ELSEVIER

International Journal of Pharmaceutics 190 (1999) 109–123

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Stability of rifampicin in dissolution medium in presence of isoniazid

C.J. Shishoo *, S.A. Shah, I.S. Rathod, S.S. Savale, J.S. Kotecha, P.B. Shah

Department of Quality Assurance, L.M. College of Pharmacy, P.O. Box 4011, Navrangpura, Ahmedabad 380 009, India

Received 17 May 1999; accepted 3 August 1999

Abstract

Rifampicin (RIF) hydrolyzes in acidic medium to form insoluble and poorly absorbed 3-Formyl rifamycin SV (3-FRSV). This study describes development of two principally different methods, Dual Wavelength UV–Vis. spectrophotometry (DW spectrophotometry) and HPTLC, to determine 3-FRSV in presence of RIF. Using DW spectrophotometry, RIF was estimated by using wavelengths 475.0 and 507.0 nm and 3-FRSV was estimated using 457.0 and 492.0 nm. In HPTLC method, a mixture of chloroform:methanol:water (80:20:2.5 v/v) was used as the mobile phase to resolve 3-FRSV from RIF and 3-FRSV was quantified at 333 nm. The linearity range for 3-FRSV was 2–10 μg/ml and 50–250 ng/spot for DW spectrophotometric method and HPTLC method, respectively, and 5–50 μg/ml for RIF using DW spectrophotometric method. Both the methods were found to be specific, accurate and reproducible. The proposed methods were successfully applied to determine the rate of degradation of RIF to 3-FRSV in dissolution medium (0.1 N HCl) and also in presence of isoniazid (INH). The rate of degradation of RIF in presence of INH was almost two times more than that of RIF alone. These methods were utilized to study the stability of RIF in market formulations of RIF and RIF with INH in dissolution medium. It has been observed that RIF degrades by 12.4% to form 3-FRSV (RIF formulations) while in presence of INH the degradation is catalyzed to about 21.5% (RIF + INH formulations), in 45 min. Thus, lower concentration of RIF may be available for absorption leading to poor bioavailability of RIF from combination dosage forms (RIF + INH) as compared to formulations containing only RIF. It is proposed that specific analytical method should be used to measure RIF in presence of 3-FRSV in a dissolution study.

Keywords: Rifampicin; 3-Formyl rifamycin SV; Isoniazid; Dual Wavelength spectrophotometry; HPTLC; Stability; Dissolution

1. Introduction

The resurgence of reported cases of tuberculosis along with the recent emergence of multidrug resistant strains of *M. tuberculosis* has prompted W.H.O. to declare the disease as "Global health emergency, a public health disaster" (Anon, 1997). Nearly 19% of tuberculosis isolates in a New York city hospital were found resistant to both isoniazid and rifampicin (Shalom et al., 1996). Rifampicin (RIF), isoniazid (INH) and

* Corresponding author. Tel.: +91-079-6443984.
E-mail address: cjsbcs@netscape.net (C.J. Shishoo)

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00286-0

pyrazinamide (PZ) are drugs of choice for the treatment of tuberculosis. They are administered separately or as a combination dosage form. The triple combination of these drugs is widely used regimen in India and elsewhere because of better patient compliance, reduction in viable bacteria population and minimization of drug resistance. However, poor bioavailability of RIF from a number of dosage forms of RIF and its combination with INH has been reported (Mouton et al., 1979; Doshi et al., 1986; Ellard et al., 1986; Acocella, 1989; Fox, 1990; Anon, 1991). This may be responsible for the treatment failure and consequent development of resistance.

Degradation of RIF is pH dependent (Gallo and Radaelli, 1976). In acidic medium RIF hydrolyzes to 3-Formyl rifamycin SV (3-FRSV) and it undergoes air oxidation in alkaline medium to form inactive quinone derivative, Rifampin quinone. 3-FRSV precipitates in acidic conditions (Maggi et al., 1966). It shows high antimicrobial activity in vitro (Maggi et al., 1965) but is inactive in vivo (USP DI, 1996). Therefore, formation of 3-FRSV in the acidic environment of stomach can be an important factor affecting bioavailability of RIF and cannot be overlooked.

With a view to determine the amount of 3-FRSV formed during the course of hydrolysis, it became important to develop a specific and sensitive analytical method to determine 3-FRSV in presence of RIF. The present study reports our results on the stability of RIF alone and in presence of INH in 0.1 N HCl and its consequent effect on the dissolution profile of RIF from these two formulations. This study underlines the fact that there is a need to improve official dissolution test method.

2. Experimental, materials and methods

2.1. DW spectrophotometric method for simultaneous estimation of RIF and 3-FRSV

2.1.1. Apparatus and instruments

Double beam Shimadzu 160A UV–Vis. spectrophotometer having two matched cells with 1-cm light path was employed for spectrophotometric determinations.

2.1.2. Materials

Analytically pure samples of RIF and 3-FRSV were procured from Themis Lab., Mumbai and analytically pure INH was gifted by Cadila Healthcare Ltd., Ahmedabad. All the chemicals including chloroform, methanol (SRL Chem.), concentrated hydrochloric acid (Ranbaxy Ltd.), anhydrous sodium sulfate (Samir Tech-Chem), potassium dihydrogen phosphate, disodium hydrogen phosphate (S.D. Fine Chemicals), used were of Laboratory reagent grade.

2.1.3. Preparation of standard stock solutions

2.1.3.1. RIF stock solution. RIF powder (10 mg) was accurately weighed and transferred to 10-ml volumetric flask. It was dissolved in 7 ml of chloroform by sonication for 5 min. The volume was adjusted to 10 ml with chloroform. 1 ml of this solution was further diluted to 10 ml with chloroform to have the final concentration of 100 μg/ml.

2.1.3.2. 3-FRSV stock solution. 3-FRSV (10 mg) was dissolved and diluted in chloroform as in case of RIF stock solution, to obtain the final concentration 100 μg/ml.

2.1.4. Selection of wavelengths for estimation of RIF

Spectrum of pure 3-FRSV was scanned in the SPECTRUM basic mode. Using the cursor function, the absorbance corresponding to 475 nm (wavelength λ_1) (the wavelength of maximum absorbance for RIF) was noted from spectrum. Then the cursor function was moved along with peak curve until the absorbance equal to that of absorbance at 475 nm (λ_1) was found. The wavelength obtained corresponding to this absorbance value was 507 nm (λ_2).

The absorbance of various dilutions of 3-FRSV in chloroform was measured at 475 and 507 nm. The difference between the two absorbance values was calculated.

2.1.5. Selection of wavelengths for estimation of 3-FRSV

Spectrum of pure RIF was scanned in the SPECTRUM basic mode. Using the cursor function, λ_1 and λ_2 were found as described in Section 2.1.4. The values obtained were 492 nm (λ_1) (the wavelength of maximum absorbance for 3-FRSV) and 457 nm (λ_2), respectively.

2.1.6. Calibration curves for RIF and 3-FRSV

Appropriate aliquots from the stock solutions of RIF and 3-FRSV were used to prepare four different sets of dilutions, series A, B, C and D, as follows,

2.1.6.1. Series A. This series comprised of mixtures of RIF and 3-FRSV having fixed concentration of 3-FRSV (2 μg/ml) and varying concentrations of RIF (5–50 μg/ml). The solutions were prepared by pipetting out 0.2 ml of 3-FRSV stock solution (100 μg/ml) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml of the stock solution of RIF (100 μg/ml) into a series of 10-ml volumetric flasks and the volume was adjusted to the mark with chloroform.

2.1.6.2. Series B. In this series mixtures of the RIF and 3-FRSV having fixed concentration of RIF (50 μg/ml) and varying concentrations of 3-FRSV (2–10 μg/ml) were prepared by pipetting out 5.0 ml of RIF stock solution (100 μg/ml) and 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the stock solution of 3-FRSV (100 μg/ml) into 10-ml volumetric flasks and volume was adjusted with chloroform to the mark.

2.1.6.3. Series C. This series consisted of different concentrations of RIF ranging from 5 to 50 μg/ml prepared by appropriate dilution of aliquots of RIF stock solution, with chloroform, same as in the case of series A.

2.1.6.4. Series D. This series included solutions having concentrations of 3-FRSV ranging from 2 to 10 μg/ml prepared by diluting appropriate volumes of corresponding stock solution with chloroform as in the case of series B.

The absorbance of the solutions of series A and C were measured at 475.0 (λ_1) and 507.0 nm (λ_2) while absorbance of the solutions of series B and D were measured at 492.0 (λ_1) and 457.0 nm (λ_2). The difference in absorbances was plotted against concentration (μg/ml).

2.2. High performance thin layer chromatography (HPTLC) for estimation of 3-FRSV

2.2.1. Instrumentation

CAMAG–HPTLC system consisting of CAMAG Linomat IV semiautomatic spotting device, CAMAG twin-trough TLC chamber, CAMAG–TLC scanner 3 and CAMAG CATS 4 software and Hamilton syringe (100 μl) was used.

2.2.2. Materials

TLC aluminum sheets pre-coated with silica gel G60 F_{254} (layer thickness 0.2 mm; 10×10 cm)(E. Merck) were used and other materials were same as described under Section 2.1.2.

2.2.3. Chromatographic conditions

Chromatographic estimations were performed using pre-coated TLC plates under following conditions: mobile phase, chloroform:methanol:water (80:20:2.5 v/v); chamber saturation time, 60 min; temperature, 25 ± 1°C; migration distance, 30 mm; slit dimension, 4×0.45 mm; wavelength of detection: 333 nm. Spotting parameters: bandwidth, 4 mm; space between two bands, 5 mm; spraying rate, 10 s/μl.

2.2.4. Preparation of combined standard solution of RIF and 3-FRSV

The standard solutions containing RIF (100 μg/ml) and varying concentrations of 3-FRSV (5–25 μg/ml) were prepared by mixing 1 ml of RIF solution (1 mg/ml) with 0.5, 1.0, 1.5, 2.0 and 2.5 ml of 3-FRSV solution (100 μg/ml) in series of 10 ml volumetric flasks and diluted to the mark with chloroform.

2.2.5. Chromatographic separation

Ten microliters of combined standard solution (100 μg/ml RIF and 20 μg/ml 3-FRSV) was applied on TLC plate under nitrogen stream using

semiautomatic spotter. The plate was dried and developed at constant temperature (25 ± 1°C) using mixture of chloroform:methanol:water (80:20:2.5 v/v) as the mobile phase. After development the plate was dried for 5 min. Photometric measurements were performed at 333 nm in the reflectance mode with CAMAG TLC scanner 3 connected to a computer running CATS 4 software incorporating the track optimization option. The purity of the chromatographic peak was determined by recording spectra at three different points of each peak: peak start, peak apex and peak end.

Quantitative determinations were carried out using the peak area.

2.2.6. Calibration curve for 3-FRSV

Ten microliters of combined standard solutions containing RIF (100 μg/ml) and 3-FRSV (5–25 μg/ml) were spotted on TLC plate and analyzed as described in Section 2.2.5.

Calibration curve was prepared by plotting respective peak areas against concentration (ng/spot) of 3-FRSV.

2.2.7. Validation of HPTLC method

2.2.7.1. Precision

Repeatability of measurement of peak area and height. Combined standard solution (RIF, 100 μg/ml + 3-FRSV, 20 μg/ml) was spotted on TLC plate and developed as described in Section 2.2.5. The separated spot was scanned for seven times without changing position of the plate and R.S.D. was computed.

Repeatability of sample application. Combined standard solution (RIF, 100 μg/ml + 3-FRSV, 20 μg/ml) was applied seven times on TLC plate by semiautomatic spotter, developed and analyzed as described under Section 2.2.5.

2.2.7.2. Accuracy. Accuracy of an analysis is determined by calculating systemic error involved. It was determined by calculating recovery of 3-FRSV by standard addition method at three different levels 50, 100 and 200 ng/spot.

2.2.7.3. Linearity. The linear response for 3-FRSV was determined in the range of 50–250 ng/spot.

2.2.7.4. Specificity. To confirm the specificity of the proposed method, the RIF tablet solution in chloroform and the mixture of RIF tablet solution with standard 3-FRSV solution were spotted on TLC plate and analyzed.

2.3. Dissolution stability

2.3.1. Dissolution stability of RIF in 0.1 N HCl

A solution of 0.1 N HCl (200 ml) was placed in the vessel of the USP dissolution apparatus No. 2 (US Pharmacopoeia XXIII, 1995) and the medium was equilibrated at 37 ± 0.2°C with stirring at 100 rpm. RIF (150 mg) was accurately weighed, dissolved in and diluted to 100 ml with 0.1 N HCl (37°C). The resulting solution was transferred immediately to the dissolution vessel at once and 5 ml of specimen was withdrawn immediately from a zone midway between the surface of the dissolution medium and bottom of the vessel (0-min sample). Specimens were withdrawn at 15-min intervals up to 60 min. An aliquot, 1 ml, was extracted immediately with 5 ml of chloroform using cyclomixer (3 min). Aqueous phase was discarded and anhydrous sodium sulfate was added to chloroform layer to remove the traces of water. The chloroform extract was analyzed for 3-FRSV (10 μl) using suitable amount of standard 3-FRSV by HPTLC method as described in Section 2.2.5.

2.3.2. Dissolution stability of RIF–INH mixture in 0.1 N HCl

Solution of 200 ml 0.1 N HCl was placed in the vessel of the dissolution apparatus and the medium was equilibrated to 37 ± 0.2°C with stirring at 100 rpm. RIF (150 mg) and INH (100 mg) were accurately weighed, dissolved in and diluted to 100 ml with 0.1 N HCl (37°C). Resulting solution was transferred immediately to the vessel at once and analyzed as described in Section 2.3.1.

2.3.3. Dissolution study of some marketed formulations (RIF and (RIF–INH) capsules/tablets)

Dissolution medium (0.1 N HCl; 900 ml) was placed in the vessel of the apparatus [USP ap-

paratus No. 1 (Basket) for capsule and No. 2 (Paddle) for tablet] (US Pharmacopoeia XXIII, 1995, 1996). The apparatus was assembled and the dissolution medium was allowed to equilibrate to 37 ± 0.2°C. A tablet/capsule was placed in the vessel/basket taking care to exclude air bubble from the surface of the dosage form unit and the apparatus was operated immediately at 100 rpm. Aliquot sample, 10 ml, was withdrawn at an interval of 15 min from the zone midway between the surface of the dissolution medium and top of the rotating blade of the paddle or basket, not less than 1 cm from the vessel wall, up to 45 min. The aliquot withdrawn for analysis was replaced with equal volume of fresh dissolution medium at 37 ± 0.2°C.

An aliquot, 1 ml, was extracted immediately with 5 ml of chloroform using cyclomixer (3 min). The aqueous phase was discarded and chloroform extract was dried over anhydrous sodium sulfate. The samples were analyzed for RIF and 3-FRSV using the proposed DW spectrophotometric method and HPTLC method. Five ml of the withdrawn sample was analyzed as per the official method (US Pharmacopoeia XXIII, 1995, 1996).

2.3.3.1. Analysis by DW spectrophotometric method. The chloroform extract was analyzed by measuring the absorbance of RIF and 3-FRSV at their characteristic wavelengths (475 and 507 nm for RIF, and 457 and 492 nm for 3-FRSV) and amount of RIF released and 3-FRSV formed were calculated by referring to the calibration curve as in Section 2.1.6.

2.3.3.2. Analysis by HPTLC method. Ten microliters of the chloroform extract was analyzed for 3-FRSV as described in Section 2.2.5.

3. Results

3.1. DW spectrophotometric method (Dual Wavelength data processing)

The utility of dual wavelength data processing program is its ability to calculate unknown concentration of a component of interest in a mixture containing an interfering component with close absorption maxima (λ_{max}). For elimination of the effects of an interfering component, two specific wavelengths are chosen: (i) The wavelength λ_1 at which maximum absorbance is observed for the pure component of interest; and (ii) second wavelength λ_2, is the wavelength at which the absorbance of the interfering component is equal to the absorbance of the interfering component at λ_1 (Fell, 1986).

In the proposed procedure the absorbance of RIF alone in a mixture of RIF and 3-FRSV was determined using dual wavelength data processing program. To remove the interference of 3-FRSV to the absorbance at 475 nm (λ_1), the wavelength of maximum absorbance for RIF, another wavelength 507 nm (λ_2) was found out at which the absorbance of 3-FRSV was equal to its absorbance at 475 nm. This was confirmed by measuring the absorbance of various dilutions of 3-FRSV in chloroform at 475 nm and 507 nm, respectively. The absorbance at these two wavelengths was found to be equal. These two selected wavelengths were employed to determine the concentration of RIF from the mixture of RIF and 3-FRSV. The difference in absorbance at these two wavelengths ($A_{475} - A_{507}$) cancels out the contribution of absorbance of 3-FRSV in measurement of RIF at 475 nm and the difference in absorbance is proportional to the concentration of RIF in the mixture.

This was also confirmed by determining the absorbance of mixtures of various concentrations of RIF, keeping 3-FRSV concentration constant, at these wavelengths. It was observed that with the increase in RIF concentration, there was corresponding increase in difference of the absorbance values, ΔA ($A_{475} - A_{507}$). It was also found that this difference in absorbance values was linear in the range of 5–50 μg/ml of RIF with correlation coefficient 0.9998 in presence of 3-FRSV (Table 1, Fig. 1).

Further, the difference in the absorbance values at 475 and 507 nm, ΔA, for RIF alone also gave linear range of 5–50 μg/ml of RIF (Table

Table 1
Determination of RIF in presence of 3-FRSV (series A) and RIF alone (series C) by the proposed DW spectrophotometry

Serial No.	Series A				Series C			
	Composition of mixture (μg/ml)		Mean ($A_{475}-A_{507}$) ± S.D. ($n=5$)	% C.V.	Composition of mixture (μg/ml)		Mean ($A_{475}-A_{507}$) ± S.D. ($n=5$)	% C.V.
	RIF	3-FRSV			RIF	3-FRSV		
1	5	2	0.024 ± 0.001	4.16	5	0	0.022 ± 0.0005	2.27
2	10	2	0.046 ± 0.001	1.08	10	0	0.046 ± 0.0007	1.51
3	15	2	0.068 ± 0.001	1.47	15	0	0.072 ± 0.002	2.77
4	20	2	0.093 ± 0.002	2.15	20	0	0.094 ± 0.002	2.12
5	25	2	0.116 ± 0.002	1.72	25	0	0.119 ± 0.002	2.12
6	30	2	0.139 ± 0.005	2.15	30	0	0.145 ± 0.002	2.75
7	35	2	0.162 ± 0.006	3.70	35	0	0.168 ± 0.004	2.38
8	40	2	0.184 ± 0.004	2.17	40	0	0.190 ± 0.006	3.15
9	45	2	0.209 ± 0.003	1.43	45	0	0.214 ± 0.009	4.20
10	50	2	0.230 ± 0.005	2.17	50	0	0.238 ± 0.003	1.26

1, Fig. 2). These results confirm the suitability of the proposed method to determine RIF in the presence of 3-FRSV.

Similarly, the interference of RIF in the determination of 3-FRSV was also nullified by selecting two wavelengths, 492 nm (λ_1), the wavelength of maximum absorbance for 3-FRSV, and 457 nm (λ_2). The difference in the absorbance values, ΔA ($A_{492}-A_{457}$) was found to be linear in the range of 2–10 μg/ml of 3-FRSV in presence of RIF and with 3-FRSV alone with correlation coefficient 0.9993 and 0.9996, respectively (Table 2, Figs. 3 and 4).

3.1.1. Regression analysis

Regression analysis for series A and C, and series B and D shows no difference in the equations and thus, indicates that there is no interference of 3-FRSV in determination of RIF and there is no interference of RIF in determination of 3-FRSV (Table 3).

3.1.2. Validation

The method was validated in terms of precision, accuracy, limit of quantitation, limit of detection, specificity, linearity range and recovery. The validation parameters are summarized in Table 4.

3.2. HPTLC method

A sensitive and specific HPTLC method was developed for quantitative estimation of 3-FRSV in presence of RIF.

A mixture comprising chloroform: methanol: water (80: 20: 2.5 v/v) was chosen as a mobile phase as it could resolve both RIF and 3-FRSV without interference from the excipients (Fig. 5).

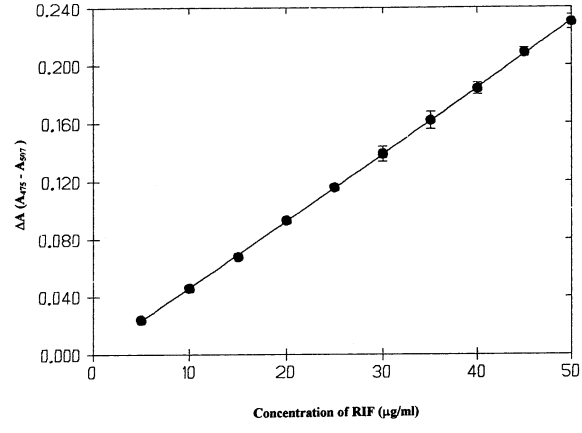

Fig. 1. Calibration curve for Series A (Solutions containing different concentrations of RIF and fixed concentration of 3-FRSV) plotted as ΔA ($A_{475}-A_{507}$) vs. concentration of RIF (μg/ml).

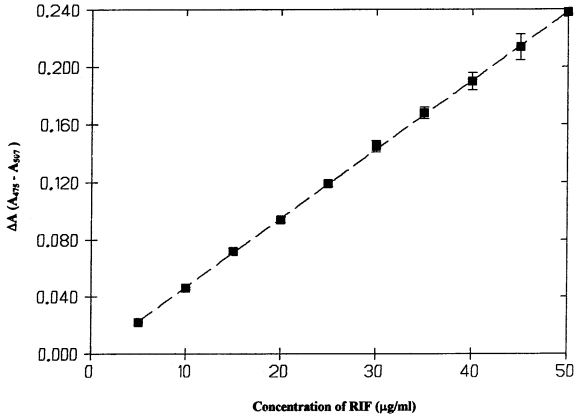

Fig. 2. Calibration curve for Series C (Solutions containing different concentrations of RIF only) plotted as ΔA ($A_{475} - A_{507}$) vs. concentration of RIF (μg/ml).

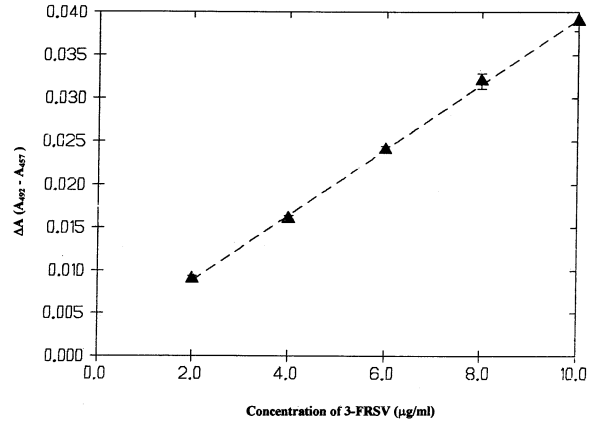

Fig. 3. Calibration curve for Series B (Solutions containing different concentrations of 3-FRSV and fixed concentration of RIF) plotted as ΔA ($A_{492} - A_{457}$) vs. concentration of 3-FRSV (μg/ml).

Methanol in mobile phase increases the solubility of the sample components, while chloroform prevents their moving with the solvent front and water helps to resolve RIF and 3-FRSV.

During HPTLC method development it was also observed that pre-saturation for 60 min of TLC chamber with mobile phase ensures good separation and reproducibility. Chromatographic plate was developed up to 30-mm migration distance. Before spotting, HPTLC plates were washed by keeping them in TLC chamber with methanol, dried and activated to remove volatile impurities.

Photometric measurements were performed in the absorbance/reflectance mode with CAMAG TLC scanner 3. The absorbance/reflectance spectra of 3-FRSV were scanned and maxima 333 nm was selected for quantification. Quantitative determinations were made by considering the peak areas from chromatograms.

3.2.1. Validation

3.2.1.1. Precision. Data of repeatability of sample application for seven times and repeatability of measurement of peak area, peak height based on seven times measurement of the same spot is shown in Table 5. For repeatability of sample application, peak height and area gave R.S.D. of

Table 2
Determination of 3-FRSV in presence of RIF (series B) and 3-FRSV alone (series D) by the proposed DW spectrophotometry

Series No.	Series B				Series D			
	Composition of mixture (μg/ml)		Mean ($A_{492}-A_{457}$) ± S.D. ($n=5$)	% C.V.	Composition of mixture (μg/ml)		Mean ($A_{492}-A_{457}$) ± S.D. ($n=5$)	% C.V.
	RIF	3-FRSV			RIF	3-FRSV		
1	50	2	0.009 ± 0.0004	4.44	0	2	0.007 ± 0.0000	–
2	50	4	0.016 ± 0.0004	2.50	0	4	0.015 ± 0.0009	6.60
3	50	6	0.024 ± 0.0004	1.66	0	6	0.022 ± 0.0004	1.81
4	50	8	0.032 ± 0.0009	2.91	0	8	0.029 ± 0.0012	4.13
5	50	10	0.039 ± 0.0012	3.07	0	10	0.037 ± 0.0012	3.24

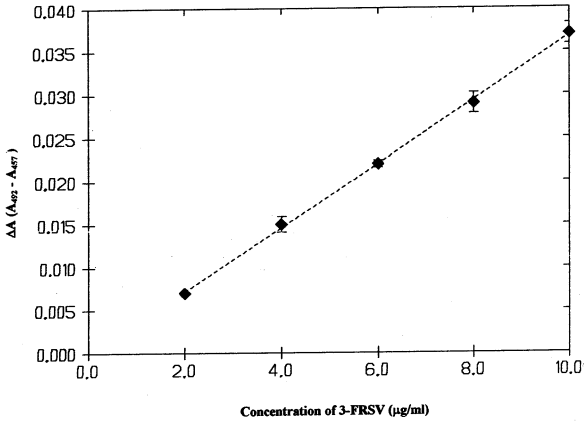

Fig. 4. Calibration curve for Series D (Solutions containing different concentrations of 3-FRSV only) plotted as ΔA ($A_{492} - A_{457}$) vs. concentration of 3-FRSV (μg/ml).

2.2 and 2.8%, respectively while for repeatability of measurement of peak height and peak area, the corresponding R.S.D. values were 0.29 and 0.26%.

3.2.1.2. Accuracy. The % recovery of 3-FRSV was found to be 100.03%, which is satisfactory.

3.2.1.3. Specificity. The method was specific. It resolved the peak of 3-FRSV ($R_f = 0.45$) from RIF ($R_f = 0.75$) even in presence of excipients (Fig. 5).

3.2.1.4. Linearity. Linearity range for the 3-FRSV was found to be 50–250 ng/spot with correlation coefficient of 0.9989 and R.S.D. ranging from 2.89 to 5.87% for different concentrations (Table 6, Fig. 6).

Table 4
Validation parameters for DW spectrophotometric method

Serial No.	Validation parameter	For RIF	For 3-FRSV
1	Linearity range	5–50 μg/ml	2–10 μg/ml
2	Coefficient of correlation	0.9998	0.9995
3	Limit of detection	1 μg/ml	1 μg/ml
4	Limit of quantitation	5 μg/ml	2 μg/ml
5	Specificity	Specific	Specific
6	Precision (% CV)	1.08–4.16	1.66–4.44
7	Accuracy %	99.0–102.68	96.80–98.69

3.2.1.5. Limit of detection. The minimum detectable quantity was found to be 20 ng/spot.

The validation parameters for the HPTLC method are summarized in Table 7.

3.3. Stability studies

3.3.1. Stability of RIF in 0.1 N HCl

Stability study of RIF was carried out by treating equivalent amount of RIF as generally encountered in marketed formulation (RIF 450 mg), with 0.1 N HCl at 37°C and % 3-FRSV formed was determined. Using the area normalization method % degradation of RIF was also calculated. The concentration of RIF and 3-FRSV was plotted against time (Figs. 7 and 8). The results indicate that the decomposition of RIF increases with increase in time (Table 8).

Table 3
Regression analysis data of the calibration curves prepared using Series A, B, C and D by Dual Wavelength spectrophotometry for RIF and 3-FRSV

Series	Composition of the sample solution		Regression equation of the curve	Coefficient of correlation
	RIF (μg/ml)	3-FRSV (μg/ml)		
Series A	5–50	2	$y = 0.0046x + 0.0002$	0.9999
Series B	50	2–10	$y = 0.0038x + 0.0012$	0.9993
Series C	5–50	0	$y = 0.0047x - 0.0010$	0.9998
Series D	0	2–10	$y = 0.0037x - 0.0002$	0.9996

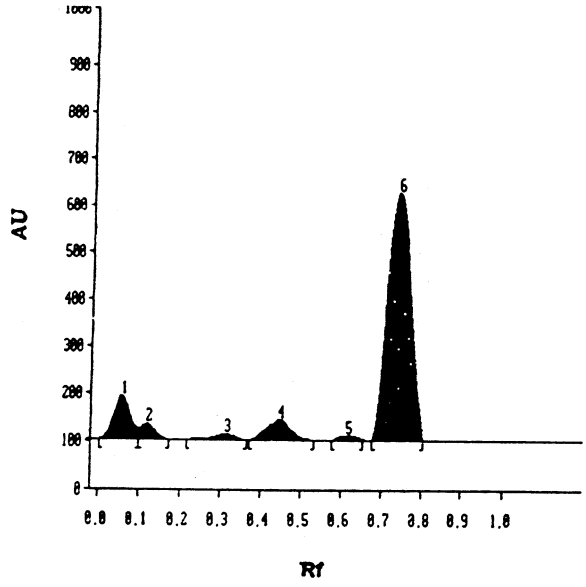

Fig. 5. Chromatogram showing resolution of RIF (6) and 3-FRSV (4) from the excipients.

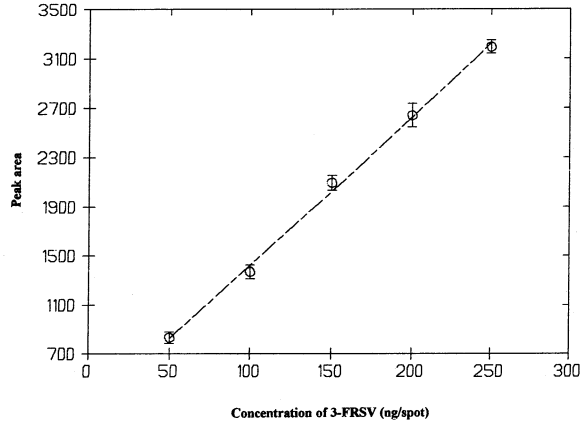

Fig. 6. Calibration curve for 3-FRSV by HPTLC method.

3.3.2. Stability of RIF in 0.1 N HCl in presence of INH

In the present work, RIF–INH mixture, in proportion equivalent to that generally encountered in dosage forms (RIF 450 mg and INH 300 mg), was treated with 0.1 N HCl and samples

Table 5
Determination of 3-FRSV by HPTLC method — Repeatability of sample application and measurement of height and area corresponding to 3-FRSV

Spot No.	Height	Area	No. of measurement	Height	Area
1	130.0	2138.5	1st	133.00	2315.10
2	132.7	2201.2	2nd	133.90	2324.60
3	135.4	2256.1	3rd	133.30	2317.80
4	138.7	2309.48	4th	133.80	2306.20
5	133.8	2184.2	5th	133.80	2308.20
6	137.3	2291.8	6th	133.20	2319.00
7	132.8	2193.5	7th	133.00	2312.20
Mean	134.37	2224.91		133.14	2314.72
R.S.D	2.2 (<3%)[a]	2.8		0.29 (<0.5%)[a]	0.26

[a] Values in parentheses indicate recommended limits for R.S.D. for validation of sample applicator and scanner of HPTLC

Table 6
Determination of 3-FRSV by HPTLC method

Serial No.	Conc. of 3-FRSV (ng/spot)	Peak area $\pm$ S.D. ($n=4$)	% CV
1	50	832.25 ± 48.92	5.87
2	100	1369.52 ± 55.05	4.01
3	150	2093.92 ± 60.60	2.89
4	200	2637.56 ± 96.18	3.64
5	250	3194.5 ± 152.98	4.78

were withdrawn at different time intervals, extracted with chloroform and analyzed by HPTLC. The results indicate that as the time increases the amount of RIF decreases (Fig. 7). Comparison of results of degradation of RIF in 0.1 N HCl for RIF alone and in presence of INH, indicates that the decrease in amount of RIF is greater in presence of INH as compared to that of pure RIF (Fig. 7). There is a significant increase in formation of 3-FRSV in presence of INH (Fig. 8). These results clearly indicate that the presence of INH catalyzes degradation of RIF to 3-FRSV in 0.1 N HCl.

Table 7
Validation parameters for the HPTLC method for determination of 3-FRSV

Series No.	Validation parameter	For 3-FRSV
1	Linearity range	50–250 ng/spot
2	Coefficient of correlation	0.9989
3	Limit of detection	20 ng/spot
4	Limit of quantitation	50 ng/spot
5	Specificity	Specific
6	*Precision (% CV)*	
	Repeatability of application	2.8
	Repeatability of measurement	0.26
7	Accuracy %	100.03

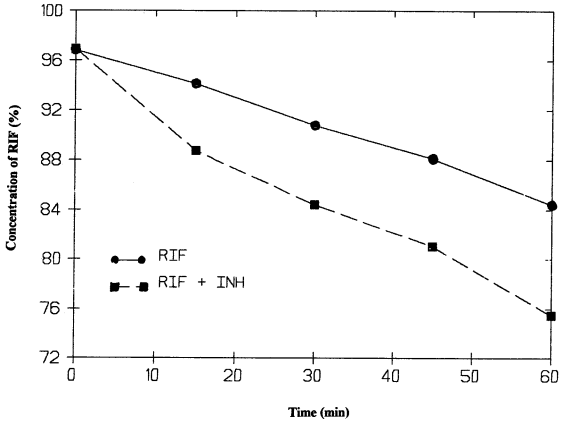

Fig. 7. Degradation of RIF alone and in presence of INH in 0.1 N HCl at 37°C.

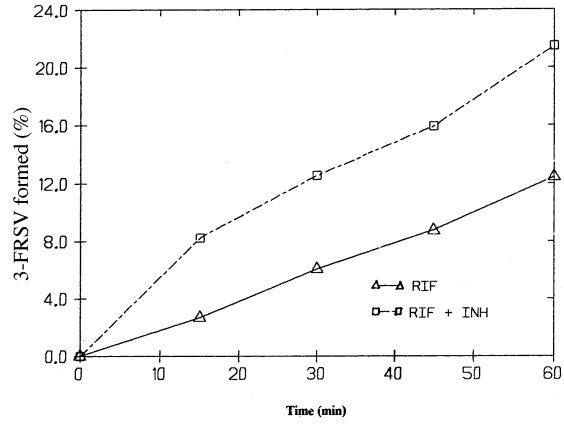

Fig. 8. Formation of 3-FRSV by degradation of RIF alone and in presence of INH in 0.1 N HCl at 37°C.

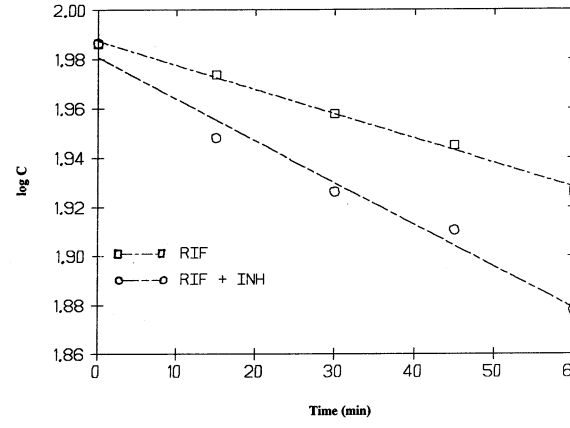

Fig. 9. Kinetics of degradation of RIF in pure form and in combination with INH in 0.1 N HCl.

Further, it was also observed that the degradation follows first order kinetics in both the cases. A plot of $\log(C_0 - C)$ (C_0 is the initial concentration of RIF at time 0 min and C is the concentration of RIF at time t min) versus time resulted in a straight line (Fig. 9) which ascertains the first order of the degradation kinetics of RIF in 0.1 N HCl. The rate constants obtained were 2.1×10^{-3} and, 4.6×10^{-3} min^{-1} for RIF alone and for RIF in combination with INH, respectively while corresponding $t_{1/2}$ values were 330 and 150 min.

Table 8
Stability of RIF alone and RIF in presence of INH in 0.1 N HCl at 37°C ($n = 3$)[a]

Time t (min)	Concentration. of RIF at time t (C)	$C_0/(C_0-C)$	$\log[C_0/(C_0-C)]$	Degradation rate constant. K (min^{-1})	Average K (min^{-1})	$t_{1/2}$ (min)
RIF alone (150 mg)						
0	(C_0) 96.83	1.0000	0.0000	–		
15	94.14	1.0286	0.0122	1.9×10^{-3}		
30	90.77	1.0668	0.0281	2.2×10^{-3}	2.1×10^{-3}	330.00
45	88.10	1.0991	0.0410	2.1×10^{-3}		
60	84.40	1.1473	0.0597	2.3×10^{-3}		
RIF+INH (150+100 mg)						
0	(C_0) 96.95	0.9988	0.0000	–		
15	88.71	1.0915	0.0380	5.8×10^{-3}		
30	84.39	1.1474	0.0597	4.6×10^{-3}	4.6×10^{-3}	150.65
45	81.04	1.1948	0.0773	4.0×10^{-3}		
60	75.45	1.2834	0.1084	4.2×10^{-3}		

[a] Values given are average of three determinations.

Dissolution study of some marketed formulations of RIF alone and RIF in combination with INH were carried out by proposed methods and as per the USP method (US Pharmacopoeia XXIII 1995, 96).

Comparison of results of proposed DW spectrophotometric method with USP method clearly indicates that concentration of RIF determined at 475 nm (USP method) is higher than that determined by proposed method (Table 9). The study of dissolution pattern of market formulations using proposed methods shows increase in amount of 3-FRSV with increase in time. The RIF–INH combination formulations show even more and appreciable amount of degradation of RIF as compared to the formulations containing RIF alone (Tables 9 and 10). The amount of 3-FRSV formed was in the range of 18.08–21.42% over the period of 45 min. It was also noticed that formulations showing faster initial dissolution show decline in total RIF concentration after maximum release, e.g. formulation UC and CB.

4. Discussion

Literature survey reveals that no systematic study has been carried out to study the effect of INH on stability of RIF in dissolution medium (0.1 N HCl).

Essentially spectrophotometric (Maggi et al., 1969; Sunahara and Nakagawa, 1972; Brechbuhler et al., 1978; Fox, 1990; Garg et al., 1991), microbiological (Furesz et al., 1967; Ellard et al., 1986; Garg et al., 1991) and HPLC (Swart and Papgis, 1992; Chouchane et al., 1995; Lau et al., 1996) methods have been used to estimate RIF in the dosage forms and biological fluids.

Because of the close absorption maxima values for RIF (475 nm) and 3-FRSV (492 nm), the spectrophotometric method is not a specific method for the determination of RIF. Even the microbiological assays fail to differentiate between RIF and 3-FRSV, as both of them are microbiologically active in vitro (Maggi et al., 1966). In HPLC method mobile phase has to be selected very carefully, otherwise RIF and 3-FRSV are likely to coelute. Earlier, using HPTLC method, concentration of RIF has been calculated by considering sum total area of three peaks viz. RIF, Rifampin quinone and 3-FRSV (Argekar et al., 1996). Thus, a specific and reliable method is necessary to estimate RIF and its degraded product, 3-FRSV, in presence of each other.

Keeping all this in view, a simple DW spectrophotometric method has been developed and

Table 9
Dissolution study of RIF capsules and capsules and tablets containing RIF alongwith INH: comparison of the data obtained by proposed method (DW spectrophotometry) and the official method (USP) ($n = 3$)[a]

Formulation	Formulation	(Official method, USP) % RIF released	(Proposed method, Dual Wavelength Spectrophotometry)		
			% RIF released	% 3-FRSV formed	Total % RIF released
RIF capsule 450 mg	A	100.42[b]	95.21	4.58	99.79
	B	97.35[b]	90.27	6.61	96.88
Capsule containing 450 mg RIF and 300 mg INH	C	92.32[c]	77.33	16.58	93.91
	D	89.35[c]	77.41	15.27	92.68
Tablet containing 450 mg RIF and 300 mg INH	E	102.27[c]	85.08	16.31	101.39
	F	98.27[c]	85.58	13.10	98.88

[a] Values given are average of three determinations.
[b] Standard solution composed of 66 mg of RIF in 200 ml of 0.1 N HCl.
[c] Standard solution composed of 66 mg of RIF and 33 mg of INH in 200 ml of 0.1 N HCl.

Table 10
Dissolution study of some marketed formulations of RIF alone and its combination with INH by proposed methods[a]

Formulation	Time (min)	Dual wavelength spectrophotometry		HPTLC
		% RIF released	% 3-FRSV formed	% 3-FRSV formed
UC tablet[b]	10	87.43	5.61	5.38
	15	93.06	9.05	8.33
	30	90.51	12.49	13.47
	45	87.93	15.07	18.91
LU capsule[b]	10	53.10	–	–
	15	73.99	8.64	8.80
	30	80.96	14.35	16.78
	45	83.58	18.94	21.42
SA capsule[b]	10	43.04	6.79	8.07
	15	60.65	11.21	13.00
	30	72.98	13.91	15.56
	45	78.19	17.48	19.42
CB tablet[b]	10	75.22	8.51	8.18
	15	88.50	10.33	9.88
	30	89.22	13.90	15.16
	45	84.72	16.59	18.08
LR capsule[c]	10	–	–	–
	15	3.77	–	–
	30	6.62	–	5.35
	45	92.58	7.79	7.94

[a] Values given are average of three determinations.
[b] Formulations containing RIF (450 mg) and INH (300 mg).
[c] Formulation containing RIF (450 mg) alone.

validated for simultaneous estimation of RIF and 3-FRSV (Table 4). Further, a HPTLC method has been developed to separate and quantify trace amounts of 3-FRSV in presence of RIF. This method has been validated and found to be sensitive, specific, accurate and precise (Table 7). The results obtained by the proposed DW spectrophotometric method and those from the proposed HPTLC method are comparable (Table 10).

Isolation of RIF and 3-FRSV from dissolution medium is necessary to prevent further hydrolysis of RIF to 3-FRSV. It is also required to avoid interference of excipients in the estimation of each component by proposed methods. Therefore, it was needed to extract the components of interest from the dissolution medium with an organic solvent showing good solubility for both RIF and 3-FRSV. Chloroform was found to be the suitable solvent for this purpose.

It is observed that RIF degrades by 12.4% in an acidic medium (0.1 N HCl) in 60 min to 3-FRSV. However, in presence of INH the degradation increases to 21.5%. This shows that degradation of RIF in 0.1N HCl is accelerated due to the presence of INH. Degradation of RIF alone and in presence of INH was found to follow the first order reaction kinetics. Corresponding rate constants were found to be 2.1×10^{-3} and 4.6×10^{-3} min^{-1}. The $t_{1/2}$ values and the rate constants of RIF alone and in presence of INH indicate that the degradation of RIF to 3-FRSV is almost more than two times faster in the presence of INH than that of RIF alone (Table 8, Fig. 9).

The present study indicates that the concentration of RIF determined at 475 nm as per the USP method is higher than that determined by proposed DW spectrophotometric method (Table 9). On the other hand, sum total of RIF released and

3-FRSV formed, determined by the proposed method is comparable with the amount of RIF released determined using the USP analysis method in the dissolution test (Table 9). This is due to the inability of the USP analysis method to differentiate the degradation product 3-FRSV formed in the dissolution medium from RIF. It, thus, determines RIF and 3-FRSV as RIF. Therefore, USP dissolution test shows higher amount of RIF than that is actually released in the dissolution medium from the market formulations. Due to this non-specificity of the USP analysis method, the formulations complying with the USP dissolution criteria are failing to pass the USP dissolution limits when analyzed by the proposed methods.

The study of dissolution profile of RIF from market formulations by proposed methods shows increase in concentration of 3-FRSV in dissolution medium with increase in time. This is due to longer exposure of RIF to the acidic medium. At the same time formulations containing RIF in combination with INH show more degradation (18.08–21.42%) as compared to those containing RIF alone (7.94%). These results clearly indicate that even in formulations, presence of INH catalyzes the degradation of RIF. Earlier Jindal et al., also have reported degradation of RIF using 0.1 N HCl as dissolution medium to the extent of 10–23% in a RIF–INH combination formulation (Jindal et al., 1994).

In case of formulations having faster release rate of RIF in dissolution medium more amount of RIF is released in short time and gets exposed to the acidic medium for longer time. Thus, such formulations show higher RIF concentrations for initial short time interval in the dissolution medium (Formulation UC, CB) followed by decrease in the concentrations. On the other hand, for the formulation (Formulation LR) showing slower initial release of RIF, less amount of RIF is available for degradation in the acidic dissolution medium and hence, shows higher concentrations of RIF as the time progresses (Table 10).

The acidic conditions in the in vitro dissolution test are simulated according to the pH in the stomach. Therefore, after oral administration of combined dosage form, appreciable amount of RIF may be degraded in stomach, which ultimately results into lower concentration of RIF available for absorption. This may lead to poor bioavailability of RIF from combined dosage forms (RIF–INH) compared to formulations containing RIF alone. Thus, due to non-specific method employed for the analysis of the dissolution samples, correlation between the in vitro dissolution and bioavailability of RIF from oral dosage forms is questionable.

Further, it is recommended that RIF oral dosage form should be taken on an empty stomach or at least from 1 h before or 2 h after meals (USP DI, 1996). But the pH at this stage remains more acidic (pH 1.4–2.1) as compared to stomach after meals (pH 4.3–5.4) (Kararly, 1995). Therefore, acidic pH of empty stomach might accelerate formation of 3-FRSV. This may be one of the important factors contributing to reduced bioavailability of RIF from RIF and RIF–INH combined dosage forms. At the same time if the concentration of RIF reduces below 9 mg/kg body weight, it results in loss of therapeutic efficacy (Long et al., 1979). Thus, poor bioavailability of RIF after oral administration of the RIF–INH combined dosage form may be an important contributing factor in the development of resistance.

Present study indicates that administration of RIF–INH combination formulation by oral route poses stability problem especially if the drug is released in stomach. Therefore, the combination dosage form may be formulated so that RIF is released not in stomach but in the upper part of intestine where the drug meets less hostile acidic pH. More important, correlation between in vitro dissolution test with in vivo bioavailability of RIF from RIF–INH combination formulations needs to be reexamined taking into consideration formation of significant amount of 3-FRSV as degradation product, which is undesirable.

5. Conclusion

RIF degrades to form 3-FRSV in 0.1 N HCl with a first order rate constant of (K) 2.1×10^{-3} min^{-1}. This degradation rate is accelerated in presence of INH to give K value of 4.6×10^{-3} min^{-1}. Since, 3-FRSV is poorly absorbed from stomach, the

degradation of RIF in acidic medium can affect bioavailability of RIF. At the same time, as the official dissolution test does not differentiate between RIF and 3-FRSV, there is a need to reexamine correlation of in vitro dissolution test with in vivo bioavailability data. Further, RIF–INH combination needs to be formulated to withstand acidic pH of stomach. In view of the fact that acidic pH of stomach is unfavorable for the optimum bioavailability of RIF from its combination dosage form, it may be advisable to formulate the formulation such that RIF is released preferably in upper part of intestine.

References

Acocella, G., 1989. Satellite symposium on quality control of antituberculosis drugs — human bioavailability studies. Bull IUATLD. 64, 38–40.

Anon, X., 1991. The resurgence of tuberculosis: a call for commitment, general policy topics. WHO Drug Inf. 5 (3), 39–40.

Anon, X., 1997. New TB drug combinations needed. Pharm. J. 258, 397.

Argekar, A.P., Kunjir, S.S., Purandare, K.S., 1996. Simultaneous determination of Rifampicin, Isoniazid and Pyrazinamide by high performance thin layer chromatography. J. Pharm. Biomed. Anal. 14, 1645–1650.

Brechbuhler, S., Fluehler, H., Riess, W., Theobald, W., 1978. The renal elimination of RIF as a function of oral dose — a convenient way to assess relative bioavailability. Arzneim. -Forsch. Drug Res. 28, 480–483.

Chouchane, N., Barre, J., Toumi, A., Tillement, J.P., Benakis, A., 1995. Bioequivalence study of two pharmaceutical forms of Rifampicin capsules in man. Eur. J. Drug Metab. Pharmacokinet. 20 (4), 315–320.

Doshi, B.S., Bhate, A.D., Chauhan, B.L., Parkar, T.A., Kulkarni, R.D., 1986. Pharmacokinetic interaction of oral RIF and INH in normal subjects. Indian Drugs 23 (12), 672–676.

Ellard, G.A., Ellard, D.R., Allen, B.W., Girling, D.J., Nunn, A.J., Seng-kee, T., Tiong-har, T., Hin-kwong, N.G., Sin-lun, C., 1986. The bioavailability of INH, RIF and Pyrazinamide in two commercially available combination formulations designed for use in the short course treatment of tuberculosis. Am. Rev. Respir. Dis. 133, 1076–1080.

Fell, A.F., 1986. Ultraviolet, visible and fluorescence spectrophotometry. In: Moffat, A.C. (Ed.), Clarke's Isolation and Identification of Drugs, 2nd edn. The Pharmaceutical Press, London, p. 229.

Fox, W., 1990. Drug combinations and the bioavailability of Rifampicin. Tubercle 71, 241–245.

Furesz, S., Scotti, R., Pallanza, R., Mapelli, E., 1967. Rifamycins: LIII. Rifampicin: a new rifamycin. 3. Absorption, distribution and elimination in man. Arzneim. -Forsh. Drug. Res. 17 (5), 534–537.

Gallo, G.G., Radaelli, P., 1976. Rifampicin. In: Florey, K. (Ed.), Analytical Profile of Drug Substances, vol. 5. Academic Press, New York, pp. 467–515.

Garg, S.K., Chakrabarti, A., Panigrahi, D., Sharma, M., Talwar, P., Kumar, N., Sharma, P.L., 1991. Comparative bioavailability and in vitro antimicrobial activity of two different brands of Rifampicin. Eur. J. Drug. Metab. Pharmacokinet. 16 (3), 723–729.

Jindal, K.C., Chaudhary, P.S., Singla, A.K., Gangwal, S.S., Khanna, S., 1994. Dissolution test method for Rifampicin-isoniazid fixed dose formulation. J. Pharm. Biomed. Anal. 12 (4), 493–497 Chem. Abs., 1994. 121, 65672g.

Kararly, T.T., 1995. Comparison of the gastrointestinal anatomy, physiology and biochemistry of humans and commonly used laboratory animals. Biopharm. Drug Dispos. 16, 351–380.

Lau, Y.Y., Hanson, G.D., Carel, B.J., 1996. Determination of RIF in human plasma by high performance liquid chromatography with ultraviolet detection. J. Chromatogr. B. Biomed. Appl. 676 (1), 147–152.

Long, M.W., Srider, D.R., Farer, L.S., 1979. US Public Health Service Cooperative trial of three RIF–INH regimens in treatment of pulmonary tuberculosis. Am. Rev. Respir. Dis. 119, 879–894.

Maggi, N., Pallanza, R., Sensi, P., 1965. New derivatives of Rifamycin SV, Antimicrob. Agents Chemother. 765–769.

Maggi, N., Pasqualucci, C.R., Ballotta, R., Sensi, P., 1966. Rifampicin*: A new orally active Rifamycin. Chemotherapia 11, 285–292.

Maggi, N., Furesz, S., Palliza, G., Pallanza, R., 1969. Rifamycins: LVII. Rifampicin deacetylation in the human. Arzneim. -Forsch. Drug Res. 19 (4), 651–654.

Mouton, R.P., Mattie, H., Swart, K., Kreukneit, J., deWael, J., 1979. Blood levels of Rifampicin, desacetylrifampicin and isoniazid during combined therapy. J. Antimicrob. Chemother. 5, 447–454.

Shalom, Y., Harvey, R.G., Blum, J., 1996. Adducts of phenanthrene-9,10-imine and of benz(a)anthracene to some nitrogen heterocycles. J. Med. Chem. 33, 680–685.

Sunahara, S., Nakagawa, H., 1972. Metabolic studies and controlled clinical trials of Rifampicin. Chest 61, 526–532.

Swart, K.J., Papgis, M., 1992. Automated high performance liquid chromatographic method for the determination of Rifampicin in plasma. J. Chromatogr. 593 (1–2), 21–24.

US Pharmacopoeia XXIII. US Pharmacopoeial Convention, Rockville, MD (1995), pp. 1382–1383.

US Pharmacopoeia XXIII, Supplement 3. US Pharmacopoeial Convention, Rockville, MD (1996), p. 2976.

USP DI, Drug Information for the Health-care Practitioners. US Pharmacopoeial Convention, Rockville, MD, vol. 1, 16th edn (1996), pp. 2567–2572.