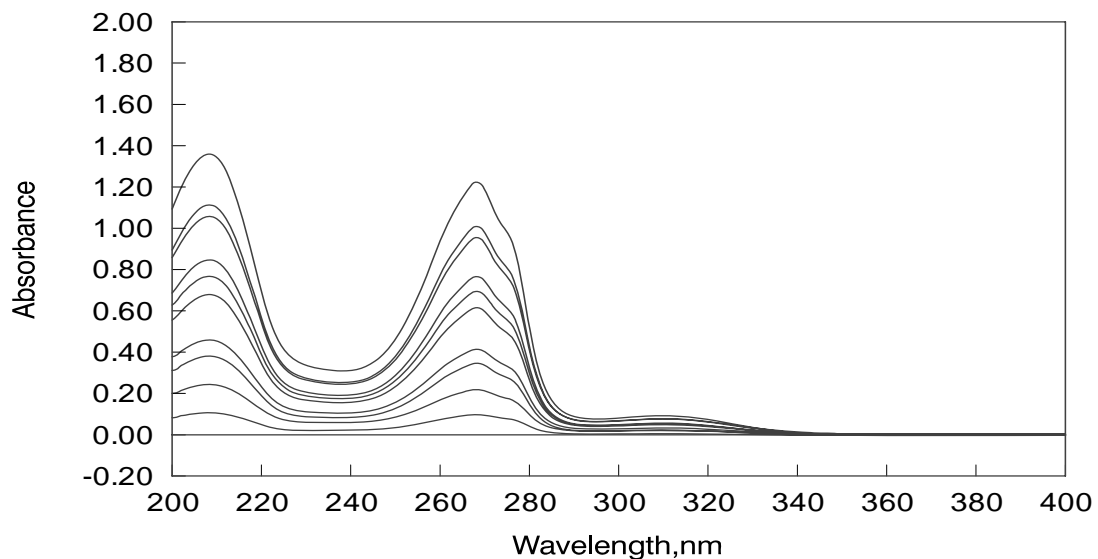


Figure 7: Overlay absorption curves of pyrazinamide in the calibration range (2-20  $\mu\text{g/ml}$ )

From the above graphs one can readily observe that there is no obvious overlapping and/or crossing of the spectra lines. The lowest concentration is at inner bottom with the weakest peak amplitude and the highest concentration is at the outer with the strongest peak amplitude. The above graphs shows that concentration and response (absorbance) increase in proportion and gives highlight to under go the studies further with the proposed chemometric techniques.

#### 4.1.2. Histogram plots of the C-values

This will make apparent the presence or absence of clusters and gross outliers in the concentration gradient. [Figures \(8 -10\)](#) show the concentration dependent histograms for the studied compounds at four randomly selected wavelengths.

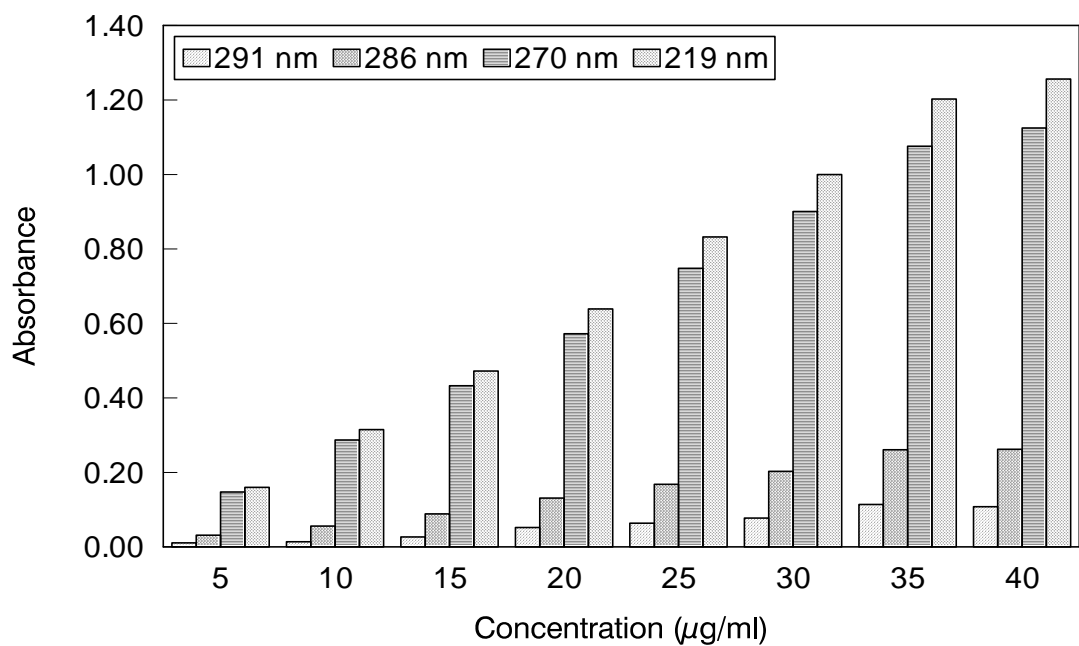


Figure 8: Histogram of C-values of isoniazid at four different wavelengths

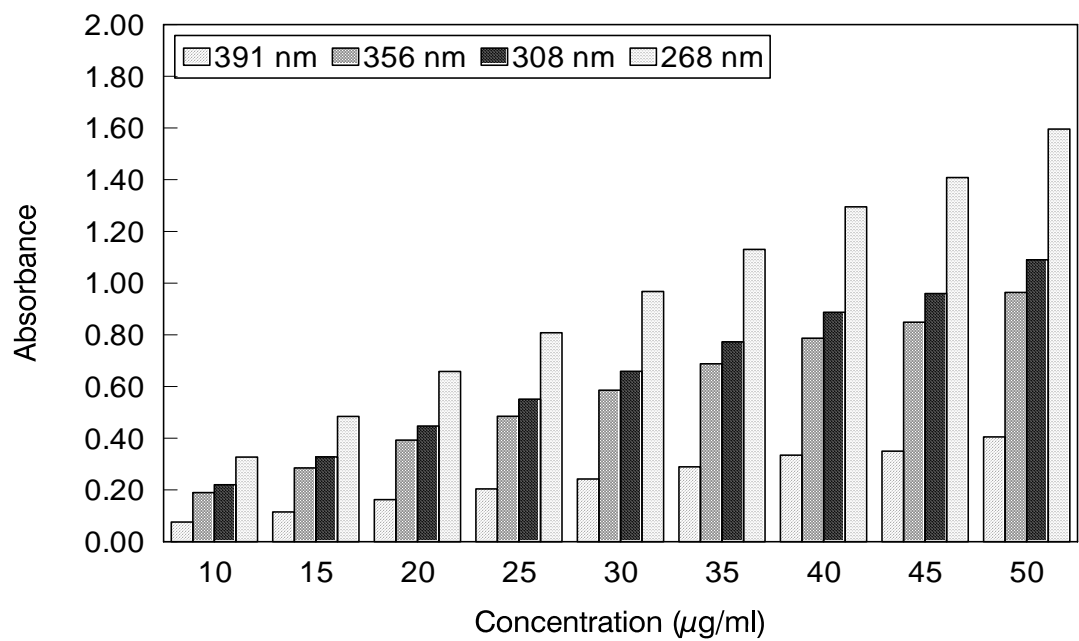


Figure 9: Histogram of C-values of rifampicin at four different wavelengths

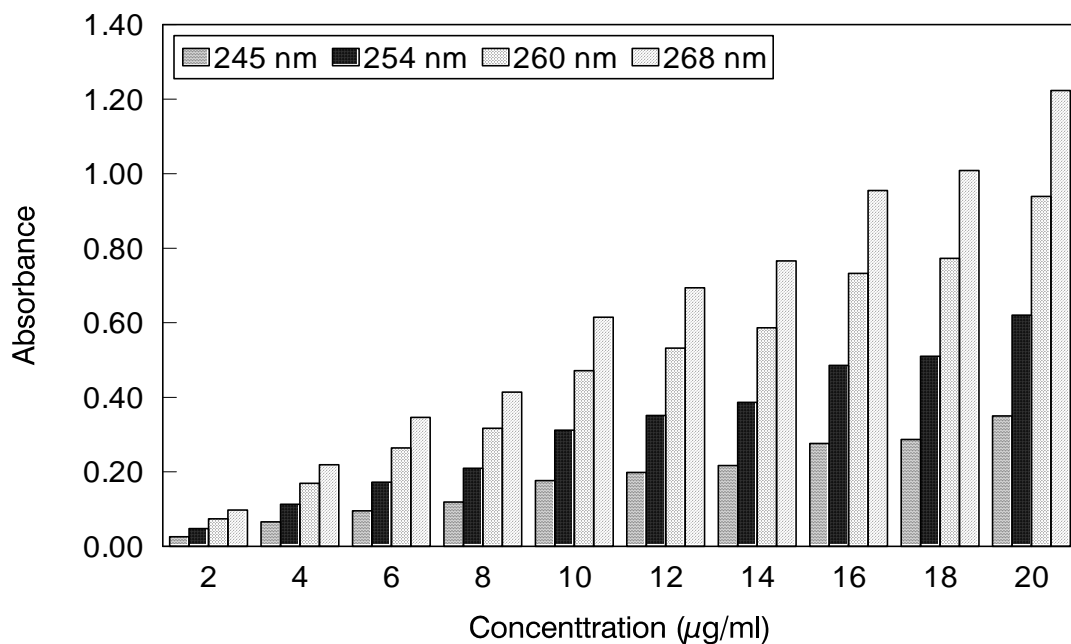


Figure 10: Histogram of C-values of pyrazinamide at four different wavelengths

It is obvious from the above histograms that in all cases the response are concentration dependent and there is no any outliers with respect to C-values. The above data (Figures 8 - 10) have shown dependable concentration-absorbance correlations that enable the studies to go further.

#### 4.1.3. Relationship of the plot between the principal components

This will provide additional insight in clustering or the presence of extreme data in absorbance. These plots are important in the case of PCR analysis because strong non-linearities if any will probably show up on one of these plots (Figures 11-14). It is recommended that, even if one has already decided to use a specific method, obtaining these plots will be advisable to check the existence of non-linearities and clusters. One has to know that at this stage exhaustive search for the presence of non-linearities, clustering and less gross outliers is not conducted. Minor non-linearities, clustering and less gross outliers do not need to be detected at this stage.

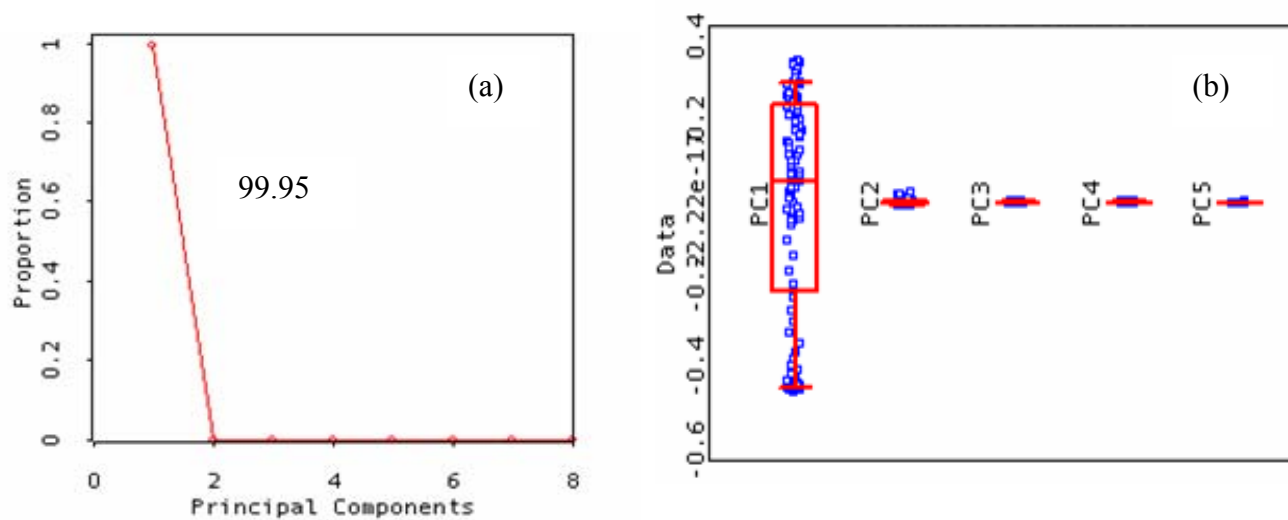


Figure 11: Scree (a) and connected box (b) Plots for principal component model of isoniazid data

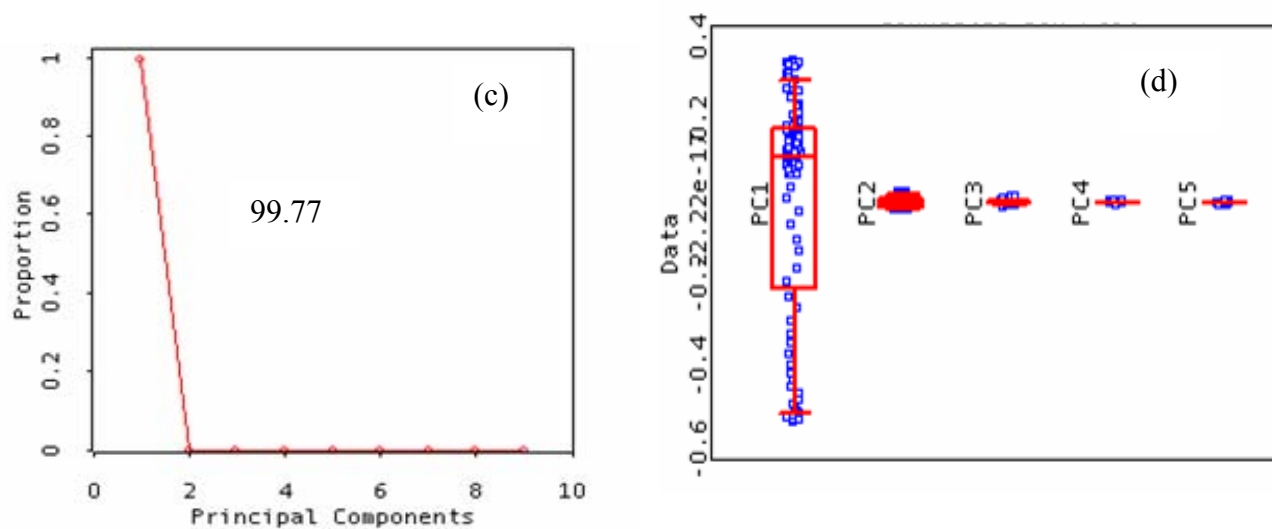


Figure 12: Scree (c) and connected box (d) plots for principal component model of rifampicin data

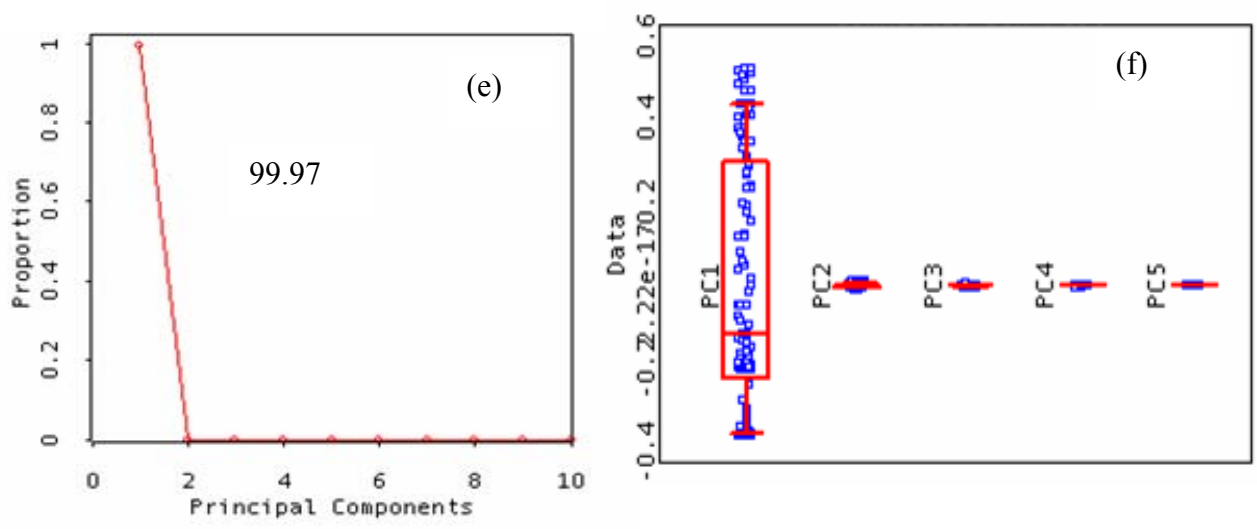


Figure 13: Scree (e) and connected box (f) plots for principal component model of pyrazinamide data

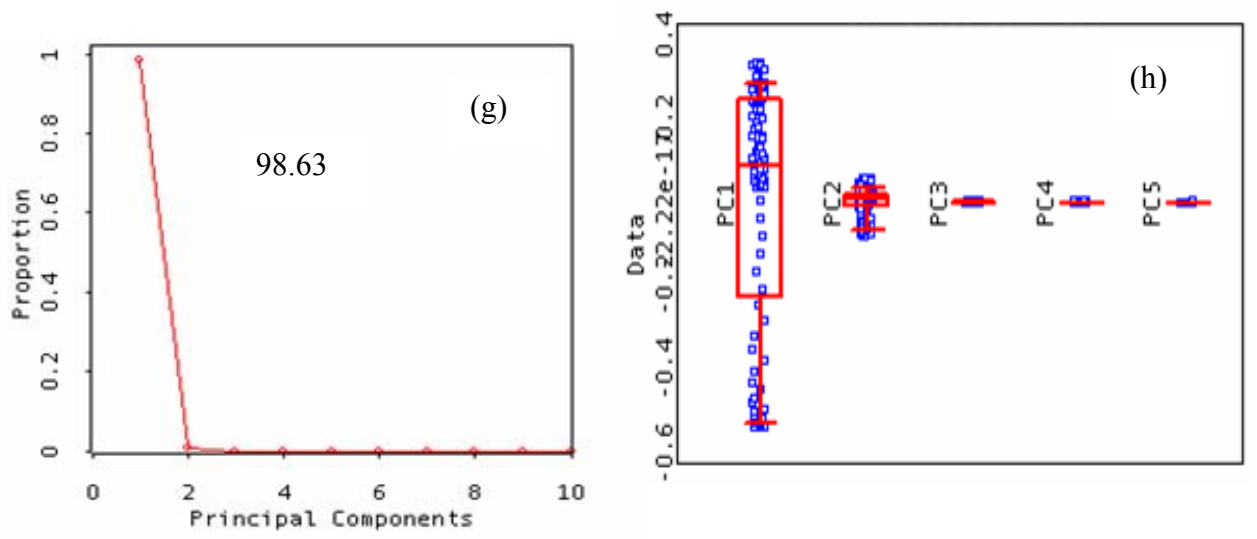


Figure 14: Scree (g) and connected box (h) plots for principal component model of rifampicin, isoniazid and pyrazinamide mixtures data

## 4.2. First Derivative spectrophotometric analysis

### 4.2.1. Zero-crossing technique

Derivative spectrophotometry has been recommended for assay of two component mixtures because it gives better resolution of spectra than that of the zero order absorption spectra (Rote and Sharma, 1997) and costly HPLC method that was used for the assay of these drugs (Shah *et al.*, 1992). In this work, first derivative spectrophotometry was examined for the assay of two component drug mixtures (rifampicin and isoniazid) due to reproducible results obtained by this technique. [Figure 15](#) shows the zero order spectra for both rifampicin and isoniazid, and shows the high degree of overlapping between them.

The first derivative spectra of the studied drugs showed good identified zero-crossing points that can be used for simultaneous determination of both drugs ([Figures 16-18](#)). The wavelengths of [320.5 and 360.5 nm](#) were used for determination of rifampicin in presence of isoniazid (zero-crossing wavelength of isoniazid) and wavelengths of [230 and 250.5 nm](#) were selected for estimation of isoniazid in presence of rifampicin (zero-crossing wavelength of rifampicin). In [Table 2](#), the correlation coefficient ( $r$ ) values obtained were close to one may show insignificance of interference come from the other constituent in this set of synthetic mixtures. Linear relationships between the derivative amplitudes and drug concentrations were obtained over the concentration range of 5-40  $\mu\text{g/ml}$  for isoniazid and 10-50  $\mu\text{g/ml}$  for rifampicin. (Linear regression ranges, intercepts (a), slopes (b), correlation and determination coefficients, limit of determination ( $\mu\text{g/ml}$ ) and limit of quantification ( $\mu\text{g/ml}$ ) are presented in [Table 2](#))

[Tables 3 and 4](#) shows the actual and predicted amounts  $\pm$  relative standard deviation of the studied drugs as given by the first derivative calculations for the spectral data obtained experimentally in the given calibration range of each drug at the wave length range from 200-400 nm. The results shows a considerable (but not perfect) degree of precision and indicate that the method can be used for analysis of binary mixtures of rifampicin and isoniazid in the given calibration domain with out prior separation. Accordingly, several prepared mixtures in the laboratory as well as commercial dosage forms were subjected to the analysis by the proposed method in order to confirm the suitability of the method for the determination of the studied drugs in pharmaceutical sample solutions.

As can be seen from the results summarized in [Tables 3 and 4](#) the concentrations predicted by the zero-crossing are considerably close to the real concentrations. The recovery in most cases is satisfactory and deviation between the estimated and true values is found between 0.1 and 3.6 % which considered acceptable results (Goicoechea and Olivieri, 1999)

The limits of detection and quantification were calculated on the bases of the following relations

$$\text{LOD} = 3 \sigma / S$$

$$\text{LOQ} = 10 \sigma / S$$

Where  $\sigma$  is the standard deviation of the intercept and S is the sensitivity (expressed here by the slopes of the calibration curves).

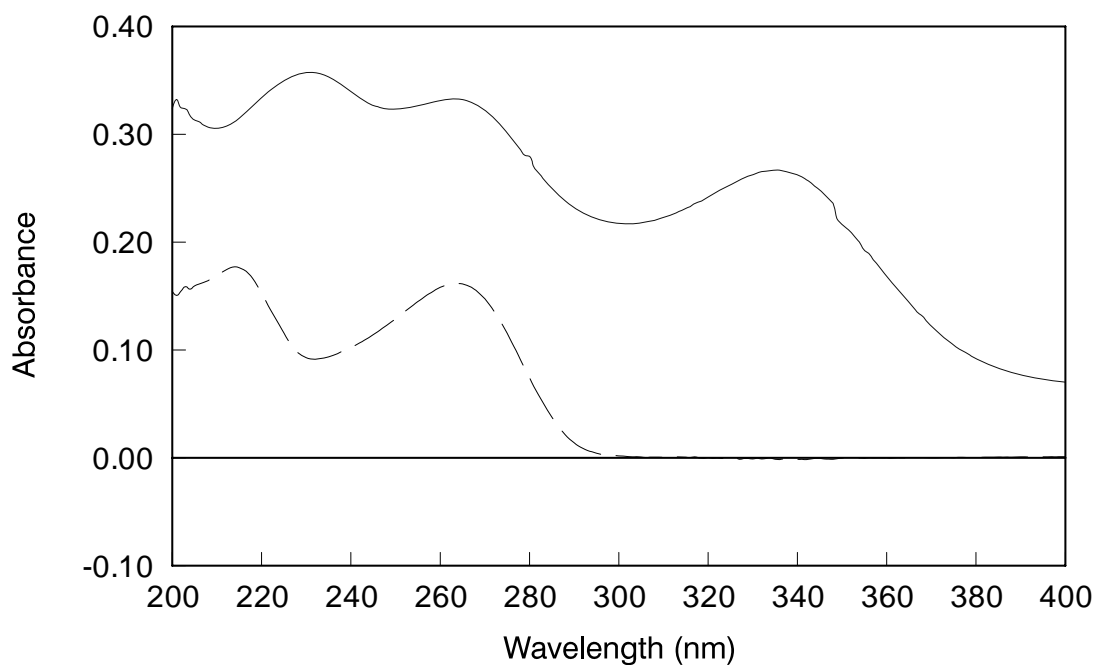


Figure 15: Degree of overlapping as indicated by the zero order absorption spectra of isoniazid (---) (5  $\mu\text{g/ml}$ ) and rifampicin (—) (10  $\mu\text{g/ml}$ )

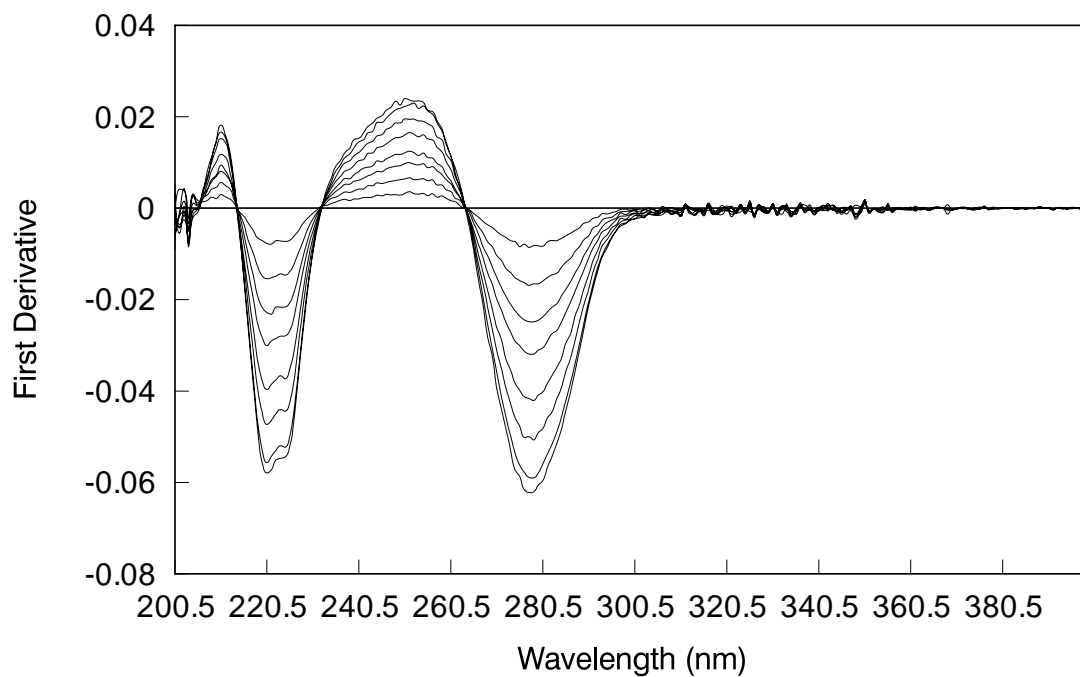


Figure 16: Overlay first derivative of isoniazid in the calibration range (5-40 µg/ml)

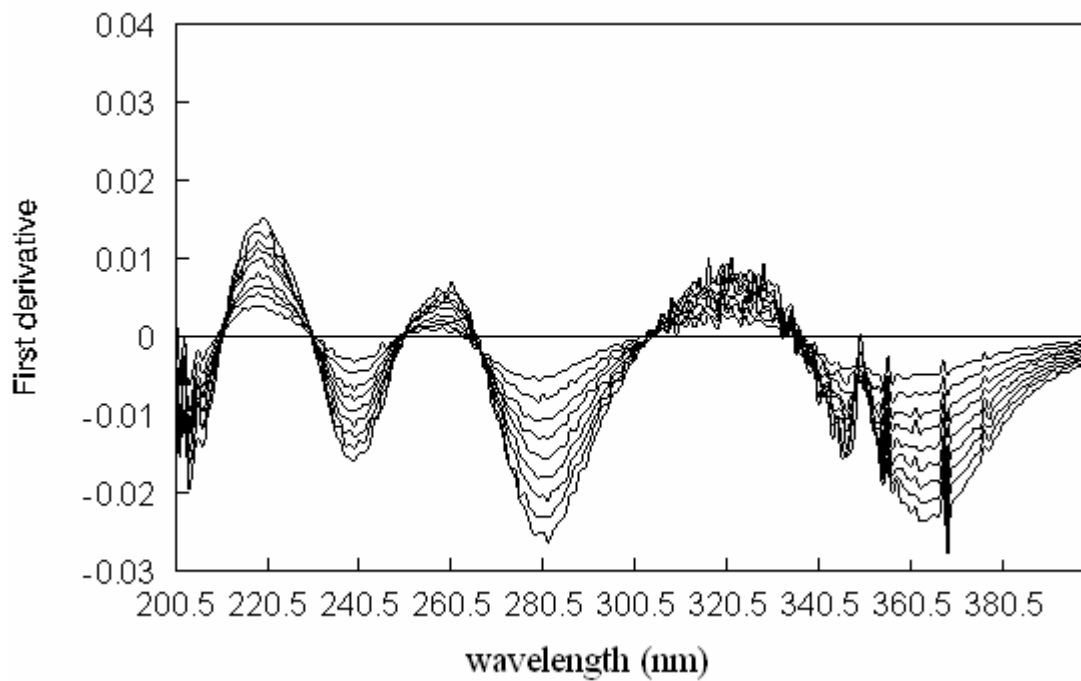


Figure 17: Overlay first derivative of rifampicin in the calibration range (10-50 µg/ml)

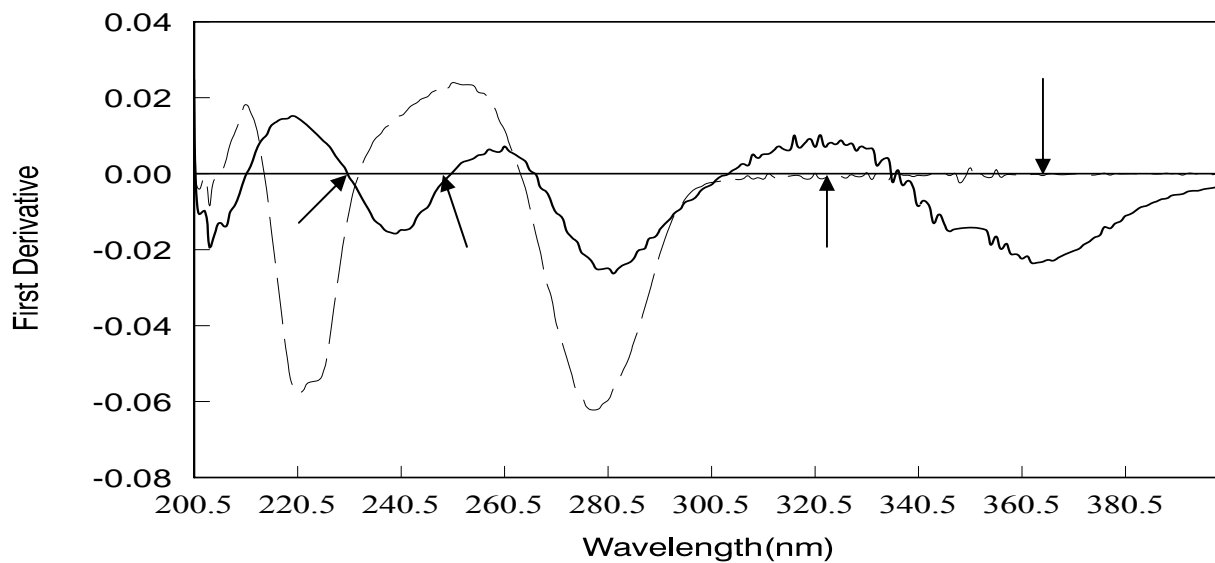


Figure 18: First derivative spectra of isoniazid (----) with zero-crossing points at 320.5, 360.5 nm and rifampicin (—) with zero-crossing points at 230 and 250.5 nm

Table 2: Spectral characteristics and analytical parameters for determination of isoniazid and rifampicin with the proposed <sup>1</sup>D spectrophotometric technique.

Standard solution of	Calibration range, µg/ml	Wavelength of determination (nm)	Linear Regression Equation Parameters					
			Intercept (a)	Slope (b)	Correlation coefficient (r)	Determination coefficient (r <sup>2</sup> )	LOD * (µg/ml)	LOQ ** (µg/ml)
Isoniazid	5-40	230	0.000341	-0.00043	0.9971	0.9942	0.81	2.69
		250.5	0.000334	0.000615	0.9974	0.9950	0.30	1.01
Rifampicin	10-50	320.5	0.0002	0.000201	0.9936	0.9873	3.760	6.535
		360.5	-0.00133	-0.00043	0.9988	0.9975	1.842	6.138

\* LOD limit of detection

\*\* LOQ limit of quantification

Table 3: Actual and predicted amounts of isoniazid given by applying <sup>1</sup>D technique for pure, synthetic mixtures with rifampicin and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	At 230 nm			At 250.5 nm		
		Found			Found		
		$\mu\text{g/ml}$	%	CV*	$\mu\text{g/ml}$	%	CV*
Pure	5	4.94	98.80	1.98	4.96	99.11	2.00
	10	9.79	98.00	0.25	9.87	98.66	1.64
	15	14.75	98.36	0.81	15.11	100.74	0.33
	20	19.60	98.00	0.09	19.64	98.20	0.17
	25	25.38	101.54	1.95	25.02	100.07	0.81
	30	29.90	99.68	1.99	30.34	101.14	1.21
	35	34.86	99.61	0.02	35.29	100.83	0.20
	40	39.22	98.04	0.53	40.42	100.76	1.25
Synthetic mixtures	5:50	4.93	98.51	1.25	5.05	100.99	1.12
	10:45	9.81	98.11	0.58	10.14	101.36	0.49
	15:40	14.92	99.48	1.48	14.94	99.61	0.87
	20:35	19.78	98.91	0.28	20.31	101.53	0.04
	25:30	25.06	100.12	1.88	25.05	100.19	0.06
	30:25	30.15	100.51	1.19	30.22	100.73	1.22
	35:20	34.95	99.87	0.31	35.26	100.74	0.72
	40:15	39.87	99.68	0.99	39.78	99.44	0.20
	20:40	19.83	99.15	1.27	20.16	100.82	0.91
	10:20	10.36	100.38	0.55	9.86	98.62	0.29
AKuriT <sup>®</sup> (INH:RIF)	10:20	9.94	99.41	0.89	9.87	98.70	0.08

\* CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

Table 4: Actual and predicted amounts of rifampicin given by applying <sup>1</sup>D technique for pure, synthetic mixtures with isoniazid and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	At 320.5 nm			At 360.5 nm		
		Found			Found		
		$\mu\text{g/ml}$	%	CV*	$\mu\text{g/ml}$	%	CV*
Pure	10	9.96	99.56	1.99	10.10	101.04	1.13
	15	14.86	99.06	0.11	14.95	99.64	0.57
	20	20.02	100.11	1.82	20.23	101.16	0.19
	25	25.42	101.70	0.31	24.87	99.47	1.03
	30	30.65	99.75	1.15	30.33	100.11	1.01
	35	35.22	100.63	1.82	35.55	101.57	1.29
	40	40.68	101.69	1.57	40.54	101.36	0.82
	45	44.92	99.71	2.00	44.29	98.42	1.40
	50	49.17	98.34	0.61	49.56	99.12	1.02
Synthetic mixtures	50:5	49.92	99.84	0.78	49.80	99.86	0.54
	45:10	45.75	101.66	1.16	45.35	100.79	0.60
	40:15	40.67	101.66	1.94	39.67	99.18	1.12
	35:20	35.09	100.25	0.23	35.47	101.33	0.14
	30:25	29.71	99.02	1.45	30.55	101.83	0.26
	25:30	25.26	101.06	1.87	24.62	98.49	1.25
	20:35	20.37	101.87	1.29	20.06	100.30	0.92
	15:40	14.92	99.44	0.97	14.94	99.60	1.02
	40:20	39.96	99.89	0.27	40.48	101.19	0.89
20:10	19.68	98.41	1.08	19.61	98.07	1.76	
AKuriT <sup>®</sup> (RIF:INH)	20:10	20.24	101.20	1.61	19.87	99.34	1.06

\*CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

#### 4.2.2. Derivative ratio technique

Derivative ratio method uses a standard divisor when the spectra of the components are overlapped. The use of standardized spectra as divisor will minimize experimental error and background noise. For the determination of isoniazid, the absorption spectra of standard solutions of isoniazid were divided (amplitude by amplitude at appropriate wavelengths) by absorption spectrum of a well-selected standard solution of rifampicin 30 µg/ml to obtain the corresponding ratio spectra (Figure 19). Then the first derivative of the obtained ratio spectra were calculated with  $\Delta\lambda = 5$  nm (Figure 20). From this figure, isoniazid can be determined in this mixture by measuring the amplitude at 222.5, 247.5, and 282.5 nm where there is no contribution or interference from rifampicin. On the other hand, for the determination of rifampicin, an analogous procedure was followed. The absorption spectra of rifampicin were divided by that of a solution of isoniazid 25 µg/ml (Figure 21), and the first derivative of the developed ratio spectra were calculated with  $\Delta\lambda = 5$  nm (Figure 22). It was shown from this figure that, rifampicin can be determined by measuring the amplitude at many wavelengths where isoniazid has no contribution. It was found that the amplitude at 322.5 and 327.5 nm give the best results. Tables 6 and 7 summarize the actual, predicted, synthetic mixtures and commercial dosage form of the studied drugs.

The influence of  $\Delta\lambda$  for obtaining the first derivative of the ratio spectra was tested to obtain the optimum wavelength interval;  $\Delta\lambda = 5$  nm was considered as the most suitable values. The effect of the divisor concentration on the calibration graphs was also studied. The results obtained from this study indicated that the divisor concentration has no significant effect on the assay results. The regression equations for both drugs were derived using the least-squares regression analysis for all used techniques and at the specified wavelengths for each drug, Table 5 summarizes the obtained results, which include the intercepts (a), slopes (b), correlation coefficients (r), determination coefficients ( $r^2$ ), limits of detection (µg/ml) and limits of quantification (µg/ml). The slopes are used as measure of sensitivity of the proposed methods, while intercepts indicate the magnitude of background interferences. Results listed in Table 5 indicate the high sensitivity and low background effects. The results confirm a good recovery in the synthetic mixtures as well as the commercial dosage form and indicates that method is suitable for this analysis in the given calibration domain for each drugs.

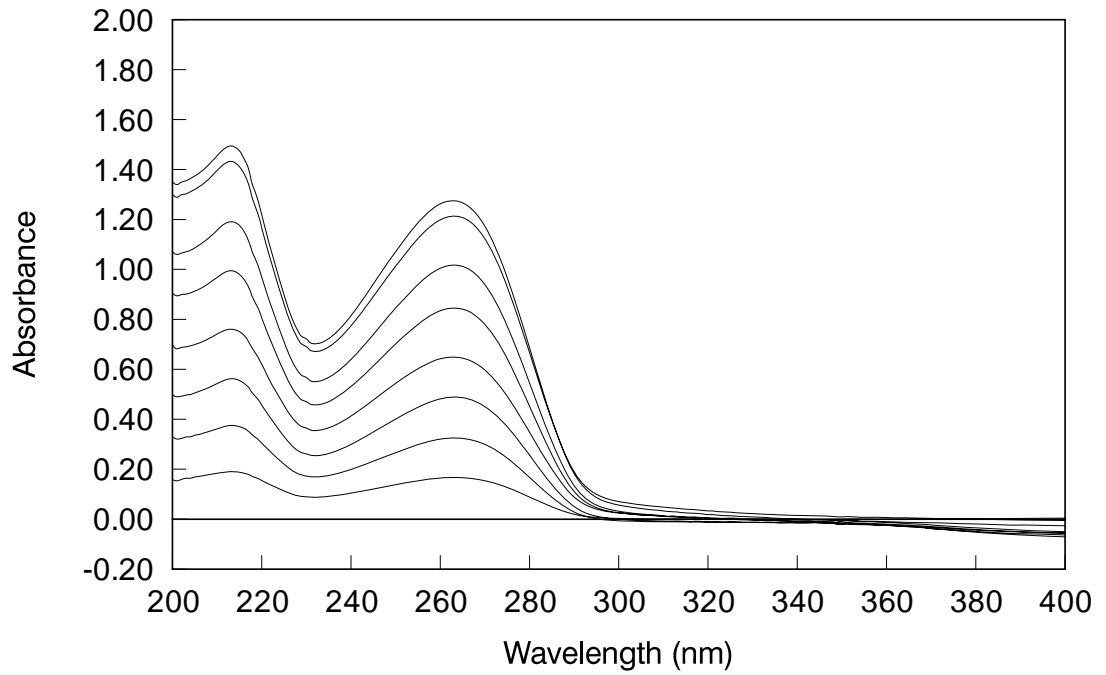


Figure 19: Ratio spectra of isoniazid (5 – 40 µg/ml) divisor is 30 µg/ml rifampicin

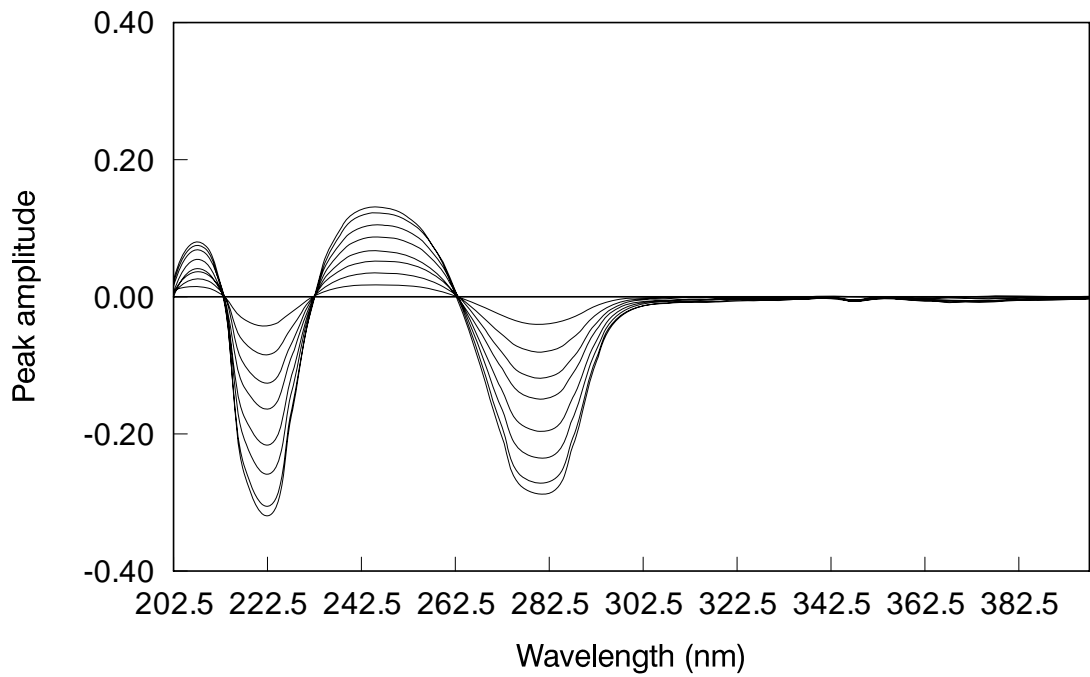


Figure 20: First derivative ratio spectra of isoniazid (5-40 µg/ml) divisor is 30 µg /ml rifampicin

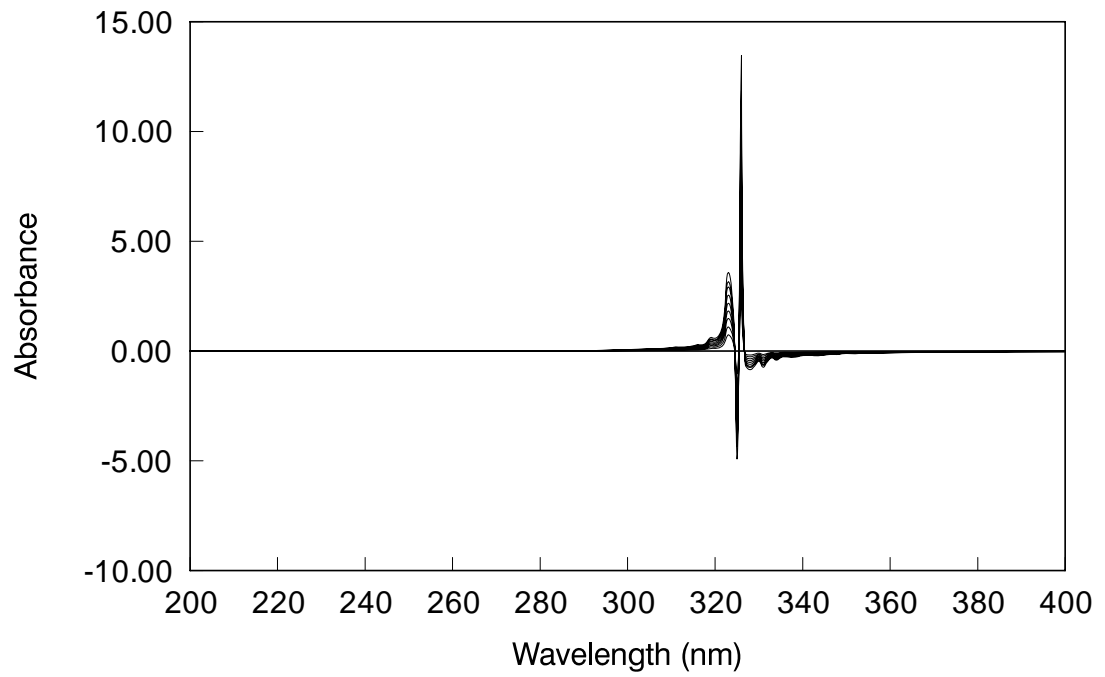


Figure 21: Ratio spectra of rifampicin (10-50 µg/ml) divisor is 25 µg/ml isoniazid

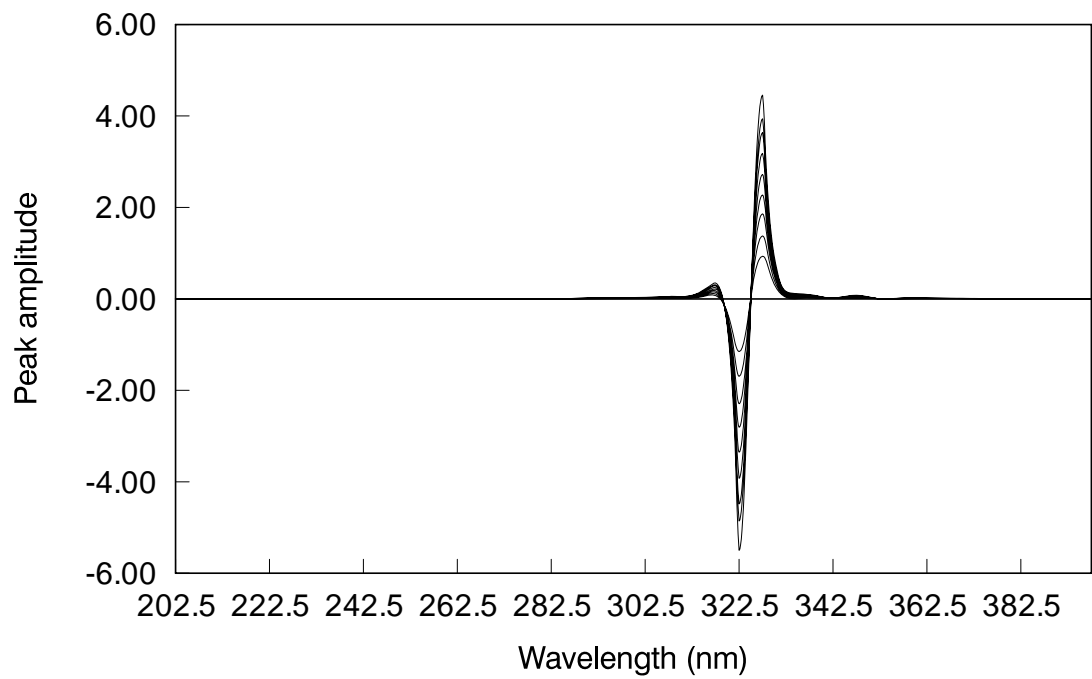


Figure 22: First derivative ratio spectra of rifampicin (10-50 µg/ml) divisor is 25 µg/ml isoniazid

Table 5: Spectral characteristics and analytical parameters for determination of isoniazid and rifampicin with the proposed <sup>1</sup>D ratio spectrophotometric technique.

Standard solution of	Calibration range, (µg/ml)	Wavelength of determination (nm)	Linear Regression Equation parameters					
			Intercept (a)	Slope (b)	Correlation coefficient (r)	Determination coefficient (r <sup>2</sup> )	LOD * (µg/ml)	LOQ ** (µg/ml)
Isoniazid	5-40	222.5	-0.0005	-0.0015	0.9961	0.9922	0.632	2.11
		247.5	0.0003	0.0006	0.9951	0.9902	0.59	1.96
		282.5	-0.0009	0.0014	0.9967	0.9937	0.351	1.17
Rifampicin	10-50	322.5	-102.25	-107.95	0.9995	0.9989	1.22	4.08
		327.5	83.52	87.49	0.9995	0.9989	1.22	4.07

\* LOD limit of detection

\*\* LOQ limit of quantification

Table 6: Actual and predicted amounts of isoniazid given by applying <sup>1</sup>D ratio technique for pure, synthetic mixtures with rifampicin and commercial dosage forms

	Real concentrations (µg/ml)	At 222.5 nm			At 247.5 nm			At 282.5 nm		
		Found			Found			Found		
		µg/ml	%	CV*	µg/ml	%	CV*	µg/ml	%	CV*
Pure	5	4.87	101.76	0.55	4.91	98.12	0.50	4.84	99.70	1.51
	10	9.91	99.10	0.75	10.10	100.96	1.66	9.96	99.69	1.49
	15	14.79	98.61	1.11	15.20	101.34	0.32	14.99	99.95	1.38
	20	19.65	98.26	0.45	19.70	98.52	1.65	19.99	99.96	0.87
	25	24.51	98.03	0.89	24.76	99.53	1.42	25.16	100.62	0.03
	30	29.55	98.49	1.58	29.45	98.17	0.31	30.39	101.31	0.29
	35	35.18	100.51	0.67	34.69	99.11	1.87	34.98	99.93	1.47
	40	38.70	99.54	0.73	39.68	98.10	0.90	39.87	99.66	0.44
Synthetic mixtures	5:50	4.99	99.78	2.00	4.77	99.76	0.01	5.04	100.08	2.00
	10:45	10.06	100.58	1.47	9.81	98.07	0.09	10.03	100.32	1.09
	15:40	14.94	99.58	0.09	15.04	100.24	0.27	14.89	99.26	1.71
	20:35	19.99	99.93	1.06	20.10	100.50	1.61	19.88	99.40	0.48
	25:30	24.69	98.74	1.76	25.20	100.78	1.34	24.74	98.97	1.38
	30:25	30.10	100.33	1.13	30.26	100.85	1.32	30.17	100.57	1.15
	35:20	35.17	100.50	0.88	35.08	100.22	0.95	35.11	100.31	0.48
	40:15	40.00	100.01	0.37	39.60	99.00	1.09	40.01	100.03	0.24
	20:40	19.88	99.39	0.05	19.96	99.81	0.19	19.99	99.96	0.78
	10:20	10.16	101.65	0.49	10.20	101.97	0.67	10.02	100.18	1.58
AKuriT <sup>®</sup> (INH:RIF)	10:20	9.94	99.42	0.71	9.89	98.86	0.64	9.87	98.72	0.55

\*CV coefficient of Variation as calculated from the corresponding calibration model (n = 3)

Table 7: Actual and predicted amounts of rifampicin given by applying <sup>1</sup>D ratio technique for pure, synthetic mixtures with isoniazid and commercial dosage forms

	Real concentrations (µg/ml)	At 322.5 nm			At 327.5 nm		
		Found			Found		
		µg/ml	%	CV*	µg/ml	%	CV*
Pure	10	9.91	99.08	1.69	9.91	99.09	1.67
	15	14.76	98.42	0.95	14.76	98.42	1.26
	20	20.29	101.47	0.06	20.29	101.47	0.50
	25	25.05	100.19	0.42	24.89	99.58	0.32
	30	30.13	100.42	0.13	30.13	100.43	0.69
	35	35.40	101.15	1.10	35.41	101.17	0.93
	40	39.48	98.70	1.19	39.72	99.31	0.89
	45	44.85	99.66	1.21	44.97	99.93	0.56
	50	49.97	99.94	1.01	49.99	99.98	0.16
Synthetic mixtures	50:5	49.08	98.15	0.08	49.21	98.41	0.03
	45:10	45.29	100.65	0.66	45.49	101.08	0.84
	40:15	40.30	100.37	0.38	40.40	101.01	0.34
	35:20	35.64	101.84	1.11	35.56	101.59	0.26
	30:25	30.86	101.05	1.02	30.35	101.16	1.73
	25:30	25.11	100.43	1.44	25.11	100.43	1.77
	20:35	20.06	100.32	1.97	20.07	100.34	1.59
	15:40	14.98	99.88	1.21	15.06	100.54	0.41
	40:20	39.93	99.83	0.82	39.58	98.94	0.86
	20:10	20.09	100.44	1.00	19.86	99.31	0.18
AKuRiT <sup>®</sup> (RIF:INH)	20:10	19.38	99.38	1.40	19.82	99.12	1.69

\*CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

### 4.3. Multivariate calibration analysis

#### 4.3.1. Classical least square analysis

Full spectrum methods usually provide significant improvement in precision over methods restricted to a small number of wavelengths and also make it available the full-spectrum residuals for examination and interpretation (Madan *et al.*, 2005). The simplest of them is the classical least squares (CLS) analysis (Mahalanabis *et al.*, 1989). It should certainly be preferred when the selection of variables is simple. In such cases, the regression coefficients for different selected collinear wavelengths may have relatively little meaning for interpretation purposes, but the model performs well, both in the calibration and prediction stages, provided that the model possesses linearity between responses and concentrations and the prediction is performed within the calibration domain. In addition, the baseline effects and noise are probably non-significant or of very low significance. Under these conditions CLS is probably the method to be recommended.

In CLS a linear relationship between the absorbance and the component concentrations at each wavelength are assumed. In matrix notation, the model for  $m$  calibration standards containing  $l$  chemical components with spectra at  $n$  digitized wavelengths is given by the equation:

$$\mathbf{A} = \mathbf{CK} + \mathbf{E}$$

Where  $\mathbf{A}$  is the  $m \times n$  matrix of calibration spectra,  $\mathbf{C}$  the  $m \times l$  matrix of component concentrations,  $\mathbf{K}$  the  $l \times n$  matrix of regression coefficient, and  $\mathbf{E}$  the  $m \times n$  matrix of spectral errors or residuals not fit by the model. By means of the calibration sample set, estimation of absorptivities is possible by solving the  $\mathbf{K}$  matrix according to the general least-squares solution indicated by the equation:

$$\mathbf{K} = (\mathbf{C}^t\mathbf{C})^{-1} \mathbf{C}^t\mathbf{A}$$

During prediction, the solution for the vector of unknown component concentrations ( $\mathbf{C}_{un}$ ) is given by the equation

$$\mathbf{C}_{un} = \mathbf{A}_{un} \mathbf{K}^t (\mathbf{K}\mathbf{K}^t)^{-1}$$

Where ( $\mathbf{A}_{un}$ ) is the spectrum of the unknown samples.

The absorption spectrum for the studied compounds is shown in (Figure 23). As can be seen, a considerable degree of spectral overlapping occurs in the region from 200 - 350 nm. The degree of spectral overlapping can be conveniently given by  $(D_i)^{0.5}$ , where  $D_i$  is the magnitude of dependency that can be calculated for a two components mixture from the equation:

$$D_i = \frac{(\sum k_2 k_2^t)^2}{\sum k_1 k_1^t \sum k_2 k_2^t}$$

Where  $K_1$  and  $K_2$  are the  $l \times n$  matrices of regression coefficients for the compounds 1 and 2, respectively.

In case of the presently studied compounds, the spectra shown in Figure 23 lead to  $D_i = 0.88$ ,  $D_i = 0.60$ ,  $D_i = 0.91$  implying a 93.84 %, 95.62 % and 77.76 % of spectral overlapping for isoniazid with rifampicin, pyrazinamide with rifampicin and isoniazid with pyrazinamide respectively of the studied mixtures this may not be resolved using normal spectroscopy (Rote and Sharma, 1996).

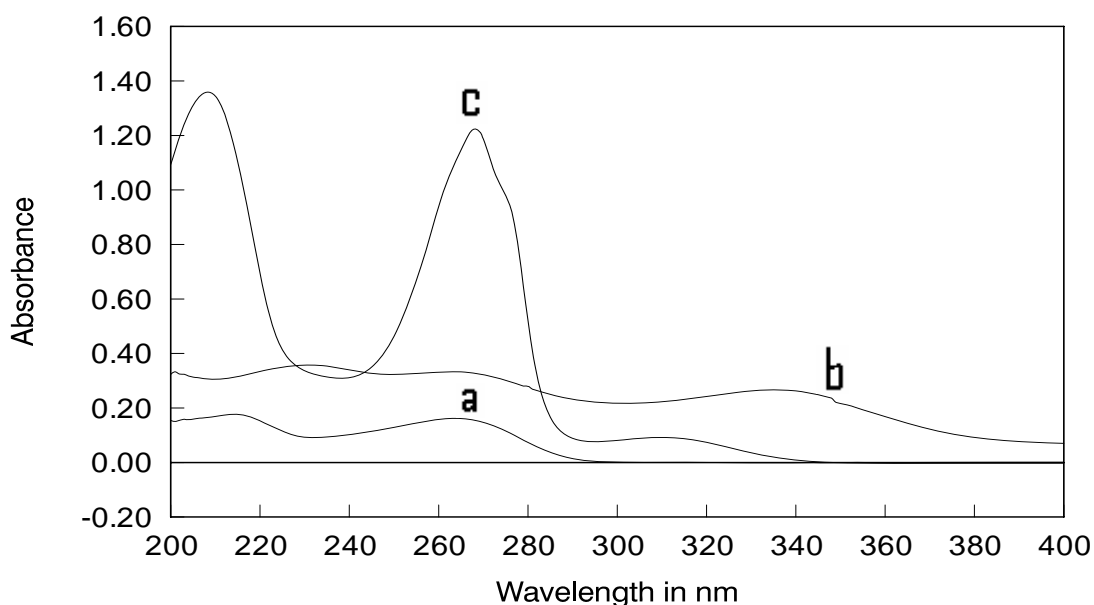


Figure 23: Degree of overlapping as indicated by zero order absorption spectra of isoniazid (a) 5  $\mu\text{g/ml}$ , rifampicin (b) 10  $\mu\text{g/ml}$  and pyrazinamide (c) 20  $\mu\text{g/ml}$

Tables (8-9) show the actual and predicted amounts  $\pm$  relative standard deviation of the studied drugs. The results confirm a good of recovery of above 98% and indicate that method is suitable for this analysis in the given domain for each drug. Several synthetic binary and ternary (in the presence of pyrazinamide ) mixtures were subjected to the CLS analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. In a previous CLS study of binary mixtures of rifampicin and isoniazid, (Mahalanabis *et al.*, 1989), it was suggested that the optimum working region involved  $\lambda < 300$  nm. However in the presently studied cases, the spectral region 200-350 nm lead to good recoveries. Tables (8-9) summarize the results obtained for the suggested synthetic mixtures.

As could be seen, the concentrations predicted by the model are very close to the real concentrations, the recoveries in all cases were satisfactory. It can be observed from this set of results that the drug mixture determination is perfectly feasible and the multivariate calibration model i.e., CLS allows a significant reduction of errors in relation to the determination by univariates.

On the other hand, the results for synthetic mixtures and commercial dosage forms with comparable concentrations were found closely matched. This indicated that, the present or added excipients and additives did not interfere with the determinations

Table 8: Actual and predicted amounts of rifampicin given by applying CLS technique for pure, synthetic mixtures with isoniazid, and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	10	10.12	101.18	1.78
	15	14.98	99.87	1.82
	20	19.78	98.90	1.97
	25	24.72	98.88	1.33
	30	30.13	100.43	0.91
	35	35.19	100.54	0.73
	40	40.27	100.68	1.98
	45	44.76	99.47	0.77
	50	50.13	100.27	0.82
Synthetic mixture	50:5	50.71	101.42	0.91
	45:10	45.07	100.16	0.76
	40:15	40.11	100.28	1.13
	35:20	35.18	100.51	1.12
	30:25	29.39	97.96	1.71
	25:30	25.01	100.06	1.90
	20:35	20.03	100.17	0.97
	15:40	14.88	99.19	0.84
	40:20	39.37	98.43	1.11
	20:10	19.78	98.90	0.86
AkuriT <sup>®</sup> (RIF:INH)	20:10	19.81	99.06	1.78

\*CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

Table 9: Actual and predicted amounts of isoniazid given by applying CLS technique for pure, synthetic mixtures with rifampicin, and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	5	4.94	98.81	1.83
	10	9.81	98.12	2.01
	15	14.75	98.35	1.92
	20	19.75	98.77	1.23
	25	25.25	100.98	2.02
	30	30.41	101.38	2.14
	35	35.70	102.00	1.94
	40	39.57	98.92	2.33
Synthetic mixture	5:50	5.04	100.78	1.98
	10:45	10.01	100.12	1.38
	15:40	14.97	99.77	1.76
	20:35	19.85	99.23	1.72
	25:30	24.68	98.71	1.76
	30:25	29.68	98.92	1.33
	35:25	34.34	98.12	0.93
	40:15	39.27	98.18	1.94
	20:40	19.69	98.44	1.92
	10:20	10.04	100.42	0.98
AkuriT <sup>®</sup> (INH:RIF)	10:20	9.93	99.31	2.03

\*CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

Table 10: Actual and predicted amounts of rifampicin given by applying CLS technique for pure, synthetic mixtures with isoniazid and pyrazinamide, and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	10	10.19	101.93	1.23
	15	14.92	99.47	1.98
	20	19.49	97.46	1.37
	25	24.74	98.96	1.82
	30	30.28	100.93	1.77
	35	35.37	101.06	1.79
	40	40.49	101.25	1.72
	45	44.73	99.40	1.88
	50	49.79	99.59	1.92
Synthetic mixture	15:5:15	14.78	98.55	2.01
	20:5:20	19.84	99.19	0.98
	25:5:25	24.56	98.36	0.87
	10:5:25	10.09	100.89	0.72
	15:5:25	15.03	100.23	1.12
	5:10:25	4.92	98.44	1.11
	10:10:25	10.18	101.77	1.73
	AkuriT-Z <sup>®</sup> (RIF:INH:PZA)	10:5:25	9.96	99.60

\*CV is the coefficient of variation as calculated from the corresponding calibration model (n = 3)

Table 11: Actual and predicted amounts of isoniazid given by applying CLS technique for pure, synthetic mixtures with rifampicin and pyrazinamide ,and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	5	4.97	99.31	1.38
	10	10.03	100.23	1.74
	15	14.84	98.92	1.98
	20	20.11	100.55	2.00
	25	25.29	101.19	2.01
	30	30.32	101.07	1.72
	35	34.79	99.41	1.39
	40	40.28	100.71	1.22
Synthetic mixture	5:15:15	5.03	100.64	1.18
	5:20:20	5.03	100.61	1.34
	5:25:25	5.04	100.93	2.00
	5:10:25	5.10	102.16	1.91
	5:15:25	5.12	102.16	1.72
	10:5:25	9.92	102.37	1.85
	10:10:25	9.96	99.56	1.57
AkuriT-Z <sup>®</sup> (INH:RIF:PZA)	5:10:25	5.05	101.03	2.03

\*CV is the coefficient of variations as calculated from the corresponding calibration model (n = 3)

### 4.3.2. Principal component regression analysis

Principal component regression method combines the principal component analysis (PCA) spectral decomposition with inverse least squares regression method to create a quantitative model for complex samples and do not require detail of all components. Unlike quantitation methods based directly on Beer's law which attempt to calculate the absorptivity coefficients for the constituents of interest from a direct regression of the constituent concentration on to the spectroscopic responses, PCR method regress the concentrations on the PCA scores (Mahalanabis *et al.*, 1989). As a first step of PCR method, the proper number of components in the PCA model must be determined. Analytically in the PCR methods, the principal components that presenting 92% of the data or more are used the next steps of calculations. The data from isoniazid, rifampicin and pyrazinamide mixtures (Figure 14) indicate that the residual variance approximately levels off after the first component and the first components accounts for about 98.63% of the total data variance fit by the whole components.

The main equation for calculating the concentration can be written as:

$$\mathbf{C} = \mathbf{F} \cdot \mathbf{A}_{\text{Proj}}$$

Where C is the concentration components and F is the regression matrix of the new coordinates, thus;

$$\mathbf{C} \cdot \mathbf{A}_{\text{Proj}} \cdot [\mathbf{A}_{\text{Proj}} \cdot \mathbf{A}_{\text{Proj}}^t]^{-1} = \mathbf{F}$$

Then the calculated F values are used to predict the concentrations in an unknown sample from its measured spectrum as follows:

$$\mathbf{C}_{\text{un}} = \mathbf{F} \cdot \mathbf{V}_c^t \mathbf{A}_{\text{un}} \text{ or } \mathbf{C}_{\text{un}} = \mathbf{F}_{\text{cal}} \cdot \mathbf{A}_{\text{un}}$$

Where  $\mathbf{F}_{\text{cal}}$  = the quantity  $\mathbf{V}_c^t \cdot \mathbf{A}$  pre-calculated at calibration time.

The obtained data showed the following characters:-

1. Absorption spectra and spectral constants of the targeted drugs agree well with the reported spectral data.
2. There was no obvious outlier, marked errors or data clustering were detected in the collected data points either for C- or A-values. (Figures 8-10).

3. Calibration space for each compound covers the expected prediction space according to the ratios used for the prepared synthetic mixtures and those amounts labeled for the commercial dosage forms.
4. There was a strong correlation between the absorbance and concentration as indicated by their calibration equations and correlation coefficients as given by the CLS and PCR of the spectral data that obtained experimentally in the range from 200 to 350 nm.
5. Correlation between the first- two principal components in case of PCR model, (PC1 and PC2), it was found that all data points occur in one and the same plan as seen in Figure 14.

Several ternary synthetic mixtures were subjected to the PCR analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. Table 12 and 13 shows the actual and predicted amounts  $\pm$  relative standard deviation of the studied drugs. As could be seen, the concentrations predicted by the model are very close to the real concentrations, the recoveries in all cases were satisfactory. The results confirm a good recovery in the synthetic mixtures as well as the commercial dosage form and indicates that method is suitable for this analysis in the given calibration domain for each drugs.

Table 12: Actual and predicted amounts of rifampicin given by applying PCR technique for pure, synthetic mixtures with isoniazid and pyrazinamide and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	10	9.91	99.13	1.07
	15	14.83	98.87	1.33
	20	19.78	98.87	1.54
	25	24.75	99.00	1.67
	30	29.62	98.73	1.63
	35	35.44	101.26	1.64
	40	39.55	98.88	1.34
	45	45.09	100.20	0.78
	50	50.13	100.26	0.97
Synthetic mixture	15 : 5 : 15	14.91	99.40	1.22
	20 : 5 : 20	19.78	98.90	1.56
	25 : 5 : 25	25.08	100.31	2.00
	10 : 5 : 25	10.07	100.66	0.97
	15 : 5 : 25	14.87	99.13	0.45
	5 : 10 : 25	4.94	98.80	1.34
	10 : 10 : 25	10.06	100.55	1.66
	15 : 5 : 15	14.91	99.40	1.56
	20 : 5 : 20	19.78	98.90	1.22
AkuriT-Z <sup>®</sup> (RIF:INH:PZA)	10 : 5 : 27	10.076	100.76	1.88

\*CV is the coefficient of variations as calculated from the corresponding calibration model (n = 3)

Table 13: Actual and predicted amounts of isoniazid given by applying PCR technique for pure, synthetic mixtures with rifampicin and pyrazinamide and commercial dosage forms

	Real concentrations ( $\mu\text{g/ml}$ )	Found		
		$\mu\text{g/ml}$	%	CV*
Pure	5	4.96	99.20	1.15
	10	9.922	99.22	1.29
	15	14.65	97.68	1.32
	20	20.18	100.90	1.54
	25	25.37	101.48	1.57
	30	29.56	98.53	1.78
	35	34.77	99.34	2.01
	40	40.12	100.30	1.44
Synthetic mixture	5 : 15 : 15	4.92	98.44	1.47
	5 : 20 : 20	4.95	99.00	1.47
	5 : 25 : 25	4.92	98.36	1.11
	5 : 10 : 25	5.01	100.16	1.95
	5 : 15 : 25	5.01	100.22	1.86
	10 : 5 : 25	10.02	100.23	0.87
	10 : 10 : 25	10.09	100.98	1.55
	5 : 15 : 15	4.92	98.44	0.96
	5 : 20 : 20	4.95	99.00	0.96
AkuriT-Z <sup>®</sup> (INH:RIF:PZA)	5 : 10 : 27	4.93	98.6	2.00

\*CV is the coefficient of variations as calculated from the corresponding calibration model (n = 3)

#### **4.4. Comparison of the results from the proposed methods**

As shown in the respective tables the actual and predicted amounts  $\pm$  the relative standard deviation of the studied drugs as given by the first derivative zero-crossing (Tables 3 and 4), first derivative ratio (Tables 6 and 7), CLS and PCR (Tables 8-13) of the spectral data that obtained experimentally in the calibration range of each drug at the wavelength range from 200-400 nm. The results confirm considerable degree of agreement between the four methods for the analysis of the drugs in binary and ternary (in the presence of pyrazinamide) mixtures and commercial dosage forms. Which indicate these methods are suitable for analysis in the given calibration domain for each drug.

Several laboratory prepared mixtures of the studied drugs were subjected to analysis by the four techniques in order to confirm the suitability of the calibration models for determination of the studied drugs in the pharmaceutical sample solutions and to verify the precision of the methods for analysis of such mixtures and matching the commercial dosage forms with those having comparable concentrations. The above tables summarize the results obtained for the suggested binary and ternary mixtures. As can be seen, the concentrations predicted by the models are considerably close to the real concentrations, the recoveries in most cases were satisfactory and the deviation range between the estimated and true concentrations were found between 0.1 and 3.6% with relative standard deviation between 0.04 and 1.94% for first derivative zero-crossing, 0.13 and 4.6% with relative standard deviation between 0.03 and 2% for first derivative ratio, 0.04 and 2.4% with relative standard deviation between 0.72 and 2.01% for CLS , 0.2 and 1.6 with relative standard deviation between 0.45 and 2% for PCR respectively. It can be observed from this set of results that the drug mixtures determination is feasible for the multivariate procedures (CLS and PCR), but the factorized multivariate calibration model (PCR) allows a more significant reduction of errors in relation to the determination by the CLS and derivative procedures, especially in the mixtures containing low concentration of isoniazid (5:50 isoniazid: rifampicin).

#### 4.5. Stability studies

Literature survey shows that microbiological (Mariappan, *et al.*, 2004 a) and colorimetric (Mariappan, *et al.*, 2004 b) methods have been used to estimate rifampicin in the formulation.

The microbiological assay fails to differentiate between rifampicin and isonicotinyl hydrazone as both of them microbiologically active *in vitro* (Mariappan, *et al.*, 2005) and colorimetric assay have been over estimate rifampicin in the presence of isonicotinyl hydrazone because of similar absorption maxima at 475 nm (Mariappan, *et al.*, 2004 b). Studies showed instability of rifampicin and isoniazid combination formulation in 0.1 M HCl as dissolution medium that lead to the degradation of rifampicin (Jindal *et al.*, 1994; Shishoo *et al.*, 1999)

In our studies the stability of rifampicin in the presence of isoniazid in mixture and formulation (RIF 150 mg and INH 75 mg) was carried out in 0.1 M HCl for 150 minutes in 30 intervals at 60 °C as shown in (Table 14). The results show that as time increases the amount of rifampicin decreases (Figure 24). These clearly indicate that the presence of isoniazid causes degradation of rifampicin due to (formation of isonicotinyl hydrazone). Further, it is also found that rifampicin degraded by about 23.2% (RIF-INH mixture) and 30.9% (RIF-INH formulation) through 150 minute at 60 °C. The degradation of rifampicin follows first order kinetics in all cases as indicated by (Figures 25 and 26). The rate constants obtained were  $7.7 \times 10^{-4}$  and  $9.60 \times 10^{-4} \text{ min}^{-1}$  for rifampicin in the laboratory prepared mixtures and formulations in presence of isoniazid respectively while the corresponding half life time ( $t_{1/2}$ ) values were 1424.7 and 1142.7 min, respectively (Table 14). The rate constants and  $t_{1/2}$  values of both cases are almost comparable but on the formulation the rate constant is to some extent accelerated this might be due to the effect of excipients and additives that present in the formulations.

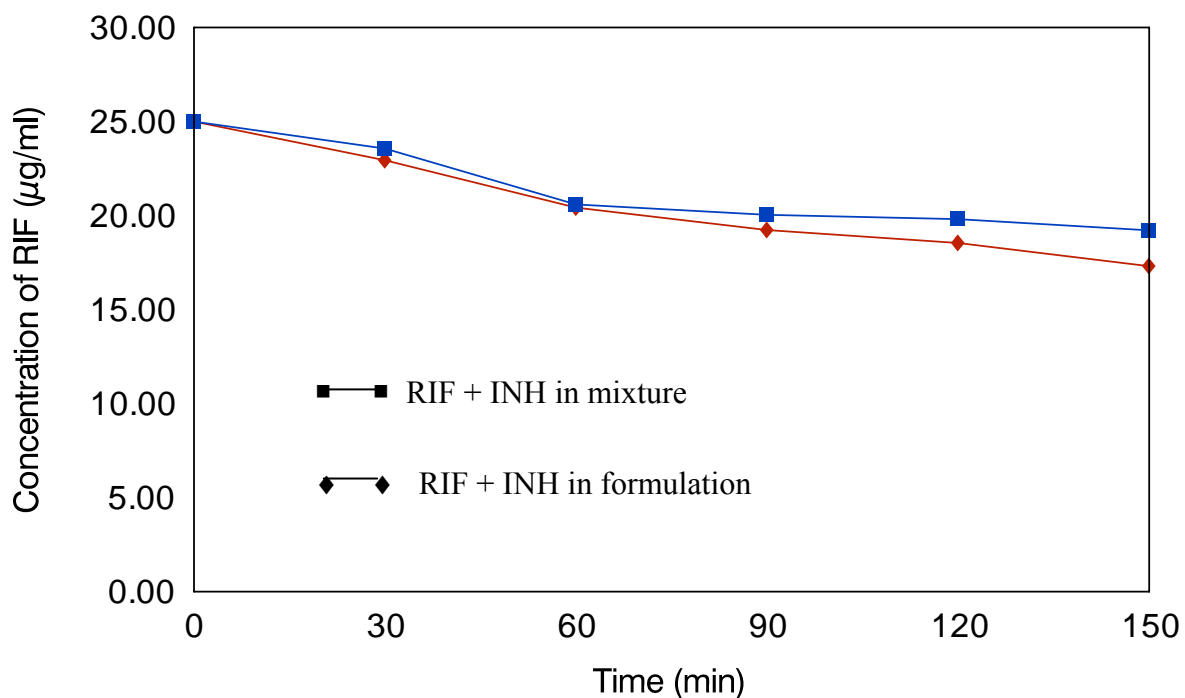


Figure 24: Degradation of RIF in combination with INH in mixture and formulation in 0.1 M HCl at 60 °C

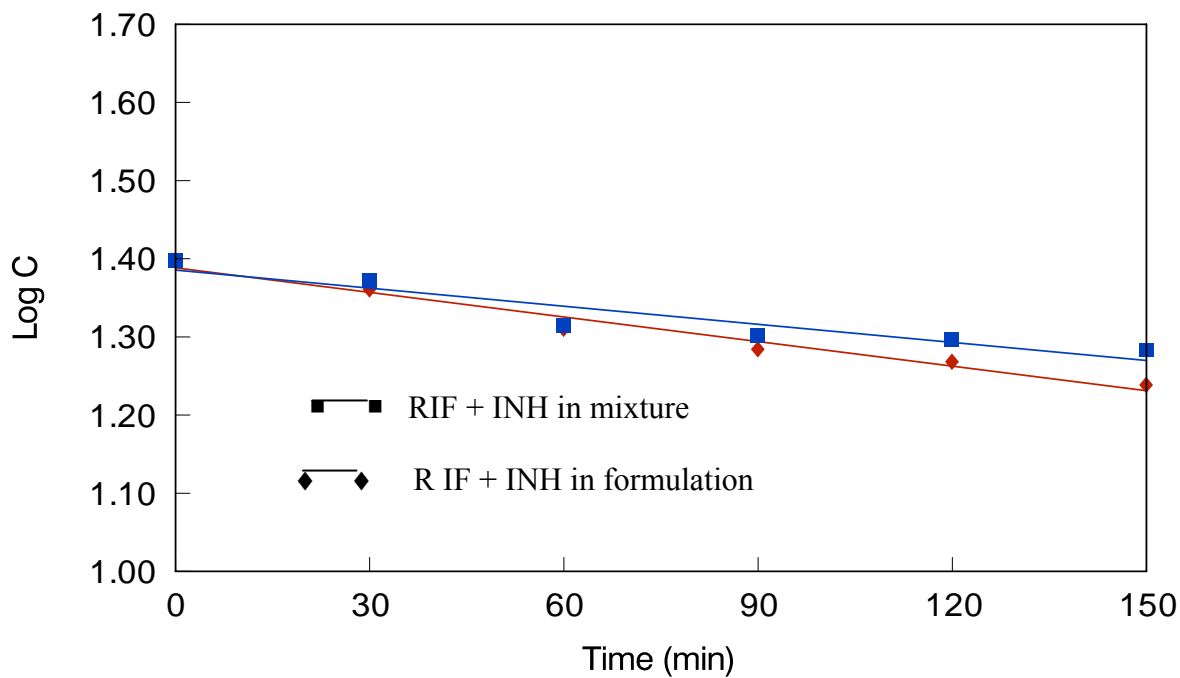


Figure 25: First order plot for the degradation of RIF in combination with INH in mixture and formulation in 0.1 M HCl at 60 °C

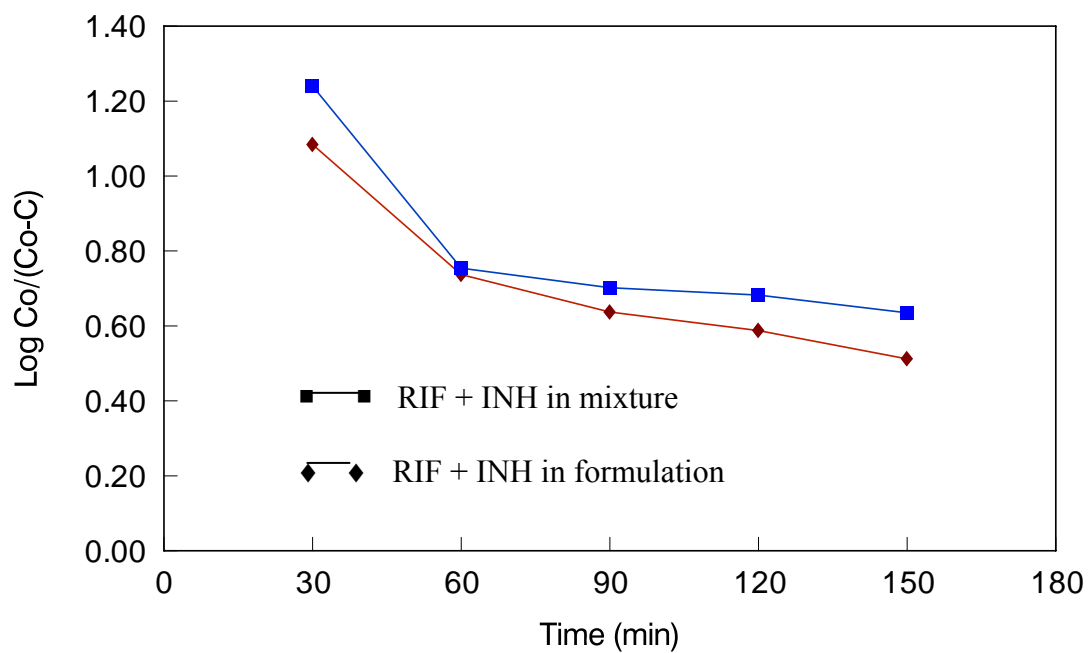


Figure 26: Kinetics of degradation of RIF in combination with INH in mixture and formulation in 0.1 M HCl at 60 °C

Table 14: Stability of rifampicin in presence of isoniazid in 0.1 M HCl at 60°C (n = 3)\*

Time t (min)	Concentration of RIF at t (C) µg/ml	$C_t / (C_0 - C)$	$\log [C_t / (C_0 - C)]$	$\log [C]$	Degradation rate constant K (min <sup>-1</sup> )	Average K (min <sup>-1</sup> )	Average $t_{1/2}$ (min)
RIF + INH mixture (150 mg and 75 mg)							
0	25.00	1.0000	0.0000	1.3979	—		
30	23.56	17.3611	1.2395	1.3722	$8.59 \times 10^{-4}$		
60	20.59	5.6689	0.7536	1.3137	$9.5 \times 10^{-4}$		
90	20.04	5.0403	0.7024	1.3019	$6.92 \times 10^{-4}$	$7.7 \times 10^{-4}$	1424.70
120	19.81	4.8170	0.6821	1.2969	$7.67 \times 10^{-4}$		
150	19.21	4.3178	0.6355	1.2835	$5.45 \times 10^{-4}$		
RIF + INH tablet (150 mg and 75 mg)							
0	25.00	1.0000	0.0000	1.3979	—		
30	22.94	12.1359	1.0841	1.3606	$9.29 \times 10^{-4}$		
60	20.42	5.4585	0.7371	1.3101	$13.72 \times 10^{-4}$		
90	19.23	4.3328	0.6368	1.2839	$9.76 \times 10^{-4}$	$9.60 \times 10^{-4}$	1142.70
120	18.54	3.8700	0.5877	1.2681	$5.29 \times 10^{-4}$		
150	17.31	3.2510	0.5120	1.2383	$9.94 \times 10^{-4}$		

\* Values given are average of three determinations, in all cases.

## 5. Conclusion

The contents of rifampicin and isoniazid in synthetic mixtures and commercial dosage forms were simultaneously determined using UV spectrophotometric measurements together with first derivative zero-crossing, first derivative ratio and multivariate calibration analysis i.e. principal components regression and classical least squares. Synthetic binary and ternary (in the presence of pyrazinamide) mixtures as well as commercial dosage forms were studied. The two compounds which produce a perfect overlapping spectrum in the zero-order spectra, the first derivative zero-crossing and first derivative ratio spectrophotometry has given good recoveries for simultaneous determination of isoniazid and rifampicin in the binary mixture as well as commercial dosage forms. The multivariate methods principal component regression and classical least squares analysis also gave excellent recoveries for the binary and ternary mixtures and commercial dosage forms. The good recoveries obtained in all cases proved that the proposed methods could be applied for the determination of studied drugs simultaneously in their synthetic mixtures and as well as commercial dosage forms with satisfactory precision. Rifampicin in presence of isoniazid in synthetic mixture degrades to form isonicotinyl hydrazone in 0.1 M HCl with a first order rate constant of ( $K$ )  $7.7 \times 10^{-4} \text{ min}^{-1}$ . This degradation rate was a little bit more accelerated in fixed dose combination products of rifampicin with isoniazid to give  $K$  value of  $9.60 \times 10^{-4} \text{ min}^{-1}$ . It was observed that the degradation of rifampicin (rate of formation of isonicotinyl hydrazone) was not significant and considerable but it may be advisable to control the quality of these drugs that are being imported in Ethiopia.

The proposed methods in this study are rapid, precise and may be useful for laboratories involved in quality control of anti-tubercular fixed dose combination products.

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