WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 13, Issue 1, 1472-1496. Research Article ISSN 2277–7105

EVALUATION OF POTENTIALITY OF ANTIMICROBIAL ACTIVITY OF TRIAZAPENTACYCLO PIPERIDINE CONTAINING BROAD SPECTRUM ANTIBIOTIC RIFABUTIN BY MINIMAL INHIBITORY CONCENTRATION TO TREAT *MYCOBACTERIUM AVIUM* COMPLEX INFECTION IN HIV PATIENT AND QUANTIFICATION OF PHARMACOKINETICS PARAMETERS IN INDIAN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY TANDEM QUADRUPLE MASS SPECTROMETRY (API-4000)

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Article Received on
07 November 2023,

Revised on 28 Nov. 2023,
Accepted on 18 Dec. 2023

DOI: 10.20959/wjpr20241-30810

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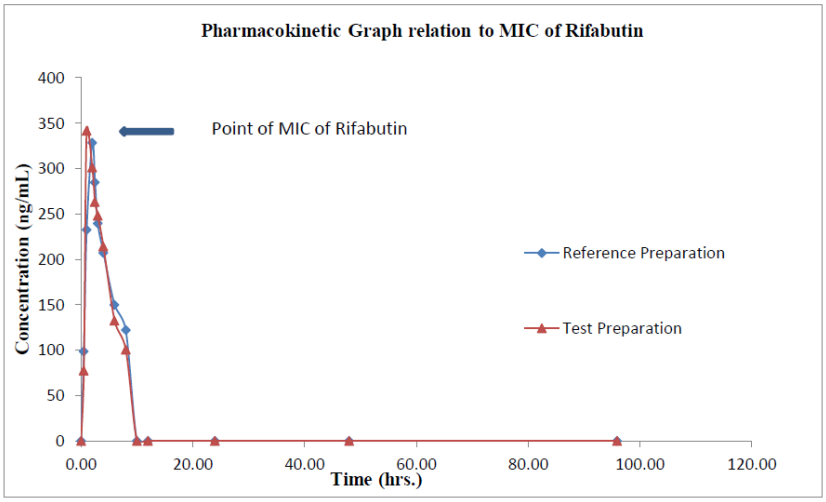
Graphical Abstract



ABSTRACT

Mycobacterium has caused epic diseases: tuberculosis is still the more important infectious killer of humans. *Mycobacterium avium-intracellulare (or Mycobacterium avium* complex*; MAC)* continues to be difficult to treat. Rifabutin is an antibiotic used as first line treatment to treat tuberculosis and prevent and treat *Mycobacterium avium* complex. This derivative of macrocyclic antibiotic rifampin is

typically only used in those who cannot tolerate rifampin such as people with HIV/AIDS on antiretroviral and for active tuberculosis it is used with other antimycobacterial medications also used for latent tuberculosis when exposed to drug resistant TB. Rifabutin inhibits the growth of most MAC isolates at concentration ranging from 0.25- 1.0mg/L and also inhibit the growth of many strains of *M. tuberculosis* at concentration of 0.015to 0.125µg/Ml and it had been reported from Pfizer Mycobutin hard gelatin capsule 150mg that rifabutin in vitro MIC99 values of ≤0.5µg/ml, determined by agar dilution method for *Mycobacterium kansasii, Mycobacterium gordonae and Mycobacterium marinum* that in case of in invitro has better MICs than rifampin. Rifabutin (**CAS NO.-72559-06-9**), is similar in structure and activity of rifampin, which is semi synthetic rifamycin. The chemical name of rifabutin is 1, 4-didehydro-1-deoxy-1-4-dihydro-5'-(2-methyl propyl)-1-oxorifamycin XIV. Molecular formula of rifabutin is C46H62N4O11, belonging to the class of ansamycins. It is found that rifabutin inhibits DNA –dependent RNA polymerase, which interact with and to penetrate the outer layers of *Escherichia coli* (E. coli) and *Bacillus subtilis*. But mammalian cells are not affected. This enzyme did not inhibited by resistant strains of E. coli, rifabutin, like rifampin. Advanced HIV patients are administered rifabutin for prevention of disseminated *Mycobacterium avium* complex. It is not known how rifabutin inhibits this reaction. Rifabutin also inhibited the DNA synthesis of rifampin resistant Mycobacterium tuberculosis by inhibiting thymidine incorporation into DNA. In the present study efforts were given to develop and validate a bioanalytical method for estimation of rifabutin in human plasma by LC-MS/MS (API-4000). The calibration concentrations of rifabutin were 62.5-4000 ng/ml which accuracy was 101.36-106.54%, 100.56-105.79%, 97.73-107.59%, 100.55-108.15% and 97.13-103.42% for freeze thaw, short term, bench top, auto sampler stability and long term stability respectively and recovery was 98.53-99.56%, matrix factor was 0.94-0.96.The developed method used for determination and quantification of pharmacokinetic parameter of rifabutin in human plasma was also validated as per the US-FDA guidelines. The validation parameters found within the specified regulatory limit, hence acceptable. The present method also has a short run time (3.50 min) and easy plasma extraction process by protein precipitation technique. This method was simple, specific, highly selective, sensitive and reproducible. The developed and validated LC-ESI-MS/MS assay method was applied to compare the oral bioavailability of two formulations (test and reference) by conducting the single oral dose, open label, randomized, two period, two sequence, crossover study of 24 healthy Indian volunteers (male) with an average age of 28.08±4.92years and average BMI of 21.88±1.54Kg/m2 under fasting condition. The pharmacokinetic parameters like Cmax,

Tmax, AUC0-t, AUC0-∞, Kel, T1/2 are determined for rifabutin to calculate the relative bioavailability of test preparation rifabutin 150mg tablet over the reference preparation of same dosage after oral administration to healthy human volunteers. In vitro dissolution of reference and test preparation showed that more than 90% drug was released for reference preparation and test preparation after 45 minute. In vitro dissolution test of rifabutin showed that after 45 minutes 93.51 (for reference), and 96.47 (for test) drug released in appropriate test condition. From the study of pharmacokinetic application of rifabutin it was observed that after oral administration of 150mg rifabutin capsule dissolution occurs in 45min. and absorb into the systemic circulation and peak plasma levels were achieved between 2.0-4.0hrs for reference preparation and 1.0-3.0 hrs for test preparation. The mean peak plasma levels of rifabutin with reference preparation, on the study day ranged between 233.940 – 385.640 ng/ml**.** While the rifabutin test preparation of ranged between 219.570 – 394.790 ng/ml. The value of pharmacokinetic parameters of rifabutin are Cmax 302.464±41.612 for reference drug and 298.270±56.790 for test drug; Tmax 2.813±0.845 for reference drug and 1.979±0.667 for test drug; AUC0-∞ 1937.949±521.650 for reference drug and 1960.553±445.403 for test drug; Kel 0.0205±0.075 for reference drug and 0.173±0.041 for test drug; T1/2 3.815±1.318 for reference drug and 4.272 ±1.171 for test drug. On the basis of comparison of the AUC0-t for rifabutin 150 mg capsule after single dose administration, the relative bioavailability of the test preparation of rifabutin 150mg was 100.22% of that of the reference preparation. According to WHO report the critical concentration (MIC) of rifabutin 500ng/ml and below this concentration rifabutin is highly sensitive. According to WHO report 13% MAC isolates from MAC infected HIV patient after treating by 500ng/mL concentrated rifabutin , so after treating test preparation rifabutin capsule approximately less than 10% MAC will be isolated from MAC infected HIV patient within 1.0-3.0 hrs. It was concluded that rifabutin 150mg test preparation highly sensitive than reference preparation in case of MAC infected HIV patient.

KEYWORDS: Rifabutin, Antimicrobial activity of Rifabutin, LCMS/MS, Pharmacokinetic application of Rifabutin.

Abbreviation: MAC*: Mycobacterium avium* complex*;* AUC: Area under Curve; MIC: Minimum Inhibitory Concentration.

1. INTRODUCTION

Mycobacterium avium complex (MAC) infection is a special type of infection caused by bacteria: *Mycobacterium avium* and *Mycobacterium intracellulare*. There are three distinct types of MAC infections identified, i.e., i) Pulmonary MAC infections - Affect the lungs and are the most common type. ii) Disseminated MAC infections – Usually seen in HIV patients the infection spreads all over the body. iii) MAC-associated lymphadenitis - Causes swelling of the lymph nodes.[1] HIV causes serious impairment of immune system the second Disseminated MAC infections is very common in HIV patients and the health condition detoriates more rapidly.[2] As a treatment regimen for HIV patients, antiretroviral therapy which is combination nucleoside reverse-tran-scriptase inhibitors and a protease inhibitor is desired. But, for patients with MAC infection use of Rifampicin markedly lowers the concentration of nucleoside reverse-tran-scriptase inhibitors and a protease inhibitor in blood.[3] Whereas, an attributable fact was found that Rifabutin instead of Rifampicin considered a better option for treatment in patients who receiving combination of antiretroviral therapy.[4] The reason behind is it does not causes enzymatic depilation of nucleoside reverse-tran-scriptase inhibitors and a protease inhibitor, so the concentration in blood remains enough to maintain therapeutic window.[5]

Rifabutin inhibits the growth of MAC, isolates at concentration ranging from 0.25-1mg/L and it also inhibit the growth of many strains of M. tuberculosis at concentration of 0.015to 0.125µg/Ml and in *in-vitro* has better MICs than rifampin.[6] It was found that rifabutin inhibits DNA –dependent RNA polymerase. Which interact by penetrating the outer layers of Escherichia coli (E. coli) and Bacillus subtilis. But mammalian eukaryotic cells are not affected. This enzyme did not inhibited by resistant strains of E. coli, rifabutin, like rifampin.[7] Advanced HIV patients are administered rifabutin for prevention of disseminated Mycobacterium avium complex. Rifabutin also inhibited the DNA synthesis of rifampin resistant Mycobacterium tuberculosis by inhibiting thymidine incorporation into DNA.[8] In terms of stability, Rifampin is not stable in blood it undergoes hydroxylation by esterase enzyme, whereas Rifabutin is not deacetalyated in blood components.[9]

However, some serious adverse reactions like uveitis, poly-arthralgia, polymyalgia and neutropenia reported in high dose of rifabutin may be associated with higher concentration of active drug and its metabolite 25-O-desacetyl rifabutin.[10] It was well known that the anti-TB drugs exerts hepatotoxicity in varying doses. Both Rifamycin group of anti-TB drugs, vis,

Rifampin, Rifabutin undergoes hepatic metabolism by CYP2C8 hepatic enzyme.[11] In this present context, we have done the quantification using LC-ESI-MS/MS API 4000, of Rifabutin in human plasma by developed and validated bioanalytical method. The following pharmacokinetic parameters; Cmax, Tmax, AUC0-t, AUC0-∞, Kel, T1/2 were estimated and relative bioavailability was postulated. In-vitro dissolution study was carried out for finding the release parameters of test samples. Moreover, in accordance to WHO report[17] the standard value for MIC (minimum inhibitory concentration) of Rifabutin was correlated with the test sample and the susceptibility to anti-TB spectrum was postulated.

2. Drug information (test and reference) and dosing

A single-stage randomization was done using the random number generator after their clinical and vital parameters examination.

Mycobutin 150 mg Capsule containing Rifabutin 150 mg manufactured by Pfizer, Newyork-10017, represented as reference product and capsule containing Rifabutin USP 150 mg represented as test product.

The 24 volunteers were received either test or reference product based on the randomization code in each clinical period as specified in the Table-1,2. Drugs were taken with 240ml of drinking water on an empty stomach with at least 8-10hrs fasting condition in single dose without chewing.

[Table-1 and 2]

Table: Demographic Data of Volunteers.

Vol. No.	Sex	Age (Yr)	Height (cm)	Weight (kg.)	BMI(kg/m^2)
1	M	19	168	67	23.74
2	M	32	163	63	23.71
3	M	35	169	60	21.01
4	M	27	162	56	21.34
5	M	25	165	57	20.94
6	M	30	170	60	20.76
7	M	28	169	62	21.71
8	M	26	173	67	22.39
9	M	25	164	60	22.31
10	M	22	171	59	20.18
11	M	33	169	66	23.11
12	M	27	170	63	21.80
13	M	26	172	62	20.96
14	M	36	177	68	21.71

15	M	32	165	62	22.77
16	M	29	171	57	19.49
17	M	21	163	60	22.58
18	M	23	170	69	23.88
19	M	27	162	55	20.96
20	M	29	172	62	20.96

Table 2: Mode of Treatment of Capsule Containing Rifabutin USP 150 mg.

Subject No.	PERIOD I	PERIOD II
1	A2	A1
2	A1	A2
3	A2	A1
4	A1	A2
5	A2	A1
6	A1	A2
7	A1	A2
8	A1	A2
9	A1	A2
10	A2	A1
11	A2	A1
12	A1	A2
13	A2	A1
14	A1	A2
15	A1	A2
16	A2	A1
17	A1	A2
18	A2	A1
19	A1	A2
20	A2	A1
21	A2	A1
22	A2	A1
23	A2	A1
24	A1	A2

2.1 Sampling schedule and blood collection

Total 14 blood samples were taken from the volunteers according to the following schedule--0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 48.0, and 96.0 hrs. The sampling was done by cubital vein puncture with installing of cubital catheter by the study team member (phlebotomist). 5ml of blood was taken at each time point.

2.2 Bioanalytical Method development by gradation LC-MS/MS

Rifabutin(**CAS NO.-72559-06-9**) which chemical formula **C46H62N4 O 11**is derivative of rifamycin S and similar in structure and activity, chemically(9S, 12E, 14S, 15R, 16S, 17R, 18R, 19R, 20S, 21S, 22E, 24Z)-6,16,18,20 tetrahydroxy-1'-isobutyl-14-methoxy-

7,9,15,17,19,21,25-heptamethyl-spiro [9,4 (epoxypentadeca[1,11,13]trienimino)-2H-furo[2',3':7,8]naphth[1,2-d]imidazole-2,4' piperidine]-5,10,26-(3H,9H)-trione-16-acetate. Or 4-Deoxo-3,4-(2-spiro(N-isobutyl-4-piperidyl)-2,5-dihydro-1H-imidazo)-rifamycin S or 1,4-Dihydro-1-deoxy-1',4-didehydro-5'-(2-methylpropyl)-1-oxorifamycin xiv. which contain one furo,imidazole, and piperidine ring. It inhibits DNA dependent RNA polymerase in E. Coli, Mycobacterium, and Bacillus subtilis. It is high lipophilic and high propensity for distribution in tissue uptake. It is red – violet powder soluble in chloroform and methanol, sparingly soluble in ethanol and very slightly soluble in water (0.19mg\ml). Its logp value is 3.2(n-octanol/water).

The exact mass of rifabutin is 846.4415(molecular wt. 847.019), H-bond donar count=5 and H-bond acceptor count=14. The PKa value of rifabutin 3.31 (acidic) and 9.5(basic) for furan, imidazole ring(pka value of imidazole 6.9) and piperidine ring(pka value of piperidine 11.2) respectively. But it is a neutral in charater. According to Lipinski's rule an orally active drug should maintain this rule or rule of 5(RO5) and increase the activity and selectivity of the compound because it is ensure that physico chemical properties of the drug are not maintained and not confirm the RO5 rule that is violate the (RO5) Lipinski's rule so this drug not so orally active. octanol/water partition coefficient (logP) value 3.88, AlogP value 4.62 (not greater than 5), rotable bond count=4 (According to Veber's rule 10 or fewer) and polar surface area=205.54 (According to Veber's rule not greater than 140Å2), therefore it is predictable that rifabutin is not so good oral bioavailability. A positive value of logP and log KoW value 3.59 denotes a higher concentration of drug molecule is present in lipid phase and the compound is lipophilic., it is soluble in DMSO, methanol and ethanol but aqueous soulubility is very low 0.017mg/mL. ACD log D at ph 7.4 value of rifabutin is 3.94 that is highly permeable through the lipid membrane that is lipophilic in natureProtein binding of rifabutin is 85%, so optimum condition of plasma extraction and method development was required for different type of PH value of the analyte.

Metoprolol used as internal standard (IS). For quantitation used positive polarity to achieve adequate response for their simultaneous analysis. Moreover positive ionization mode is selective and highly sensitive for compounds with low electron affinity. Thus positive ionization mode was selected to fragment the analyte and IS to obtain intense and consistent product ions.

The protonated precursor ions **[M+H]+** at m/z 847.6(highest peak), 848.7(2nd peak), 815.7(3rd peak), were observed in Q1 MS in which selected parent ion 847.6 for rifabutin and characteristic product ions or fragment ions found in Q3 MS were at m/815.4,816.0,814.6. However the most stable and consistent fragment ion selected was **m/z 815.4[(M-CH3OH+H)+] for** furo, imidazole, and piperidine ring. **[Figure: 1]**

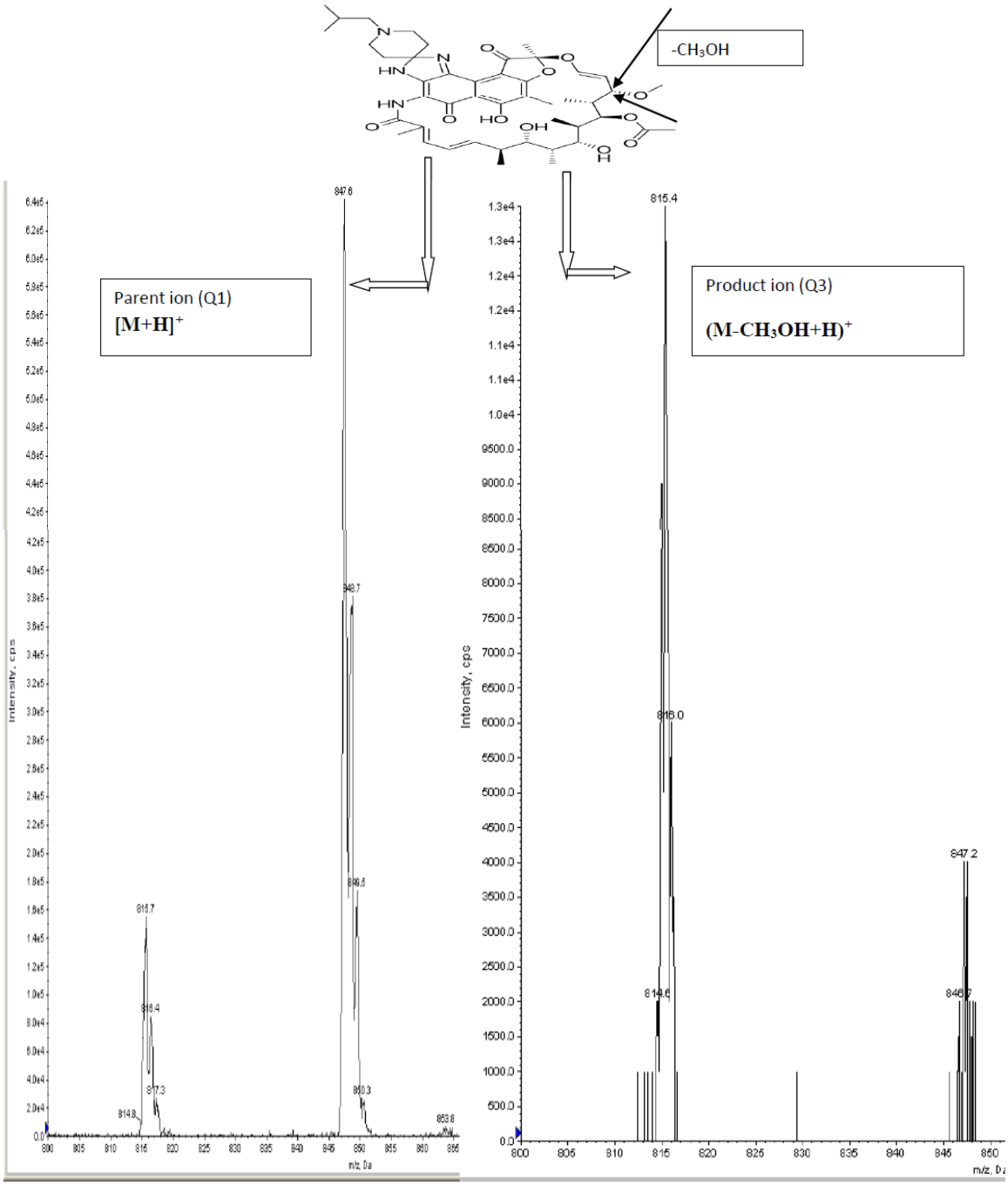


Figure: 1 Parent ion (Q1) and Product ion (Q3) of Rifabutin.

For the internal standard The protonated precursor ions **[M+H]+** at m/z 268.2(highest peak) 269.3(2nd peak), 241.2(3rd peak), 22.9(4th peak)) were observed in Q1 MS for metoprolol and characteristic product ions or fragment ions found in Q3 MS were **m/z116.1**,159.3,133.1,74.1,98.0,121.2 . However the most stable and consistent fragment ion selected was **m/z 116.1 for** five consecutive rings. The chromatographic elution of the analytes on a Phenomenex Kinetex 5µ C18 100A 50*3mm column was initiated as a rapid, sensitive and rugged analytical method covering the dynamic linear range. Mobile phase selection was necessary for analysis of drug depending on its pka value. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were very which was important for chromatographic resolution with adequate response to achieve the desired sensitivity.

Initially, acetonitrile/methanol with 1 mM ammonium acetate buffer (pH 6.5) gave response for rifabutin. However, the response was not reproducible. The signal of the lower limit of quantification concentration was changed when buffer concentration increased from 1mM to 10mM Further, the chromatography was better with a higher response using an methanol-buffer as compared to a acetonitrile -buffer combination. Moreover, lowering the methanol content in the mobile phase resulted in an increase in the retention of rifabutin and thereby the analysis time. Subsequent efforts were directed to optimize the pH of the mobile phase and the concentration of the buffer solution as they had significant impact on analyte retention, peak shape, and resolution. At pH above 5.0 the resolution of rifabutin was affected, which further deteriorated with increase in PH. Thus, to achieve greater reproducibility and better chromatography, low pH buffers were tried.

Reproducibility and peak shape of the lower concentration of the drug was better in 0.1% formic acid but the signal noise ratio was not adequate. A superior signal to noise ratio (≥ 22) and baseline resolution was obtained for the analytes by 10 Mm ammonium acetate buffer with 0.1% (v/v) formic acid together with Milli Q water having apparent pH 3.50 at a flow rate of 0.6000 mL/min.

In the present study, the chromatographic part was performed by gradation method in which 10 % and 90 % organic solvent was used for 0.01 min to 0.90 min and 0.90 min to 2.50 min of total run time whereas 90 % aqueous solvent was used from 2.50 min to rest of the total run time (3.50min) for washing purpose **[Figure: 2]**. The chromatographic elution time for rifabutin and IS (metoprolol) was found 2.98, and 2.64min respectively, in a total run time of

3.50min. The representative MRM chromatograms were showed in **[Figure-3a, 3b, 3c, 3d, 3e]**

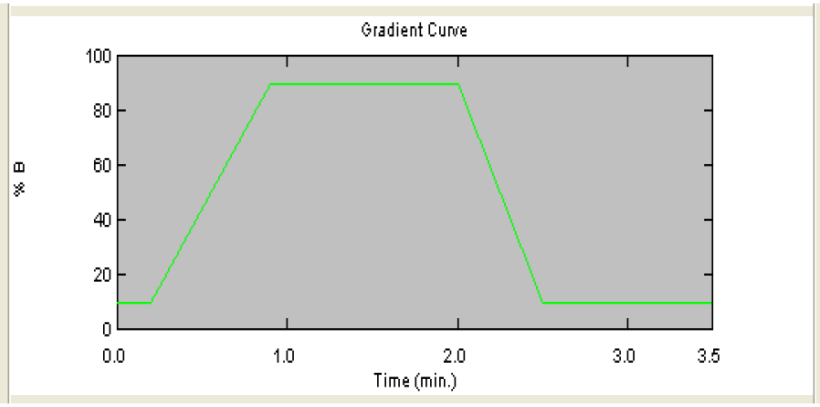


Figure: 2 Gradient Curve of Method of Rifabutin.

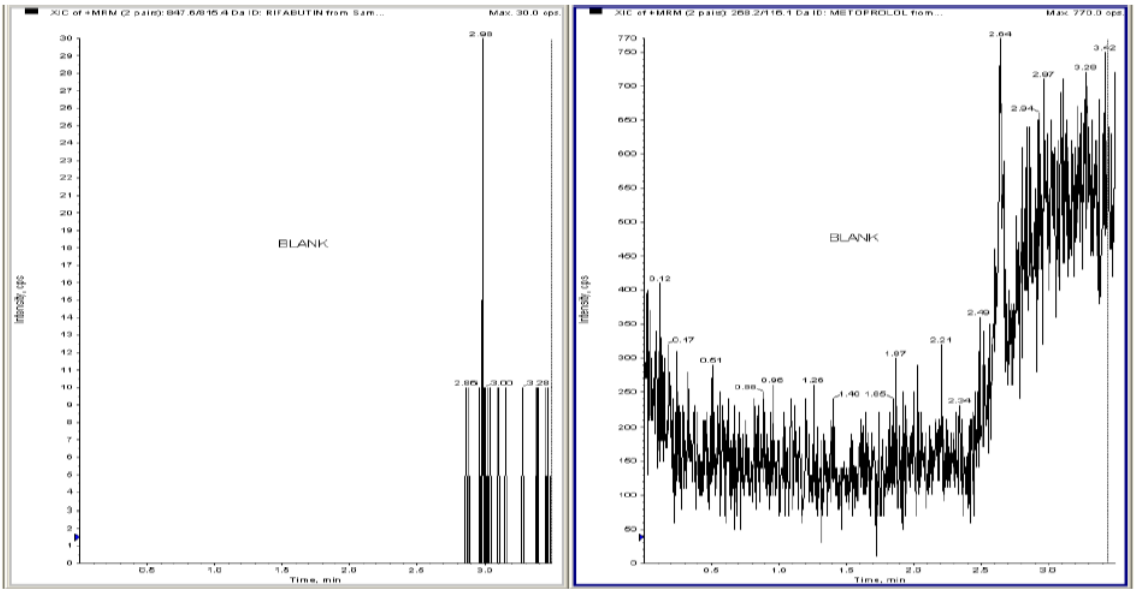


Figure: 3a. Blank Sample Chromatogram of Rifabutin.

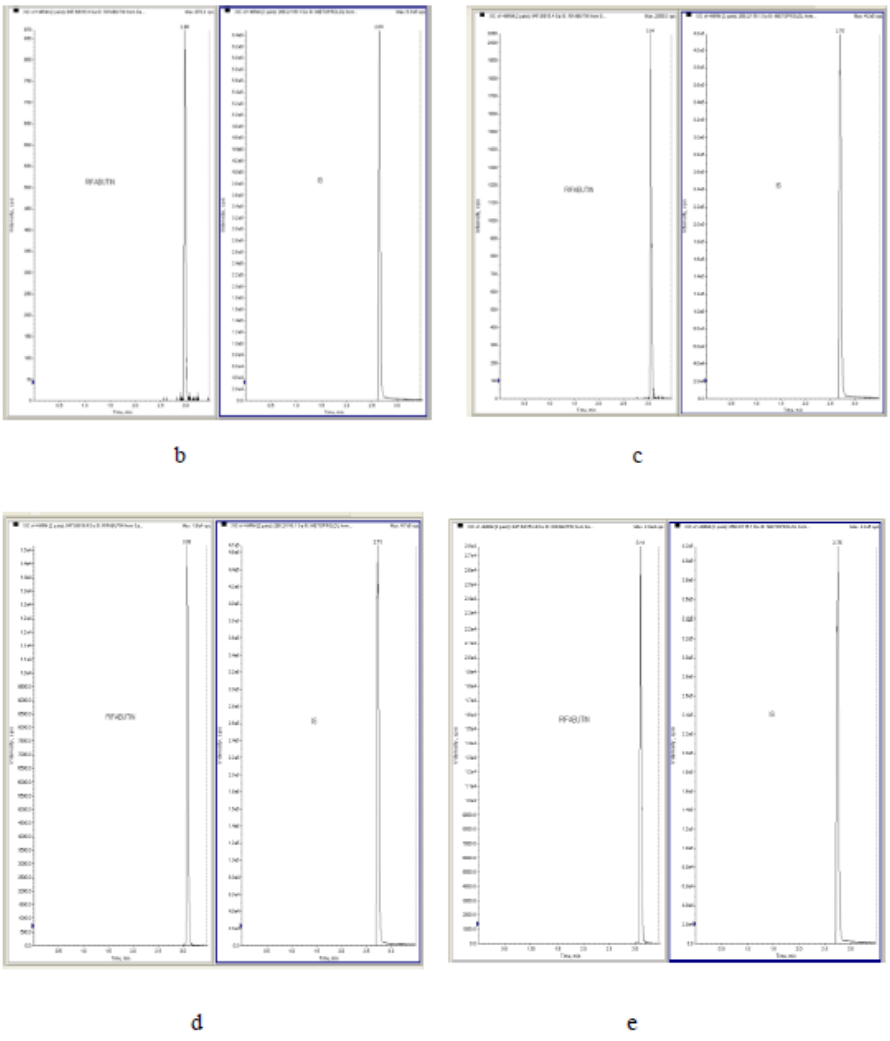


Figure: 3b, 3c, 3d, 3e. LLOQ, LQC, MQC, HQC Sample Chromatogram of Rifabutin.

2.4. Plasma extraction and sample preparation

Plasma extraction was performed by Protein precipitation technique, 100 µl of plasma was taken and precipitated with 400 µl of MeCN containing 8000ng/ml metoprolol (IS) and vortexed for 10 min, followed by Centrifugation for 10 mins at 12,000 rpm at 40C. 300µl supernatant was taken and transferred to auto sampler vials for injection.

2.5. Stock solution and calibration standards preparation

Stock solutions of rifabutin and IS (metoprolol) were prepared by dissolving accurately weighed samples in the DMSO to obtain concentrations of 1mg/ml. The stock solutions were then gradually diluted with methanol: water: 50: 50 (v/v) to obtain calibration samples of 62.5, 125, 250, 500, 1000, 2000, 4000. ng/ml for rifabutin.

2.6 Method validation

The method validation of rifabutin was conducted in accordance with the guidelines US-FDA for selectivity, sensitivity, linearity, precision, accuracy, recovery and stability.

2.6.1 Specificity, selectivity and linearity

Method specificity and selectivity was illustrated by the chromatograms of blank plasma recorded for samples near the Cmax for 2.00 to 3.50hr. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The calibration curve of the three individual day linearity was determined by known calibration standard concentration by regression analysis. Representative calibration curve of rifabutin was higher regression value which quatified from human plasma and was depicted in the linearity graph.

2.6.2 Precision and accuracy

Precision are two types, between–run precision and within-run precision accuracy were determined from the three concentrations which are low, medium and high QC samples (LQC, MQC and HQC) and another concentration was applied that is lower limit of quantification (LLOQ). Total six concentrations were assayed of each quality control samples and minimum five concentrations assay were reported. The QC samples concentrations were determined from three different calibration curves that were assayed with QC samples.

The QC samples concentrations were determined from calibration curves LIN1 of rifabutin. Precision determined at each concentration level as percent coefficient of variation (%CV) and it should not exceed 15% except for LLOQ which should not exceed 20% while accuracy was measured as the percent nominal.

2.6.3 Stability

This method was validated by stability analysis of the analyte rifabutin like freeze thaw, short term (ST), long term (LT), auto sampler (AS) and bench top (BT) stability had been performed as per the regulatory guidelines (US-FDA). As per guidelines the freeze thaw stability percentage should be within 80–120%. As per guidelines the both the ST and LT stability percentage should be within 90–110% and the AS stability percentage should be within 85–115%.

2.6.4 Matrix effect and recovery

Suppression of ionization of analyte rifabutin and IS by this method was very low which was proved by matrix effect by measuring the peak areas of the analyte and IS from the prepared plasma of LQC, MQC and HQC samples. The matrix effect percentage should be within 85–115% as per US-FDA guidelines.

The percentage recovery was determined by measuring the peak areas of the analyte and IS from the prepared plasma low, medium and high quality control samples. The peak areas of the plasma LQC, MQC, HQC samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the analyte and IS.

2.7. Dissolution method of rifabutin

For the dissolution study of reference and test preparations, one tablet each reference / test was taken in 6 dissolution vessels containing 900 ml. dissolution medium and set at the above mentioned conditions. After 10, 15, 30, 45 minutes aliquot of the fluid was withdrawn for both reference and test preparation and the release of the reference and test preparation was estimated by a standard procedure of dissolution at 279 nm using a UV Spectrophotometer.

Preparation of Stock Solution: To prepare 1mg/ml of stock solution, 10mg of Rifabutin API was dissolved in dissolution medium and the volume was made up to 10ml with medium.

Preparation of Standard Solution: To prepare appropriate concentration of standard solution, adequate volume of stock solution was taken and diluted to make volume of 10ml with dissolution medium.

Preparation of Sample Solution: The aliquot of 5 ml of fluid was withdrawn for both reference and test preparations and filtered, dilute to adequate concentration for analysis.

3. RESULTS AND DISCUSSION

3.1 Method validation

3.1.1 Specificity, selectivity and linearity

Plasma calibration standards (ng/ml) of rifabutin were prepared- 62.5, 125, 250, 500, 1000, 2000, 4000 and LQC, MQC, HQC concentrations were prepared 187.5, 1500, 3000 ng/ml. The proposed assay was found linear. The representative calibration curve was showed as linearity graphs **(Figure-4).** Back calculated concentrations of the calibrant samples of the

linearities for rifabutin were also represented in **Table-3**. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were found 19.0 ng/ml and 62.5 ng/ml respectively for rifabutin.

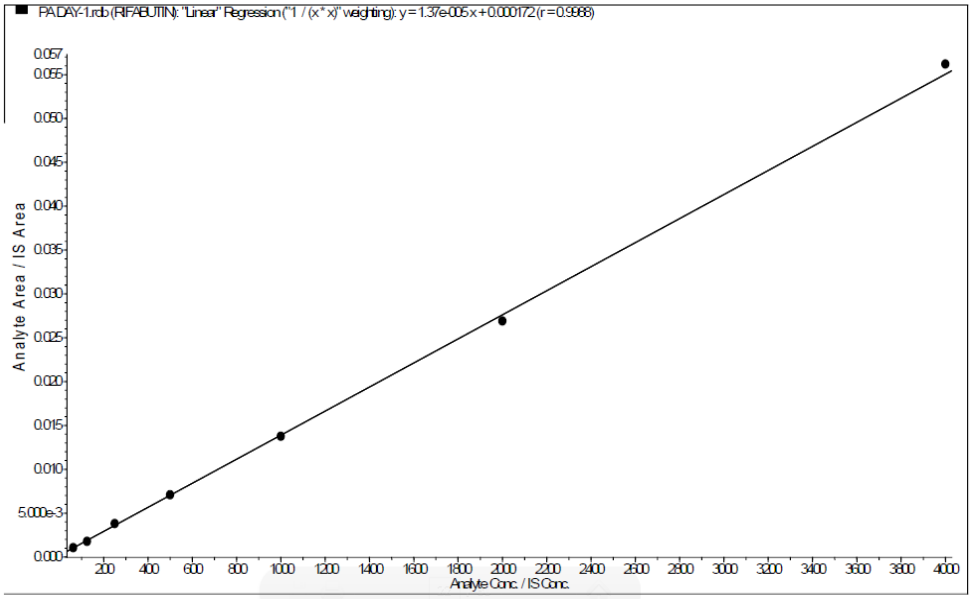


Figure: 4 Calibration Curve of Rifabutin.

Table 3: Pre Study Linearity of Detector Response.

Linearity	Concentration (ng/ml)						
	62.5	**125**	**250**	**500**	**1000**	**2000**	**4000**
LIN 1	63.82	116.82	264.21	503.54	990.23	1948.01	4083.85
LIN 2	60.54	129.69	267.5	480.67	1015.07	1969.48	3850.59
LIN 3	61.40	127.50	267.46	463.08	970.84	2082.92	3956.96
Average	**61.920**	**124.670**	**266.390**	**482.430**	**992.047**	**2000.137**	**3963.800**
S.D	**1.701**	**6.886**	**1.888**	**20.287**	**22.171**	**72.492**	**116.780**
% C.V.	**2.747**	**5.523**	**0.709**	**4.205**	**2.235**	**3.624**	**2.946**
% NOMINAL	**99.07**	**99.74**	**106.56**	**96.49**	**99.20**	**100.01**	**99.10**

PRE STUDY LINEARITY OF DETECTOR RESPONSE

LINEARITY	STATISTICS		
LINEARITY CODE	**SLOPE (m)**	**INTERCEPT(c)**	**R square**
LIN 1	0.00001	0.00017	0.9988
LIN 2	0.00001	0.00018	0.9987
LIN 3	0.00002	0.00020	0.9983
MEAN	**0.000015**	**NOT APPLICABLE**	**0.9986**
S.D.	**0.000001**		**0.000265**
C.V.%	**5.69**		**0.03**

3.1.2 Precision and accuracy

Between – run precision values (%CV) ranged from **4.430%** to **6.364%**. Between – run accuracy values (% nominal) were **97.60%** for LLOQ, **101.76%** for low QC (QCL), **99.93%** for medium QC (QCM) and **98.14%** for high QC (QCH) samples.

Within-run precision values (%CV) ranged from **2.529%** to **6.001%**. Within-run accuracy values (% nominal) were **101.78%** for LLOQ, **102.68%** for low QC (QCL), **99.95%** for medium QC (QCM) and **92.89%** for high QC (QCH) samples.

Table 4: Precision and Accuracy.

	Between Run			Within Run		
	Mean± SD	**C.V%**	**Absolute bias (%)**	**Mean± SD**	**C.V%**	**Absolute bias (%)**
LLOQ(62.5ng/ml)	**61.001±2.702**	**4.430**	**97.60**	**63.612±2.249**	**3.535**	**101.78**
LQC(187.5ng.ml)	**190.791±9.448**	**4.952**	**101.76**	**192.528±4.869**	**2.529**	**102.68**
MQC(1500ng/ml)	**1498.970±91.128**	**6.079**	**99.93**	**1499.222±89.961**	**6.001**	**99.95**
HQC(3000ng/ml)	**2944.104±187.351**	**6.364**	**98.14**	**2786.562±82.836**	**2.973**	**92.89**

3.1.3 Stability

The stability study data were elaborated in Table-5.

Bench top stability

The QC samples of plasma were kept for 24 hrs. in room temperature on sample preparation bench and then processed and analyzed and compared with fresh prepared plasma sample and percentage stability was within **97.73%** to **107.59%** for rifabutin**.**

Autosampler stability

The auto sampler stability of low and high quality control samples were determined by comparing fresh prepared plasma samples QC against samples QC kept in auto sampler at 15˚c for 24 hrs. The auto sampler stability of rifabutin was resulted in **100.55%** to **108.15%** for QCL and QCH respectively.

Freeze thaw stability

The stability of low, medium and high quality control samples were determined after three freeze thaw cycles comparing against freshly thawed samples of the same concentration. The stability of rifabutin ranges between **101.35% - 106.54%** after three cycles.

Short term stability

The stability of low, medium and high quality control samples were determined after keeping the samples in the freeze (2˚-8˚c) for 24 hrs and comparing against fresh plasma samples of the same concentration. The stability of rifabutin ranges between **100.56%** to **105.79%** after three cycles.

Long term stability

The long term stability of low and high quality control samples were determined by comparing fresh plasma samples of the QC concentrations against plasma samples of the same QC concentrations prepared and freezes before 7 days. The long term stability of rifabutin was resulted in **97.13**% to **103.42**% for QCL and QCH respectively.

Table 5: Stability Study (Freeze thaw, Short term, Auto sampler, Bench top stability, Long term stability.

		Inj No.	LQC (187.5ng/ml)	MQC (1500ng/ml)	HQC (3000ng/ml)
	FreshlyThawed	1	172.32	1429.32	2909.35
		2	195.88	1360.83	2786.99
		3	189.14	1422.72	3037.55
		4	168.33	1399.99	2959.71
		5	191.54	1438.21	2730.74
		Mean	**183.44**	**1410.21**	**2884.87**
Freeze thaw stability	After 3 cycle	1	174.62	1422.21	3058.81
		2	205.92	1404.1	3174.7
		3	190.12	1365.9	3175.36
		4	173.72	1496.17	2880.79
		5	185.18	1485.43	3077.8
		Mean	**185.91**	**1434.76**	**3073.49**
	% Stability		**101.35**	**101.35**	**101.74**
Short term stability	After 24hours.	1	181.19	1524.66	3086.47
		2	198.09	1424.41	3207.71
		3	175.37	1454.57	2893.01
		4	188.87	1410.68	2867.59
		5	178.87	1481.45	3205.33
		Mean	**184.48**	**1459.15**	**3052.02**
	% Stability		**100.56**	**103.47**	**105.79**
Auto sampler stability	After 24 hours in auto sampler (15°c)	1	192.86	1387.11	3005.28
		2	188.81	1422.94	2982.31
		3	174.01	1557.86	3322.15
		4	181.42	1495.33	3148.14
		5	185.13	1463.33	3141.93
		Mean	**184.45**	**1465.31**	**3119.96**
	% Stability		**100.55**	**103.91**	**108.15**

Bench top stability	After 24 hours in laboratory room temperature	1	185.11	1443.13	3171.75
		2	178.17	1496.51	3115.71
		3	171.25	1424.49	3087.14
		4	176.42	1535.75	3045.02
		5	185.43	1465.96	3099.77
		Mean	**179.28**	**1473.17**	**3103.88**
	% Stability		**97.73**	**104.46**	**107.59**
Long term stability		1	178.88	1498.3	2907.7
		2	177.96	1443.57	2794.66
		3	191.48	1393.31	2767.46
		4	187.59	1473.16	2838.88
		5	181.58	1483.56	2701.24
		Mean	**183.50**	**1458.38**	**2801.99**
	% Stability		**100.03**	**103.42**	**97.13**

3.1.4 Matrix effect and recovery

The Matrix Effect of Internal Standard (Metoprolol) ranges between **96.64% - 98.17%** and for rifabutin**,** it ranges between**93.88%** to **95.85%** after three cycles and determined by measuring the peak areas of the drug from the prepared plasma low, medium and high quality control samples. The peak areas of the plasma low, medium and high quality control samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the rifabutin.

The percentage recoveries were determined by measuring the peak areas of the drug from the prepared plasma low, medium and high quality control samples. The peak areas of the plasma low, medium and high quality control samples were compared to the absolute peak area of the unextracted standards containing the same concentrations of the rifabutin. The results were presented in **Table.** Recovery after extraction was **96.67% - 97.92%.** The peak areas of the plasma Low, Medium and high quality control samples were compared to the absolute peak area of the unextracted standard containing the same concentrations of the IS. Recovery after extraction was **98.53%** to **99.56%** for rifabutin.

Table 6: Matrix effect (area). (N = 5)

	Matrix effect IS		**Matrix effect Rifabutin**	
	% of ME	**Matrix factor**	**% of ME**	**Matrix factor**
LQC (187.5/ml)	96.64±3.21	0.96±0.03	95.85±1.67	0.96±0.02
MQC (1500ng/ml)	97.08±2.24	0.97±0.02	93.88±2.49	0.94±0.03
HQC (3000ng/ml)	98.17±2.31	0.98±0.02	95.68±2.14	0.96±0.02

Table 7a: Recovery of IS.

INJ No.	Diluent Sample			In Plasma		
	QCL 187.5 ng/ml	QCM 1500 ng/ml	QCH 3000 ng/ml	QCL 187.5 ng/ml	QCM 1500 ng/ml	QCH 3000 ng/ml
1	2241360.23	2227234.62	2067891.54	2231463.33	2217334.26	2048719.41
2	2284269.20	2212589.21	2099777.24	2264399.30	2205288.82	2082786.51
3	2251007.65	2237515.94	2125944.33	2241117.96	2225705.59	2116904.73
4	2181426.90	2312765.36	2021354.66	2171562.90	2307757.16	1992849.51
5	2100082.60	2069865.29	2125512.64	2096082.68	2055358.09	2045601.51
Mean	**2211629.32**	**2211994.08**	**2088096.08**	**2200925.23**	**2202288.78**	**2057372.33**
% Recovery				**99.52**	**99.56**	**98.53**

Table 7b: Recovery of Rifabutin.

INJ No.	Diluent Sample			In Plasma		
	QCL 187.5 ng/ml	QCM 1500 ng/ml	QCH 3000 ng/ml	QCL 187.5ng/ml	QCM 1500 ng/ml	QCH 3000 ng/ml
1	5929.57	47859.56	88939.43	5868.22	46895.63	82166.10
2	6870.06	46535.30	84598.71	6742.05	45466.44	82769.50
3	6820.36	48986.41	84931.19	6539.48	46880.01	83128.31
4	6545.31	48076.19	83516.50	6407.07	47845.32	82926.84
5	6215.31	48590.51	80122.27	6151.70	44961.50	78456.99
Mean	**6476.12**	**48009.59**	**84421.62**	**6341.70**	**46409.78**	**81889.55**
% Recovery				**97.92**	**96.67**	**97.00**

3.2. In vitro dissolution test result of rifabutin

In vitro dissolution of Reference and Test preparation showed that more than **90%** drug was released for Reference preparation and Test preparation after 45 minute.

In vitro dissolution test of rifabutin showed that after 45 minutes 93.51 (for reference), and 96.47 (for test) drug released in above test condition.

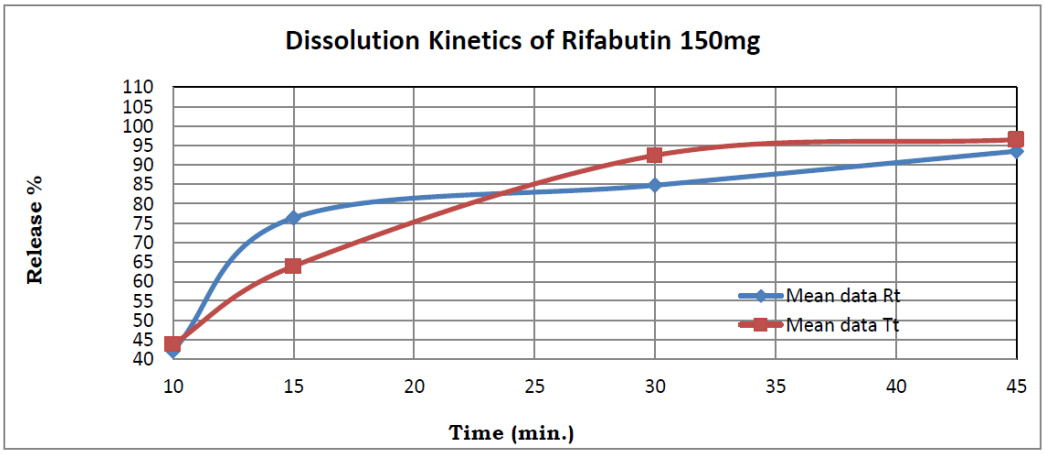


Figure 5: Graphical Presentation of Comparative Dissolution.

Table: 8a. Dissolution Data for Reference Preparation.

Time (min)	% Cumulative Release						Mean %
	1	2	3	4	5	6	
10	41.41	41.47	41.68	42.39	42.88	42.96	**46.13**
15	75.63	76.48	76.43	76.58	76.55	76.50	**76.36**
30	83.18	83.49	83.90	84.32	86.56	86.97	**84.73**
45	93.97	92.79	93.05	93.51	93.77	93.98	**93.51**

Table 8b: Dissolution Data for Test Preparation.

Time (min)	% Cumulative Release						Mean %
	1	2	3	4	5	6	
10	46.85	40.28	46.81	40.27	46.91	41.68	**43.8**
15	66.24	54.53	70.07	54.53	68.90	69.20	**63.91**

Table: 8c. Release Percentage of Rifabutin Test Sample and Reference Sample in Different Time Points.

Time	Mean data	Mean data
(Min)	Rt%	Tt%
10	42.13167	43.8
15	76.36167	63.91167
30	84.73667	92.43
45	93.51167	96.47667

3.3 Comparative pharmacokinetic study in human volunteers

The comparative pharmacokinetic study was carried out in 24 healthy human Indian volunteers under fasting condition with single dose administration. The volunteers were exposed in both the preparations *i.e.* test and reference.

Administration of the Reference preparation, mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg**)** as a single dose in the fasting state produced the maximum plasma concentration of **302.464+41.612 ng/ml** (CMax) at the time **2.813+0.845 hour** (tMax) whereas the Test preparation of capsule containing rifabutin usp 150 mg as a single dose in the fasting state produced the maximum plasma concentration **298.270+56.790 ng/ml** (CMax) at the time **1.979+0.667 hour** (tmax).

Administration of the Reference preparation, mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg**)** produced the area under plasma concentration time curve (AUC0-t) **1320.863+247.798 ng hr/ml**, whereas administration of the Test preparation of capsule containing rifabutin usp 150 mg produced the area under plasma concentration curve (AUC0-t) **1323.775+268.167 ng hr/ml**.

When administered as a single dose, in the fasting state, the Reference preparation, mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg**)**, produced the area under plasma concentration time curve upto infinity (AUC0-α) **1937.949+521.650 ng hr/ml**., whereas administration of the Test preparation of capsule containing rifabutin usp 150 mg produced area under plasma concentration time curve upto infinity (AUC0-α) **1960.553+445.403 ng hr/ml**. (Table 5 & 6).

Administration of the Reference preparation mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg) produced the plasma elimination half life, (t1/2) **3.815+1.318 hr** whereas administration of the Test preparation of capsule containing rifabutin usp 150 mg produced the plasma elimination half life (t1/2) **4.272+1.171 hr.**

Administration of the Reference preparation mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg) produced the plasma elimination rate constant (Kel) **0.205+0.075 hr-1**, whereas administration of the Test preparation of capsule containing rifabutin usp 150 mg produced the plasma elimination rate constant (kel) **0.173+0.041 hr-1.**

On the basis of comparison of the AUC0-t for rifabutin 150 mg capsule after single dose administration, the relative bioavailability of the Test preparation of capsule containing rifabutin usp 150 mg was **100.22%** of that of the Reference preparation, mycobutin 150 mg capsule (each capsule containing rifabutin 150 mg).

90% confidence interval for CMax, values of Test preparation of capsule containing rifabutin usp 150 mg were **0.88437 - 1.08685** of that of the reference preparation. 90% confidence interval for AUC0-t values of Test preparation of capsule containing rifabutin usp 150 mg were **0.90433 - 1.09940** of that of the reference preparation. 90% confidence interval for AUC0-inf values of Test preparation of capsule containing rifabutin usp 150 mg were **0.86335 - 1.15376** of that of the reference preparation.

Table 9a: Mean comparative plasma pharmacokinetic profile of Rifabutin (150mg)

Pharmacokinetic parameters		**RIFABUTIN**	
		Reference Preparation(A1)	**Test Preparation (A2)**
Cmax (ng./ml.)	Mean	**302.464**	**298.270**
	□ S.D.	**41.612**	**56.790**
Tmax (hr.)	Mean	**2.813**	**1.979**
	□ S.D.	**0.845**	**0.667**
AUC 0-t	Mean	**1320.863**	**1323.775**

(ng. hr./ml.)	□ S.D.	**247.798**	**268.167**
AUC 0-inf (ng. hr./ml.)	Mean	**1937.949**	**1960.553**
	□ S.D.	**521.650**	**445.403**
kel (hr.$^{-1}$)	Mean	**0.205**	**0.173**
	□ S.D.	**0.075**	**0.041**
T1/2 (hr.)	Mean	**3.815**	**4.272**

Table 9b: 90% confidence Interval with the Test and Reference Preparation.

Cmax	Untransformed Data	**0.88437 - 1.08685**
	Ln transformed Data	**0.97808 - 1.01403**
AUC 0-t	Untransformed Data	**0.90433 - 1.09940**
	Ln transformed Data	**0.98443 - 1.01574**
AUC 0-□	Untransformed Data	**0.86335 - 1.15376**
	Ln transformed Data	**0.97805 - 1.02710**

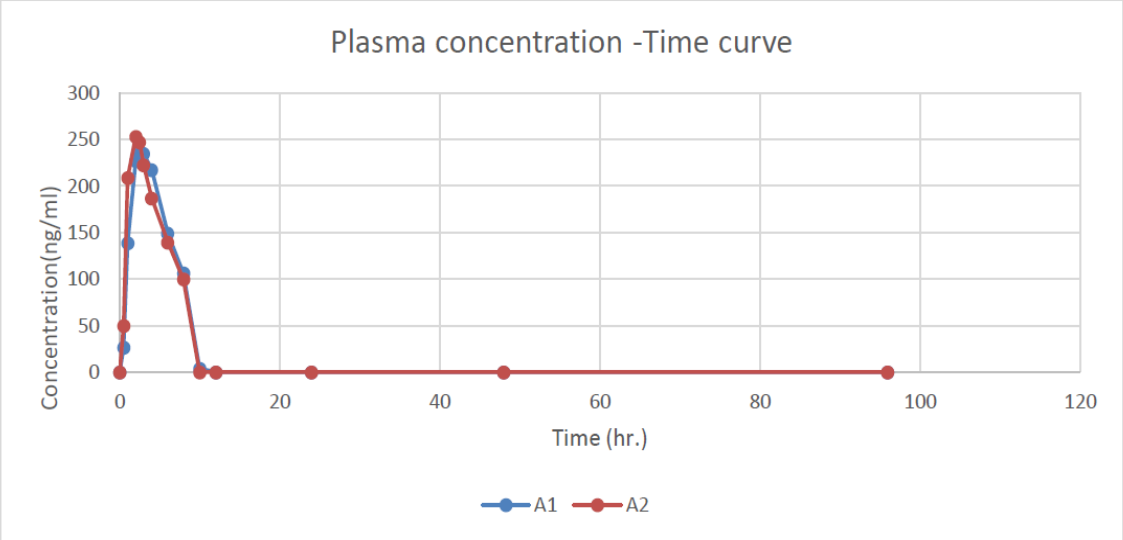


Figure 6: Mean Plasma Concentration –Time Profile Graph.

4. Overall conclusion

The literature survey reveals that there were already plenty of published articles describing the methods for determination of rifabutin in human plasma. Method development and validation for determination of rifabutin in human plasma is still not available. The present study deals with development of a LC-MS/MS (API-4000) method using gradation technique. The developed method for determination and quantification of rifabutin in human plasma was also validated as per the US-FDA guidelines. The validation parameters found within the specified regulatory limit, hence acceptable. The present method also has a short run time (3.5 min) and easy extraction process. Therefore, the developed method was found to be simple, specific, highly selective, sensitive and reproducible. This was applied in the

analysis of the volunteer plasma samples obtained from the comparative pharmacokinetic study.

The clinical phase of the comparative pharmacokinetic study under the frame work of bioequivalence study was carried out in accordance with the supervisions of the Ethics Committee and all other pertinent requirements of the ICH [Step 6] 'Guidance on Good Clinical Practice'. Total twenty four healthy human volunteers of 27.15 ± 4.61 years (average age) and 62.42 ± 4.89 kg/m2 (average BMI) were exposed to the drugs in a crossover manner (table-1). None of the volunteers complained of any adverse reaction during the entire clinical study period.

The single dose bioequivalence study of capsule containing rifabutin usp 150 mg was conducted in 24 adult healthy, human, male volunteers with two preparations of rifabutin 150 mg capsule. Values of CMax, tMax and AUCo-t, were comparable for the reference and the test preparation in the fasting state.

Rifabutin was detected in plasma from **0.5** hour to about **10.0** hours in the Reference preparation as well as in the Test preparation. Peak plasma levels of rifabutin were achieved between **2.0 – 4.0** hrs for reference preparation & **1.0 - 3.0** hours for the Test Preparation respectively.The mean peak plasma levels of rifabutin with Reference preparation, mycobutin 150 mg capsule **(**each capsule containing rifabutin 150 mg**)** on the study day ranged between **233.940 – 385.640 ng/ml.** While the Test preparation of capsule containing rifabutin usp 150 mg ranged between **219.570 – 394.790 ng/ml**. On the basis of comparison of the AUC0-t for rifabutin 150 mg capsule after single dose administration, the relative bioavailability of the Test preparation of capsule containing rifabutin usp 150 mg was 100.22% of that of the reference preparation, mycobutin 150 mg capsule (each capsule containing rifabutin 150 mg). Rifabutin inhibits the growth of most MAC isolates at concentration ranging from 0.25-1mg/L and also inhibit the growth of many strains of *M. tuberculosis* at concentration of 0.015to 0.125µg/Ml and in invitro has better MICs than rifampin. In vitro dissolution of reference and test preparation showed that more than 90% drug was released for reference preparation and test preparation after 45 minute.

In vitro dissolution test of rifabutin showed that after 45 minutes 93.51 (for reference), and 96.47 (for test) drug released in appropriate test condition. From the study of pharmacokinetic application of rifabutin it was observed that after oral administration of

150mg rifabutin capsule dissolution occurs in 45min. and absorb into the systemic circulation and peak plasma levels were achieved between 2.0-4.0hrs for reference preparation and 1.0-3.0 hrs for test preparation. According to WHO report the critical concentration (MIC) of rifabutin 500ng/ml and below this concentration rifabutin is highly sensitive. According to literature 13% MAC isolates from MAC infected HIV patient after treating by 500ng/mL concentrated rifabutin, so after treating test preparation rifabutin capsule approximately less than 10% MAC will be isolated from MAC infected HIV patient within 1.0-3.0 hrs. It was concluded that rifabutin 150mg test preparation highly sensitive than reference preparation in case of MAC infected HIV patient.

5. Conflict of interest

None.

6. ACKNOWLEDGEMENT

Author Tapan Kumar Pal is also thankful to Indian Council of Medical Research (ICMR), New Delhi for financial assistance under Emeritus Medical Scientist scheme. The authors are acknowledging the Department of Science and Technology (DST), New Delhi (India) for providing the necessary instrumental facilities at Bioequivalence Study Centre, Jadavpur University. Authors are also thankful to M/S, TAAB Biostudy Services, Kolkata-700032, India.

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