

**ASSAY OF RIFAMPICIN IN PHARMACEUTICAL FORMULATION: COMPARISON OF SPECTROPHOTOMETRIC AND MICROBIOLOGICAL METHODS****SARBJIT SINGH JHAMB<sup>1</sup> AND RAMAN PREET SINGH<sup>2,\*</sup>,<sup>†</sup>**

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**ABSTRACT**

*Micrococcus luteus* was employed for the assay of rifampicin in fixed-dose combinations (FDCs) of anti-tubercular drugs containing rifampicin, isoniazid, pyrazinamide and ethambutal. The microbiological assay was compared with spectrophotometric assay for estimation of rifampicin in the FDCs as well as in dissolution studies of the FDCs. The microbiological method was found to be more sensitive and accurate as compared to spectrophotometric assay and did not show any interference in the presence of other anti-tubercular drugs. The limits of detection and quantification for microbiological method were 0.1 µg/ml and 0.64 µg/ml, respectively.

**KEYWORDS**

Rifampicin, *Micrococcus luteus*, microbiological assay, pharmaceutical formulations, fixed dose combination

**INTRODUCTION**

Rifampicin is used as a drug of choice for treatment of tuberculosis along with other drugs. The pharmacopoeal methods of analysis of rifampicin are spectrophotometric method and high performance liquid chromatography (HPLC). However, spectrophotometric estimation may give erroneous results due to interference by formulation excipients whereas the HPLC method is relatively expensive and requires laborious procedures for sample preparation. Here, we describe a microbiological method for

assay of rifampicin using *M. luteus*. A direct correlation was observed between concentration of rifampicin and zone of inhibition. We have compared the spectrophotometric and microbiological method for analysis of rifampicin in four anti-tubercular FDCs and their dissolution samples.

## MATERIALS AND METHODS

### **Drugs, chemicals and culture media**

Anti-tubercular FDCs were obtained from a pharmacy store. The details of formulations are

summarized in Table 1. The culture media for bacterial culture were obtained from HiMedia, India. All chemicals used in the study were of analytical grade.

**Table 1**  
**Details of four FDCs containing rifampicin**

Formulation code	Contents
Formulation A	Rifampicin 150 mg, Isoniazid 75 mg, Pyrazinamide 400 mg and Ethambutal 275 mg
Formulation B	Rifampicin 150 mg, Isoniazid 75 mg, Pyrazinamide 400 mg and Ethambutal 275 mg
Formulation C	Rifampicin 150 mg, Isoniazid 75 mg, Pyrazinamide 400 mg and Ethambutal 275 mg
Formulation D	Rifampicin 150 mg, Isoniazid 75 mg, Pyrazinamide 400 mg and Ethambutal 275 mg

### **Dissolution studies**

Tablets were individually weighed and subjected to dissolution using USP apparatus II in 900 ml of 0.1 N HCl, 0.01N HCl and phosphate buffer (PB, pH 6.8) at agitation rates of 30, 50, 75 and 100 rpm. Samples were withdrawn after regular intervals and an equal volume of the dissolution medium was replaced. Rifampicin degrades at lower pH, hence reference vessel method was employed<sup>1</sup> to calculate the amount of drug released.

The presence of food can reduce the bioavailability of rifampicin. Hence, to determine the effect of food on dissolution, dissolution was carried out in 20% vegetable oil in PB at 50 rpm.

### **Spectrophotometric analysis**

Samples obtained at different time points were suitably diluted with PB and absorbance was obtained in a UV-Visible spectrophotometer at 475 nm.

### **Microbiological analysis**

#### **Bacteria and inoculum**

Freeze-dried cultures of *M. luteus* ATCC 9341 (MTCC 1541) were revived on nutrient agar and stored at 4°C until further use.

#### **Preparation of inoculum**

Stock culture of *M. luteus* was transferred to fresh nutrient agar media and incubated at 37±1°C for 16-18 hours. The culture was then harvested in sterile saline and adjusted to 25-30% transmittance at 530 nm. Inoculum optimization was done to obtain clear zones of inhibition.

#### **Inoculation of medium**

Sterile, molten medium (Antibiotic assay medium 1) was cooled to 40-50°C and inoculated with the culture suspension (1 ml suspension for 100 ml medium). The inoculated medium was poured in 100 mm petri plates and allowed to solidify at room temperature.

### **Preparation of standard and sample solution**

Stock standard solution of rifampicin was prepared in methanol (5.12 mg/ml) and dilutions were performed in PB to obtain a standard plot.

The sample solutions for assay of rifampicin in FDC tablets were prepared as follows. Individual tablets were crushed and rifampicin was extracted with methanol by gentle shaking for 30 minutes. The volume of methanol was adjusted to obtain approximately 5 mg/ml rifampicin. The methanol extract was suitably diluted in PB and rifampicin concentration was determined by spectrophotometric as well as microbiological method.

The dissolution samples were used without further processing.

### **Inoculation of standard and test dilutions**

The analysis was carried by two methods: disc diffusion and cylinder-plate method. For disc diffusion method, 6 mm paper discs were placed on surface of inoculated plates and constant volume (5 and 10  $\mu$ l) of standard and test dilutions was applied; 5  $\mu$ l was found to be the optimum volume for disc diffusion method. For bore-well method, cavities were made with the help of cylinders (8 mm diameter) and filled with a constant volume of 100  $\mu$ l of standard and test dilutions. The plates were kept at room temperature for 30 minutes and then incubated at 37°C overnight (17-18 hours). After incubation, the zone of inhibition was measured with digital vernier calipers and rifampicin concentration was obtained from the standard plot.

### **Microbiological assay validation**

#### **Linearity**

The standards and test samples were analyzed by microbiological method using 4 different

concentrations of rifampicin and regression line was obtained using Microsoft Excel 2003.

#### **Precision**

Reproducibility or intra-assay variability of the assay procedure was determined by analyzing test samples and standard samples on the same day. The precision or inter-assay variability of the assay was determined by analyzing test samples and standard samples on three consecutive days.

#### **Accuracy and recovery**

Accuracy of the assay procedure was determined by adding known amounts of rifampicin to the test samples. The test solution of rifampicin was mixed with an equal volume of reference rifampicin solution and analyzed by the microbiological assay. Alternatively, the test samples were mixed with an equal volume of distilled water and analyzed by the microbiological assay. The values obtained were compared with the reference standard.

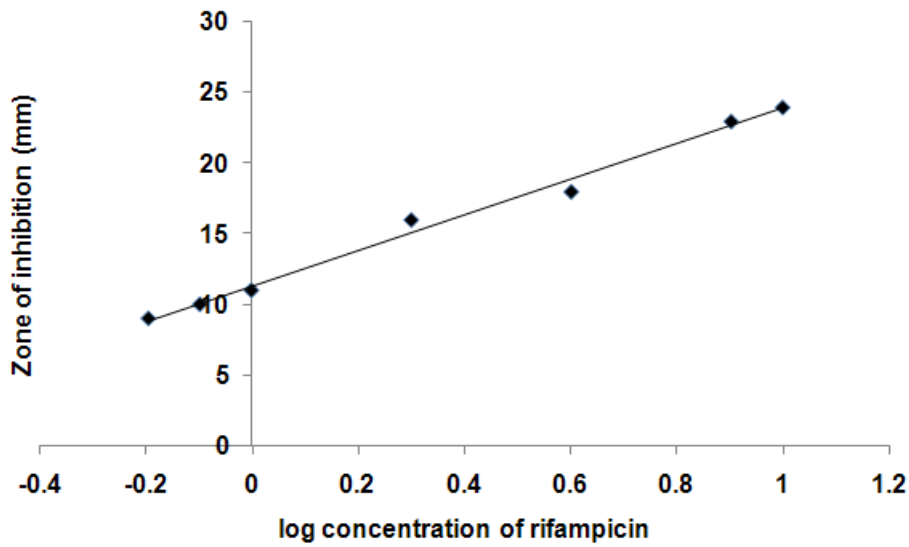
#### **Comparison of spectrophotometric and microbiological methods**

The spectrophotometric and microbiological methods were compared by Bland-Altman and Passing-Bablok difference plots<sup>2-5</sup>.

## **RESULTS**

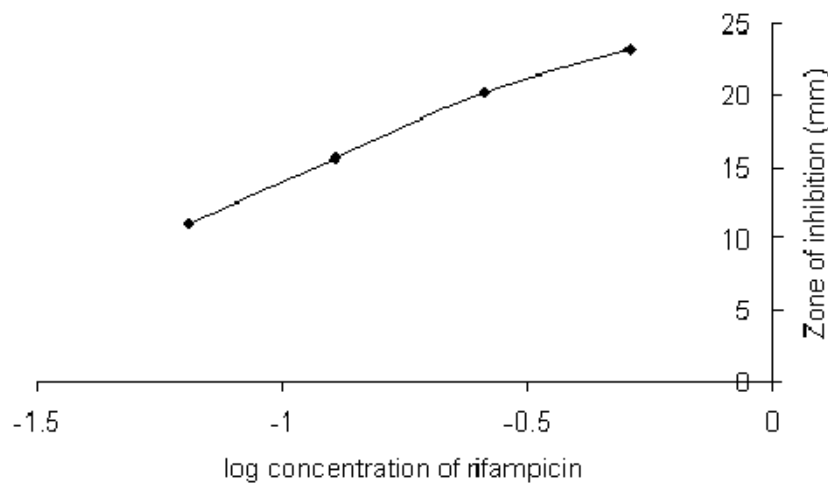
### **Standard curve between log concentration of rifampicin and zone of inhibition**

The standard curve between log concentrations of rifampicin and zone of inhibition was obtained. This curve was linear ( $r^2 = 0.99$ ) over a broad range of concentrations (0.64  $\mu$ g/ml to 10  $\mu$ g/ml). The standard curves were plotted by disc diffusion (Figure 1) or cylinder-plate method (Figure 2).



**Figure 1**

**Calibration curve of rifampicin by disc diffusion method using *M. luteus* as test organism**



**Figure 2**

**Calibration curve of rifampicin by cylinder-plate method using *M. luteus* as test organism**

**Validation of microbiological assay**

The assay was performed in quadruplicates and repeated three times. The intra-assay variability

was found to be <5% while inter-assay variability was found to be 6-9%. The recovery of rifampicin was found to be >95% (Table 2).

**Table 2**  
**Recovery of rifampicin in spiked test samples**

Spiked amount of drug (mg/ml)	Recovered drug (mg/ml)	% Recovery
0.25	0.24	96
0.5	0.49	98
1.0	0.96	96

#### **Sensitivity of microbiological assay**

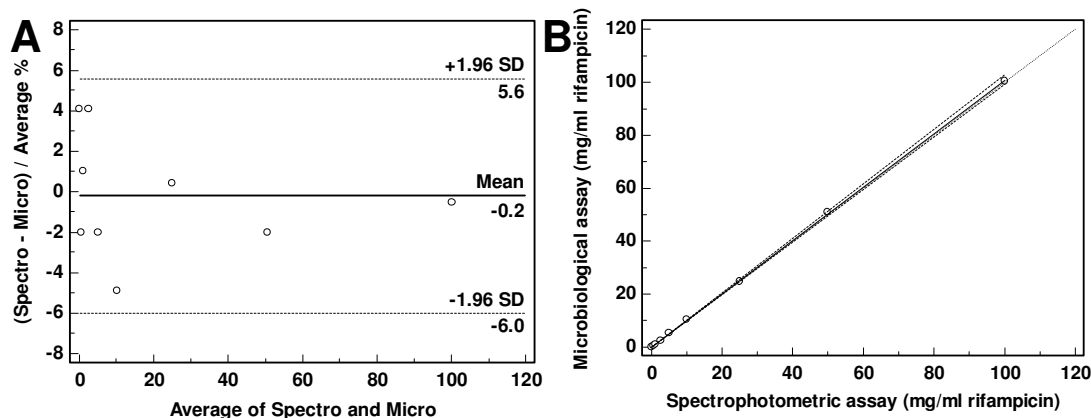
The sensitivity of assay was found to be 0.1 µg/ml for disc diffusion method (0.0005 µg per 5 µl sample) and 0.0064 µg/ml for cylinder-plate method (0.00064 µg per 100 µl sample).

#### **Limit of quantification**

The log concentration of rifampicin versus zone of inhibition plot was found to be linear over a range of 0.64-2.56 µg/ml for disc-diffusion method and 0.064-0.512 µg/ml for cylinder-plate method, thus, showing linearity over a range of 0.0032-0.128 µg and 0.0064-0.0512 µg, respectively. Hence, the limits of quantification were 0.0032 and 0.0064 µg for disc-diffusion and cylinder-plate method, respectively.

#### **Comparison between spectrophotometric and microbiological assay of rifampicin for drug samples**

The Bland-Altman (Figure 3A) and Passing-Bablok (Figure 3B) difference plots showed that concentration of rifampicin standards (after suitable dilutions) determined by spectrophotometric and microbiological assay were in close agreement over a range of 0.1-100 mg/ml. Bland-Altman plot showed that difference in rifampicin concentration determined by the two assay procedures was <5% at all the concentrations tested. Passing-Bablok plot showed no significant deviation from linearity ( $P>0.10$ ) in rifampicin concentration determined by the two assay procedures.



**Figure 3**

**Comparison of spectrophotometric and microbiological assay of rifampicin in rifampicin standards by Bland-Altman (A) and Passing-Bablok (B) difference plots**

#### **Comparison between spectrophotometric and microbiological assay of rifampicin for dissolution samples**

Dissolution samples were obtained at different time points and percent release of rifampicin was compared by spectrophotometric and

microbiological methods. The microbiological assay was found to be sensitive and accurate. The results of comparative analysis of samples for formulations A, B, C and D are summarized in tables 3-6. The difference in rifampicin concentration determined by the two assay

procedures was insignificant (Student's *t*-test;  $P>0.05$ ). Bland-Altman and Passing-Bablok analysis also showed no significant difference in the rifampicin concentration determined by the two assay procedures (data not shown).

**Table 3**  
**Comparison of spectrophotometric and microbiological assay of rifampicin in dissolution studies of Formulation A**

Dissolution parameters		Percent release	
Medium	rpm	Spectrophotometric assay	Microbiological assay
0.01N HCl	30	77.26	73.51
0.01N HCl	75	98.89	92.25
0.01N HCl	100	100.69	98.12
0.1N HCl	30	85.77	87.06
0.1N HCl	75	100.48	96.10
0.1N HCl	100	99.01	100.28
20% oil in PB	50	92.02	93.75

**Table 4**  
**Comparison of spectrophotometric and microbiological assay of rifampicin in dissolution studies of Formulation B**

Dissolution parameters		Percent release	
Medium	rpm	Spectrophotometric assay	Microbiological assay
0.01N HCl	30	80.21	79.83
0.01N HCl	75	97.58	96.63
0.01N HCl	100	100.05	101.67
0.1N HCl	30	87.67	89.31
0.1N HCl	75	98.43	97.89
0.1N HCl	100	105.31	102.31
20% oil in PB	50	94.97	94.88

**Table 5**  
**Comparison of spectrophotometric and microbiological assay of rifampicin in dissolution studies of Formulation C**

Dissolution parameters		Percent release	
Medium	rpm	Spectrophotometric assay	Microbiological assay
0.01N HCl	30	62.63	63.72
0.01N HCl	75	100.16	95.95
0.01N HCl	100	95.20	96.12
0.1N HCl	30	72.95	80.50
0.1N HCl	75	101.32	92.06
0.1N HCl	100	101.77	93.67
20% oil in PB	50	80.21	85.74

**Table 6**  
**Comparison of spectrophotometric and microbiological assay of rifampicin in dissolution studies of Formulation D**

Dissolution parameters		Percent release	
Medium	rpm	Spectrophotometric assay	Microbiological assay
0.01N HCl	30	70.62	72.6
0.01N HCl	75	102.53	98.26
0.01N HCl	100	104.72	98.60
0.1N HCl	30	75.69	81.99
0.1N HCl	75	96.19	101.12
0.1N HCl	100	104.38	104.14
20% oil in PB	50	84.31	86.43

**Comparison of spectrophotometric and microbiological assay of rifampicin in rifampicin tablets**

Sample dilutions prepared from pre-weighed tablets of the four FDCs were assayed by both the methods. There appeared to be a direct correlation between concentration of rifampicin and zone of inhibition. Further, there was no

significant difference between the values obtained by either of the methods (Table 7). Student's *t*-test, Bland-Altman and Passing-Bablok analysis showed no significant difference in rifampicin concentration determined by the two assay procedures (data not shown).

**Table 7**  
**Comparison of spectrophotometric and microbiological methods for assay of rifampicin in formulations**

Formulation code	Assay (%) $\pm$ SD	
	Spectrophotometric assay	Microbiological assay
Formulation A	108.3 $\pm$ 2.9	107.8 $\pm$ 1.3
Formulation B	108.7 $\pm$ 3.5	106.3 $\pm$ 3.8
Formulation C	109.0 $\pm$ 1.4	110.6 $\pm$ 2.1
Formulation D	96.4 $\pm$ 2.4	100.4 $\pm$ 3.1

**Interference with other anti-tubercular drugs and pharmaceutical excipients**

Results of microbiological assay were compared with spectrophotometric assay for standard drug and in the four FDCs. None of the anti-tubercular drugs showed interference in the assay. Further, commonly used pharmaceutical excipients did not show any interference with the microbiological assay (data not shown).

The method described here for estimation of rifampicin in dissolution medium and tablets is simple and accurate. Microbiological assay was found to be more sensitive than spectrophotometric method and required very small amount of sample (5  $\mu$ l). The limit of detection and limit of quantification were lower for microbiological method at 0.1  $\mu$ g/ml and 0.64  $\mu$ g/ml respectively. On the other hand, spectrophotometric method requires relatively large amount of sample (0.5-1.0 ml) and is less

**DISCUSSION**



sensitive with limits of detection and quantification above 1 µg/ml.

Further, microbiological method showed a linear correlation between concentration and zone of inhibition over a wide range of concentrations, from 0.64-2.56 µg/ml for disc-diffusion method and 0.064-0.512 µg/ml for cylinder-plate method. Although cylinder-plate method seemed to be more sensitive to lower concentrations, but disc-diffusion method is more sensitive when compared on the basis of total amount of drug added (0.0005 µg for disc-diffusion and 0.00064 µg for cylinder-plate method). The higher sensitivity of cylinder-plate method could be attributed to the large volume of drug added in wells as compared to small volume required to impregnate the paper strips in disc-diffusion method, 100 µl and 5 µl respectively.

The microbiological assay was carried out in presence of other anti-tubercular drugs (isoniazid, pyrazinamide and ethambutal). No interference was observed in the estimation,

either in the presence of other drugs or excipients.

Although previous studies using *M. luteus*<sup>6-16</sup>, *Staph. aureus*<sup>12, 17-20</sup> and *Bacillus subtilis*<sup>21-24</sup> have demonstrated the microbiological assay of rifampicin in various biological fluids, this is the first study to demonstrate that *M. luteus* can be used for assay of rifampicin in pharmaceutical formulations and in dissolution studies. Further, the microbiological assay may be useful in detection and assay of trace amounts of rifampicin which may escape detection from a relatively less sensitive spectrophotometric assay.

In conclusion, the method described in the present study can be used for estimation of rifampicin in formulations and dissolution medium. The method is sensitive, linear, precise and accurate. The method requires small volume of sample and the presence of other drugs and excipients does not interfere with the estimation of rifampicin.

## REFERENCES

1. US Pharmacopoeia. Rifampicin Capsules. In *United States Pharmacopoeia 26*, United States Pharmacopoeial Convention Inc., Rockville, MD, 2003, pp. 1640-1641.
2. Atiqi R, C van Iersel, and TJ Cleophas, Accuracy assessments of quantitative diagnostic tests for clinical research, *Int J Clin Pharmacol Ther*, 47: 153-8, (2009).
3. Ludbrook J, Statistics in biomedical laboratory and clinical science: applications, issues and pitfalls, *Med Princ Pract*, 17: 1-13, (2008).
4. Hanneman SK, Design, analysis, and interpretation of method-comparison studies, *AACN Adv Crit Care*, 19: 223-34, (2008).
5. Cleophas TJ, J Droogendijk, and BM van Ouwkerk, Validating diagnostic tests, correct and incorrect methods, new developments, *Curr Clin Pharmacol*, 3: 70-6, (2008).
6. Buniva G, V Pagani, and A Carozzi, Bioavailability of rifampicin capsules, *Int J Clin Pharmacol Ther Toxicol*, 21: 404-9, (1983).
7. Furesz S, et al., Rifampicin: a new rifamycin. 3. Absorption, distribution, and elimination in man, *Arzneimittelforschung*, 17: 534-7, (1967).
8. Boman G, Serum concentration and half-life of rifampicin after simultaneous oral administration of aminosalicylic acid or isoniazid, *Eur J Clin Pharmacol*, 7: 217-25, (1974).
9. Boman G and AS Malmberg, Rifampicin in plasma and pleural fluid after single oral doses, *Eur J Clin Pharmacol*, 7: 51-8, (1974).
10. Ericsson H, Assay of antibiotics in small amounts of fluid, *Scand J Clin Lab Invest*, 12: 423-32, (1960).



11. Zwolska Z, et al., Bioavailability of rifampicin, isoniazid and pyrazinamide from fixed-dose combination capsules, *Int J Tuberc Lung Dis*, 2: 824-30, (1998).
12. Dans PE, et al., Rifampin: antibacterial activity in vitro and absorption and excretion in normal young men, *Am J Med Sci*, 259: 120-32, (1970).
13. Acocella G, et al., Serum and urine concentrations of rifampicin administered by intravenous infusion in man, *Arzneimittelforschung*, 27: 1221-6, (1977).
14. D'Oliveira JJ, Cerebrospinal fluid concentrations of rifampin in meningeal tuberculosis, *Am Rev Respir Dis*, 106: 432-7, (1972).
15. Sippel JE, et al., Rifampin concentrations in cerebrospinal fluid of patients with tuberculous meningitis, *Am Rev Respir Dis*, 109: 579-80, (1974).
16. Garnham JC, et al., Serum concentrations and bioavailability of rifampicin and isoniazid in combination, *Br J Clin Pharmacol*, 3: 897-902, (1976).
17. Dickinson JM, et al., Assay of rifampicin in serum, *J Clin Pathol*, 27: 457-62, (1974).
18. Mitchison DA, et al., Sensitive microbiological method for the detection of rifampicin in urine, *Tubercle*, 55: 245-8, (1974).
19. Mitchison DA, BW Allen, and AB Miller, Detection of rifampicin in urine by a simple microbiological assay, *Tubercle*, 51: 300-4, (1970).
20. Furesz S, Chemical and biological properties of rifampicin, *Antibiot Chemother*, 16: 316-51, (1970).
21. Outman WR, et al., Intraocular penetration of rifampin in humans, *Antimicrob Agents Chemother*, 36: 1575-6, (1992).
22. DA Grove and WA Randall, Assay methods of antibiotics, A laboratory manual, *Medical Encyclopedia Inc.*, New York: 188-196, (1967).
23. Lees KA and JPR Tootil, Microbiological assay of large plates, Part-1 General considerations with particular reference to routine assay, *Analyst*, 80: 95-110, (1955).
24. Mannisto P, Absorption of rifampin from various preparations and pharmaceutical forms, *Clin Pharmacol Ther*, 21: 370-4, (1977).