



Development and Validation of a Simple and Sensitive RP-HPLC Method for Determination of Rifampicin in Bulk and Tablets

A. H. MEMON, N. MEMON

Faculty of Pharmacy, University of Sindh, Jamshoro, Pakistan

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Abstract: A simple, precise, sensitive, economic, reproducible and validated RP-HPLC method was developed for the quantitative determination of Rifampicin in bulk and tablet dosage form. Analysis was carried out using acetonitrile: 0.1% orthophosphoric acid (80:20) mobile phase at 0.4 mL/min flow rate and Zorbax Eclipse Plus C18 column as stationary phase with detection wavelength of 480 nm. The retention time was found to be 4.7 min. The method showed good linearity in the range of 1.95-250 µg/mL with correlation coefficient of 0.9996. Limit of detection and limit of quantification was found to be 0.607 and 1.841 µg/mL, respectively. The method was validated for specificity, linearity, precision, accuracy, and limit of detection and quantification following ICH guidelines.

Keywords: Rifampicin, RP-HPLC, Linearity, Retention time, Accuracy.

1. INTRODUCTION

Tuberculosis (TB) one of the oldest recorded human suffering, continues to be a leading cause of death due to infectious disease. Nearly 1/3rd of the world's population is suffering from TB infection, and nearly 2 million human lose their lives annually due to TB infection (Issar 2003; Sung *et al.*, 2009). After HIV syndrome, TB is considered the world's second most common death cause infectious disease (Lisat *et al.*, 2006). The emergence of multi drug resistant cases of TB also causes a major challenge to control of the disease worldwide (Bjørn *et al.*, 2001). Rifampicin (RIF) is a potent antibiotic against gram-positive bacteria including *Mycobacterium*. Currently, it is frequently used in the chemotherapy of TB along with pyrazinamide, isoniazid, ethambutol and streptomycin (Conte *et al.*, 2000; Mitchison 2005).

RIF is considered to be the cornerstone in the current treatment of TB (Burman *et al.*, 2001), owing to its potent sterilizing ability (Takushi *et al.*, 2011). RIF is a bactericidal drug for *Mycobacterium tuberculosis*. After oral administration of RIF (600 mg) its plasma peak concentration (8-12 µg/mL) reached within 1-2 h (Sriram *et al.*, 2012). RIF specifically inhibits DNA dependant RNA polymerase, the enzyme responsible for DNA transcription (Akos *et al.*, 2001; Elizabeth *et al.*, 2001; Wehrli, 1983; Yu *et al.*, 2013). Various HPLC methods (Ashutosh *et al.*, 2014; Hemanth *et al.*, 2004; Ignacio *et al.*, 2004; Sriram *et al.*, 2012) are reported for the determination of RIF. The proposed method is quite unique in terms of retention time, non-interfering wavelength and cost of mobile phase (Table 1).

2. MATERIAL AND METHODS

Solvents and Chemicals

Rifampicin (purity >98%) was used as received from Tokyo Chemical Industry, Co., Ltd., Japan. HPLC grade acetonitrile (ACN), methanol (MeOH) and 85% orthophosphoric acid (H₃PO₄) was purchased from Sigma Aldrich, USA. Double distilled deionized water was used. All other chemicals and reagents were of analytical grade.

3. Instrument and Chromatographic Conditions

Equipment: HPLC system (Agilent 1260), USA, Column: Eclipse Plus C18 column (4.6×250 mm, 5µm) Zorbax, USA. Mobile phase: Mixture of ACN: 0.1% H₃PO₄ (80: 20 v/v), Flow rate: 0.4 mL/min, Wavelength: 480 nm, column temperature: 30 °C.

4. Preparation of Standard Stock Solution

Accurately weighed quantity (10 mg) of RIF was taken into (10 mL) volumetric flask, dissolved in MeOH, sonicated, filtered through 0.45µm filter paper and diluted with MeOH to obtain (1000 µg/mL) standard stock solution. This was then serially diluted to 1.95-250 µg/mL.

5. Preparation of Sample Solution

Ten tablets of three different pharmaceutical companies were purchased from local market for the analysis of RIF. Accurately weighed amount of powdered tablets equivalent to 10mg of RIF for each product namely; Rifinah (Pacific Pharmaceuticals Ltd., Pakistan), Rimactal (Novartis Pharma, Ltd., Pakistan) and Lederrif (Wyeth Pharma, Ltd., Pakistan) was

**Corresponding author: memon_h79@yahoo.com

dissolved in separate (10 mL) volumetric flask containing sufficient volume of MeOH by well shaking and then filtered through Whatmann No. 1 filter paper before adjusting to the final volume (10 mL) with MeOH. This solution was considered as the stock solution and separate aliquots (100 µg/mL) of stock solution were taken from each product for the determination of RIF.

Method Development

Different mobile phases were used during in the development of suitable HPLC method for better analysis of RIF in tablets and bulk. These included ACN: 0.1% H₃PO₄, MeOH: 0.1% H₃PO₄, ACN: H₂O, ACN: 0.1% formic acid, ACN: ammonium acetate buffer, MeOH: H₂O. The suitable mobile phase was determined on the basis of the low retention time, sensitivity of the assay, ease of preparation, and cost of solvents.

Method Validation

The method was subjected to validate various parameters like specificity, linearity, precision, limits of quantification and detection (LOQ and LOD), and accuracy of method as described in (ICH, 1997).

Specificity

The specificity of the method was determined by observing interference of other anti-TB drugs present in the pharmaceutical formulations. Isoniazid, Pyrazinamide and Ethambutol were injected at the selective wavelength under the same conditions set for RIF. The test results obtained were compared with the results of those obtained for RIF.

Linearity

Linearity was determined by injecting standard mixture (10 µL) in the concentration range (1.95-250 µg/mL). The calibration curve was obtained by plotting peak height versus concentration, and linear correlation coefficient (R^2) was determined by regression calculation of calibration graph.

Precision

Precision of the method was performed as intraday, interday and intermediate precision. To study the precision, five standard solutions of RIF were injected in five replicates. Relative standard deviation (RSD) was calculated as (% RSD), and was within the acceptable criteria $\leq 2\%$.

Accuracy

Accuracy was determined in terms of recovery (%) of RIF, added to known amount of pre-analyzed tablets at the concentration of 1 mg/mL. The % recovery was calculated at 250, 125 and 62.5 µg/mL of RIF. Peak height of the RIF in the pre-analyzed tablets solution (A), reference compound (B) and their combinations (C) were recorded. Percentage recovery was calculated using following equation (Aisha *et al.*, 2012) and mean

$\pm$ SD ($n=3$) was calculated (Table 2). The %recovery ranging from 99.2 to 101.2% was obtained by the proposed method using following equation. %Recovery = $((C-A) / B) \times 100$.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ for the assay were calculated using equations as described in (ICH, 1997), $LOD = (3.3 \times \delta) / S$, and $LOQ = (10 \times \delta) / S$. Where δ = standard deviation of the Y intercept of linear regression equations and S = slope of linear regression equations.

6. RESULTS AND DISCUSSION

Method Development

Different solvents and solvent mixtures were investigated, but ACN: 0.1% H₃PO₄ (80: 20) produced well defined sharp peaks with better symmetry and retention time 4.7 min (Fig. 1A). The peak shape and sensitivity was poor and retention time was recorded 7.8 min in with ACN: H₂O (80: 20) as mobile phase. MeOH: 0.1% H₃PO₄ (80: 20) did not produce sharp peak. Other mobile phases produced lower sensitivity and increased retention time so, they were not considered. Finally, mobile phase composition (ACN: 0.1% H₃PO₄ (80: 20)) and flow rate (0.4 mL/min) was selected which produced better peak shape and height.

Method Validation

Specificity

In therapeutic treatment, RIF is often co-administered with the other anti-tuberculosis drugs like isoniazid, pyrazinamide, and ethambutol. When RIF samples were spiked with these drugs no interference was observed in the chromatogram using developed method as no peak signal appeared at this selected wavelength of these drugs. Moreover, well-shape peaks of RIF also showed the specificity of method (Fig. 1B-1D).

Linearity

The calibration plot of peak height versus concentration was linear at range investigated (1.95-250 µg/mL). The low % RSD indicates that method is precise. The linear regression of calibration plot shows good linear relationship between peak height and concentrations at selected range. The linear regression equation observed as $y = 2398.3x + 9180.5$ and the regression coefficient was 0.9996.

Precision

The %RSD was determined to check the precision of peak height and retention time. The standard mixtures were analyzed at five different concentrations (15.26-250 µg/mL), and the intraday, interday and intermediate precision was determined ($n=3$).

RIF was eluted at 4.7 ± 0.02 min with a %RSD < 2%. The %RSD of the peak height was calculated at concentrations (15.26-250 $\mu\text{g/mL}$). These results indicate good reproducibility of retention time and concentration. Results of system precision studies are shown (Table 3).

Accuracy

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were calculated using slope and standard deviation method by utilizing linear regression equations of the reference compounds (Table 4). The

amount found in three different brands of tablet was calculated and shown in (Table 5).

7. CONCLUSION

A precise and accurate RP-HPLC method was developed for the quantification of RIF at low retention time, good peak symmetry and phenomenal linearity. The method was proved to be better to most of the other reported methods for the RIF. The proposed method can be used for the quantification of RIF in bulk and tablets dosage form without intrusion of other anti-tubercular drugs.

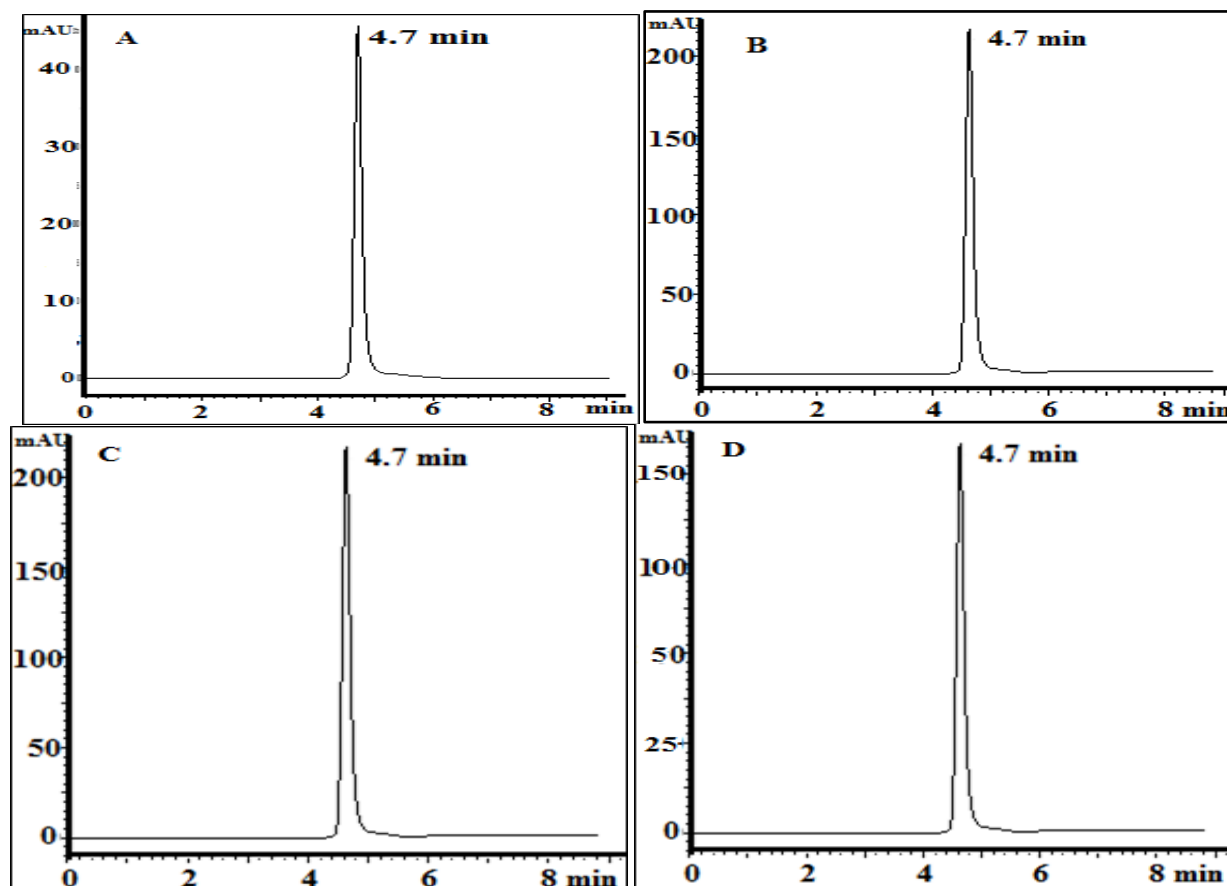


Fig-1: Representative chromatogram of RIF obtained with (A) ACN: 0.1% H_3PO_4 (80: 20), (B) Rifinah tablets, (C) Rimactal tablets and (D) Lederrif tablets

Table-1 Comparison of proposed method with reported methods.

S. #	Linearity ($\mu\text{g/mL}$)	Retention time (min)	Mobile phase	Column	Wave length (nm)	References
1	1.95-250	4.7	ACN: 0.1% H_3PO_4 (80:20) at 0.4 mL/min, pH independent	Luna C18 column (4.6× 250 mm, 5 μm , Phenomenex, Torrance, USA)	480	Proposed paper
2	10-50	2.4	ACN: H_3PO_4 (50:50) at 1 mL/min Potassium dihydrogen Phosphate buffer (pH adjusted to 3.0).	ODS C18 (4.6× 150 mm, 3.5 μm).	238	(Ashutosh <i>et al.</i> , 2014)

3	0.25-15	4.8	0.05 M phosphatebuffer: ACN (55:45) at 1.2 mL/min.	Luna C18 column (4.6×250mm, 5 µm, Phenomenex, Torrance, USA).	254	(Hemanth <i>et al.</i> , 2004)
4	1-50	4.0	H ₂ O: ACN (40:60) at 1 mL/min.	Ultrabase-C (4.6×250 mm, 5 µm) (Scharlau, Barcelona, Spain)	333	(Ignacio <i>et al.</i> , 2004)
5	0.31-25.4	6.8	ACN: 10 mM potassium dihydrogen phosphate (40:60) pH adjusted to 3.2 at 1 mL/min.	Luna C18 ODS-2 (4.6×150 mm, 5 µm, Phenomenex, Torrance, USA)	337	(Sriramet <i>et al.</i> , 2012).
6	5-200	15.4	MeOH: ACN: monopotassium phosphate (0.075 M): citric acid (1M): (28:30:38:4) at 2 mL/min.	Chrom C18 column (4.6×150 mm, 5 µm, China).	254	(Jianfang <i>et al.</i> , 2008).
7	0.5-250	7.8	ACN: 0.05 M buffer solution of potassium phosphate monobasic (38:62)adjusted to pH 3.7 with H ₃ PO ₄ at 1 mL/min	PurosphereStar C18 (150×4.6 mm, 5 µm Merck, Germany).	335	(Luis and Grande 2014).

Table-2 Regression analysis of calibration curve for the proposed method.

Parameters	Optimized conditions
Linearity range (µg/mL)	1.95-250
Regression equation (Y=ax+b)	2398.3x + 9180.5
Slope (a)	2398.3
Intercept (b)	9180.5
Correlation coefficient (R ²)	0.9996
Relative standard deviation (%)	0.01
Retention time	4.7
Recovery (%)	99.2-101.2

Table-3 Results of Precision study of RIF.

Precision	Concentration (µg/ mL)	Peak height		Retention time (min)	
		Average	%RSD	Average	%RSD
Intraday	250	67088	0.37	4.684	0.11
	125	35588	0.16	4.676	0.11
	62.5	18082	0.18	4.674	0.11
	31.25	9528	0.28	4.672	0.09
	15.62	5048	0.10	4.674	0.11
Interday	250	68214	0.85	4.688	0.27
	125	36152	0.89	4.678	0.28
	62.5	18484	0.51	4.674	0.19
	31.25	9723	0.60	4.676	0.11
	15.62	5181	0.75	4.676	0.12
Intermediate	250	67651	1.17	4.686	0.06
	125	35869	1.12	4.677	0.03
	62.5	18288	1.55	4.674	0.00
	31.25	96239	1.45	4.674	0.06
	15.62	5115	1.83	4.675	0.03

Table-4 Summary of Rifampicin calibration data.

S.#	Parameters	*a	*b	LOD (µg/mL)	LOQ (µg/mL)
1	Intraday	264.72	1420.10	0.60	1.82
2	Interday	268.77	1504.22	0.61	1.85
3	Intermediate	266.83	1462.16	0.60	1.84

*The regression equation is (y =ax+b), where (a) is the slope and (b) is the y intercept. The data are presented as average ±SD (n=3).

Table-5 Assay of Rifampicin tablets.

S.#	Samples	Amount labelled (mg) / tablet	Amount found (mg) / tablet	% Assay ±SD
1	Rifinah	300	297	99.0
2	Rimactal	450	449.5	99.8
3	Lederrif	600	603	100.5

REFERENCES:

- Aisha, A., K. Abu-Salah, M. Siddiqui, Z. Ismail, A. A. Majid, (2012). Quantification of α -, β - and γ mangostin in *Garcinia mangostana* fruit rind extracts by a reverse phase high performance liquid chromatography. *Journal of Medicinal Plant Research*, 6(29), 4526-4534.
- AkosSomoskovi, M. P. Linda, M. Salfinger, (2001). The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. *Respiratory Research*, 2(3), 164-168.
- Ashutosh, K. S., D. Manidipa, S. Rao, B. Chaitanya, P. A. Durga, G. P. Sri Ramya, M. Ujjwala1, P. R. N. G. Raj, P. G. Babu, (2014). Rapid and sensitive RP-HPLC analytical method development & validation for estimation of Rifampicin in bulk as well as in pharmaceutical formulation by using PDA detector. *Indo\American Journal of Pharmaceutical Research*, 4(3), 1561-1572.
- Blomberg, B., S. Spinaci, B. Fourie, R. Laing, (2001). The rationale for recommending fixed-dose combination tablets for treatment of tuberculosis. *Bulletin of the World Health Organization*, 79(1), 61-68.
- Burman, W. J., K. Gallicano, C. Peloquin, (2001). Comparative pharmacokinetics and pharmacodynamics of the rifamycinantibacterials. *Clinical Pharmacokinetics*, 40(5), 327-341.
- Conte, J. E. J., E. Lin, E. Zurlinden, (2000). Liquid chromatographic determination of Rifampicin in human plasma, bronchoalveolar lavage fluid, and alveolar cells. *Journal of Chromatographic Science*, 38(2), 72-76.
- Elizabeth, A. C., N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb, (2001). Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. *Cell*, 104(6), 901-912.
- Hemanth, K. A. K., I. Chandra, R. Geetha, C. K. Silambu, V. Lalitha, G. Prema, (2004). A validated high-performance liquid chromatography method for the determination of rifampicin and desacetyl rifampicin in plasma and urine. *Indian Journal of Pharmacology*, 36(4), 231-233.
- Hickey, A. J., D. A. Edwards, (2009). Formulation and Pharmacokinetics of Self-Assembled Rifampicin Nanoparticle Systems for Pulmonary Delivery. *Pharmaceutical Research*, 26(8), 1847-1855.
- ICH., (1997). Guidance for Industry, Q2B: Validation of Analytical Procedures: Methodology. Paper presented at the International Conference on Harmonisation, USA.
- Ignacio, C., J. B. P. Maria, R. Noelia, J. R. Maria, C. D. V. Maria, (2004). High-performance liquid-chromatographic determination of rifampicin in plasma and tissues. *Journal of Chromatography A*, 1031(1-2), 289-294.
- Issar, S., (2003). *Mycobacterium tuberculosis* Pathogenesis and Molecular Determinants of Virulence. *Clinical Microbiology Reviews*, 16(3), 463-496.
- Jianfang, L., S. Jin, Z. Wei, G. Kun, H. Zhonggui, (2008). HPLC determination of rifampicin and related compounds in pharmaceuticals using monolithic column. *Journal of Pharmaceutical and Biomedical Analysis*, 46(2), 405-409.
- Kaneko, T., C. Cooper, K. Mdluli, (2011). Challenges and opportunities in developing novel drugs for TB. *Future Medicinal Chemistry*, 3(11), 1373-1400.
- Lisa, C. T., V. Pillay, M. P. Danckwerts, (2006). Tuberculosis chemotherapy: current drug delivery approaches. *Respiratory Research*, 7(1), 1-18.
- Luis, M.-E., M. O. Grande, (2014). Validation of a liquid chromatography method for rifampicin determination in human plasma. *Peruvian Journal of Experimental Medicine and Public Health*, 31(1), 56-61.
- Mitchison, D. A., (2005). The diagnosis and therapy of tuberculosis during the past 100 years. *American Journal of Respiratory and Critical Care Medicine*, 171(7), 699-706.
- Sriram, S., T. B. Prasanthi, S. Tata, R. J. Vijaya, (2012). Development and Validation of High Performance Liquid Chromatographic Method for The Determination of Rifampicin in Human Plasma. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(5), 362-367.
- Sung, J. C., D. J. Padilla, L. Garcia-Contreras, J. L. Verberkmoes, D. Durbin, C. A. Peloquin, K. L. Elbert, Wehrli, W., (1983). Rifampin: mechanisms of action and resistance. *Reviews of infectious diseases*, 5, 407-411.
- Yu Pang, J. Lu, Y. Wang, Y. Song, S. Wang, Y. Zhao, (2013). Study of the Rifampin Monoresistance Mechanism in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, 57(2), 893-900.