ORIGINAL ARTICLE

A Modified and Cost-Effective HPLC Method for Determination of Plasma Concentrations of Rifampicin in Pulmonary TB Patients

Abida Shaheen[1], Tausif Ahmed Rajput[2], Fahad Azam[3]

ABSTRACT

Objective: To evaluate the pharmacokinetics of standard doses of rifampicin (RMP) in fixed dose combination in pulmonary tuberculosis patients by a modified high performance liquid chromatography (HPLC) method

Materials and Methods: This descriptive study was conducted after approval from Ethical Committee, Army Medical College Rawalpindi and was funded in part by National University of Sciences and Technology (NUST), Islamabad. Twenty adult patients with newly diagnosed pulmonary TB consented to participate in the study. RMP plasma concentrations were assayed by a simple and sensitive HPLC method in the initial phase of pulmonary tuberculosis treatment. The method was modified to use naproxen as an internal standard and validated according to International Conference on Hormonization (ICH) guidelines.

Results: The calibration curve of rifampicin was linear within the range of 0.781–50 µg/ml. Both intra-day and inter-day variability and accuracy demonstrated good reproducibility at all quality control levels. The developed method was found to be simple, precise and accurate for estimation of rifampicin in plasma. The pharmacokinetic parameters of RMP showed marked inter-individual differences and sub-therapeutic levels.

Conclusion: Evaluation of pharmacokinetics of standard doses of rifampicin in fixed dose combination in pulmonary tuberculosis patients by a modified high performance liquid chromatography (HPLC) method is precise, accurate and cost-effective. It may be used for monitoring plasma RMP levels in TB patients who are slow to respond or are non-responders and have less availability of resources.

Keywords: Tuberculosis, Rifampicin, Pharmacokinetics, High performance liquid chromatography, Accuracy, Precision, Cost effectiveness

INTRODUCTION:

The menace of tuberculosis (TB) with 9.2 million new cases and 1.5 million deaths annually, is a major public health issue. Pakistan, one of the highest burden countries contributes about 44% of the total TB burden in WHO Eastern Mediterranean region.[1] Despite TB declaration as national emergency since 2001 and expansion of directly observed treatment, short course (DOTS) throughout the health services, emergence of multidrug resistance is a matter of great concern and major public health challenge in Pakistan.

Poor bioavailability of rifampicin, in fixed dose combination (FDC) formulations with other anti-TB drugs such as isoniazid, pyrazinamide and ethambutol has raised serious concerns and impediment in wide-spread use of FDCs despite several advantages over separate formulations. This drop in bioavailability of rifampicin may lead to serious consequences such as increased treatment failure rates and selection of both isoniazid and rifampicin resistant strains of M. tuberculosis in the context of its high sterilizing activity, relapse preventing properties and prevention of emergence of resistance to its companion drugs.[2] A number of HPLC methods have been described in literature for pharmacokinetic (PK) analysis of rifampicin. However most of these methods used sophisticated instruments, expensive chemicals and internal standards. A simple, reliable and sensitive method is also been documented in the literature[3] but rifampicin used in this method as internal standard is quite expensive and is not available in our setup locally. The specified HPLC method for monitoring of rifampicin plasma levels in patients could be modified to make it more economical and applicable with the use of easily available and cost-effective internal standard, while maintaining the specificity, sensitivity and precision of original method, according to ICH guidelines.[4]

In this respect modification should be carried out by keeping in mind that after its incorporation the modified method should become easily reproduce-able for therapeutic drug monitoring of rifampicin. Especially in FDCs in TB patients whom response to treatment is very slow. With this background, present study was designed to evaluate the pharmacokinetics of standard doses of rifampicin (RMP) in fixed dose combinations (FDCs) in pulmonary tuberculosis patients by a modified high performance liquid chromatography (HPLC) method in our local setup.

MATERIALS AND METHODS:

This descriptive study was carried out in Pharmacology

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Received: 10-09-2015
Revised: 02-11-2015
Accepted: 05-11-2015

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and Therapeutics department, Centre for Research in Experimental and Applied Medicine, Army Medical College, Rawalpindi, according to guidelines of Helsinki Declaration of 1975 and its amendments.[5] The study was funded in part by National University of Sciences and Technology (NUST), Islamabad Pakistan. After approval from Ethical Committee, Army Medical College, Rawalpindi twenty patients with newly diagnosed, active pulmonary tuberculosis were included in the study following informed consent. They were between 18 to 65 years of age and details about their phase of treatment and product details (FDC products or single drug products) were recorded on a proforma. Patients with deranged liver or kidney function tests, GIT diseases, diabetes mellitus, hepatitis B and C infection, history of drug addiction or alcohol intake, anti-HIV therapy, MDR-TB, pregnancy or lactation were excluded from the study. The patients were started on the standard anti-tuberculosis chemotherapy that is FDC Rifinah containing isoniazid (300mg) and rifampicin (600 or 450 mg), single drug products of pyrazinamide (maximum dose 1500 mg), ethambutol (maximum dose 850 mg) in accordance with DOTS, Pakistan National Tuberculosis Program guidelines based on the WHO DOTS TB control strategy. Eight patients weighing less than 50 kg received 450 mg of RMP/day orally and twelve patients more than 50 kg took 600 mg of RMP/day orally.

Analytical Procedure (Bioanalysis)
1. Instrumentation and Materials:
The HPLC system by Perkin Elmer Series 200 with autosampler and ultraviolet (UV) detector was used. Chromatographic separation was accomplished using a Schimadzu reverse phase-C_{18}, stainless steel column (250 x 4.6 mm, 5 µm particle size) with a guard precolumn of the same packing material provided by Schimadzu Corporation, Kyoto Japan. The chromatograms were recorded on connected computer. The chemicals and solvents used in this study were of analytical and HPLC grade. Methanol and Sodium phosphate were purchased from Merck, Germany (C/O MS Traders, Pakistan). Phosphoric acid was purchased from Sigma Aldrich, Germany (C/O MS Traders, Pakistan). Naproxen (Internal standard) was provided by Ind-Swift Pharmaceuticals (C/O Guddia International, Pakistan) and Rifampicin by Schazoo Zaka Pvt Ltd.
2. Chromatographic Conditions and Preparation of Standards:
The UV detector was set at a wavelength of 254 nm. The final mobile phase composition optimized was methanol and 0.01 M phosphate buffer of pH 5.2, adjusted with 2% o- phosphoric acid (65:35 v/v). The mixture was filtered through 0.45 µm filter (Millipore, Sartorius, Goettingen, Germany) under vacuum and then sonicated. The mobile phase was pumped isocratically at a flow rate of 1.5 ml/min during analysis, at ambient temperature. The volume of injection was fixed at 50 µl. The chromatograms were recorded and integrated on connected computer. Rifampicin stock solution was prepared by dissolving it in methanol to make a 1 mg/ml solution containing 0.5 mg/ml of ascorbic acid to prevent oxidation of RMP. Calibration stock of RMP was suitably diluted to give working stock solution of 100 µg/ml and from this, calibration standards were prepared to contain concentration of 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50 µg/ml of rifampicin. Standard solutions were prepared fresh daily. Rifampicin used in this method as internal standard was quite expensive and not available locally, so it was modified to use naproxen as internal standard and validated according to requirements of International Conference on Hormonization (ICH) for validation of analytical procedures A stock solution of Naproxen was prepared by dissolving it in 65: 35 methanol: sodium phosphate buffer with a target concentration of 1mg/ml. From this stock solution dilutions were prepared to make 10, 50, 100, 200µg/ml working internal standard solutions. The solutions of internal standard were stored at -20°C between use and they were stable for, at least, two weeks.

3. Calibration Curve and Plasma Sample Processing:
Solutions of rifampicin and naproxen were made. The curve covered the concentration range of 0.781 to 50µg/ml for rifampicin using seven standard concentrations. Each concentration of standards was run in triplicate to make the calibration curve. The calibration curve was generated by plotting the ratio of the peak area of rifampicin and naproxen against the rifampicin concentration in standard solutions. The curve was based on simple linear model relating the rifampicin concentration to the HPLC response. Plasma samples were processed by adding 200 µl of calibration standards of different concentrations and 200 µl of naproxen (200 µg/ml) into 1.5 ml eppendorf tubes from working stock solutions. These mixtures were dried and mixed with 5 µl of methanol, then 95 µl of plasma was added and vortexed for 60 seconds. Spiked plasma was then extracted with 500 µl of methanol by vortexing for 3 min. The samples were centrifuged at 10,000 revolutions per minute for 15 minutes, and 300 µl of supernatant was taken into another micro centrifuge tube and vacuum dried in eppendorf concentrator. The residue thus obtained was reconstituted in 200 µl of mobile phase and finally aliquots were loaded on the autosampler tray and volumes of 50 ìl were injected onto the HPLC. The drug and internal standard were detected at 254 nm.[3]

4. Method Validation Procedures:
Pooled quality control samples were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. All control samples were aliquoted into polypropylene vials and stored at approximately -80°C. Quality control samples were run as replicates of blank plasma spiked with a low concentration (3.125µg/ml), a middle concentration (12.5µg/ml) and a high concentration (25 µg/ml) of rifampicin along with a fixed concentration (200 µg/ml) of internal standard. The identification of rifampicin was made on the basis of retention time on chromatograms obtained from plasma samples spiked with standard solutions of rifampicin and comparing with blank plasma samples. The solution was considered

stable if in the described storage conditions variation in the concentration is inferior to 2 percent. The retention time for rifampicin was 8.5 minutes and it was 10.5 minutes for naproxen. The solution is considered stable if in the described storage conditions variation in the concentration is inferior to 2%. The limit of detection (LOD) is defined as the lowest concentration of the analyte in a sample which can be detected but not necessarily quantitated with precision. Linearity was assessed by calibration curve constructed using 7 standard solution concentrations covering the range of 0.781–50 µg/ml. Standard curves were analyzed in triplicate. The lower limit of quantitation (LLOQ) for rifampicin was selected as the lowest concentration of the standard curve at which the rifampicin peak was identifiable and discrete with suitable precision (coefficient of variation (CV) of less than 20 percent) and accuracy (determined concentration being within 20 percent variation of the nominal concentration). The acceptance criterion for precision of analytical method recommended by Food and Drug Administration for each calculated standard concentration is a 15 percent coefficient of variation from added concentration value except at the LLOQ, where it should not deviate by more than 20 %. In accordance with ICH recommendations, precision is determined at two levels, i.e., repeatability and intermediate precision. The acceptance criterion for accuracy of the method is that the mean measured concentration should be within 80–120 of the actual concentration. The precision and accuracy of the plasma assay for rifampicin was evaluated by analysis of 5 replicates of quality control samples at three different concentrations (within the calibration range), for 3 days.

5. Application of Analytical Method:

After validation, this method was applied to determine the pharmacokinetics of rifampicin in 20 pulmonary tuberculosis patients. On the scheduled day of pharmacokinetic assessment, patients abstained from the intake of any eatable or drugs from 11 p.m. on the day before the sampling until 1 hour after the intake of anti-TB drugs. Blood sampling was done before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hours after witnessed drug ingestion. Each sample was transferred to lithium-heparinized tubes placed in ice, and was immediately centrifuged at 4000 rpm for 10 min. Plasma was harvested into labeled eppendorf tubes containing ascorbic acid (0.5 mg/ml), and stored at -80°C within 1 hour of collection, until analysis. Plasma samples of the patients were processed in the similar manner as calibration standards along with internal standard.

6. Pharmacokinetic Data Analysis:

Data of drug plasma concentration versus time for rifampicin was tabulated using Microsoft Excel 2007 computer program. The data was used to calculate pharmacokinetic parameters i.e., elimination half life, area under plasma concentration time curve, volume of distribution and plasma clearance, by computer program, APO, MWPHARM version 3.60, a MEDIWARE product Holland.

The non-compartmental pharmacokinetic model was used to compute the pharmacokinetic parameters of

rifampicin. The pharmacokinetic parameters for RMP were derived individually for each subject from the plasma concentration versus time data. Concentration-time curves were plotted for each series of drug assays. From these plots, the maximum concentration of drug in plasma was defined as the Cmax, and the time to reach this maximum concentration as Tmax. The area under the plasma concentration-time curve until 12 hours (AUC_{0-12} hr.mg/l) for rifampicin was determined by the (linear/logarithmic) trapezoidal rule up to the last data point. The AUC extrapolated to infinity (AUC_{0-8} hr.mg/l) was calculated using the relation AUC_{0-12} + Cm/kel, where Cm is the last measured concentration of rifampicin and kel is the slope of the least squares linear regression of the log concentration-time curve. The computer program, APO, MWPHARM version 3.60, a product of MEDIWARE, Holland, was used to calculate pharmacokinetic parameters. The non-compartmental pharmacokinetic model was used to compute the pharmacokinetic parameters of rifampicin derived individually for each subject from the plasma concentration versus time data.

RESULTS:

Method Validation:

The calibration curve of rifampicin in plasma using least square regression equation was linear within the range of 0.781–50 µg/ml (Figure 1). The correlation coefficient and intercept were, y = (33366) x + (32760), r^2 = 0.998. The retention time for rifampicin was 8.5 minutes and it was 10.5 minutes for naproxen (IS) (Figure 2a & 3a). The LOD was determined by diluting solutions of known concentrations of RMP until the response was three times the noise. The LOD of rifampicin in plasma samples was 0.5µg/ml. For rifampicin analysis in plasma by this method LLOQ was 0.781µg/ml. The coefficient of variation was found to be 6.8%. The accuracy was found to be 92.73%. The stability of samples was demonstrated by subjecting the three different concentrations of rifampicin to three freeze-thaw cycles and storage for 24 hours at room temperature. The freeze-thaw cycles showed little effect on the stability of the samples as the percent accuracy was in the range of 96.40-101.12 % and variability ranged between 1.16 to 1.90 %. Rifampicin was stable in plasma, enriched with 1 mg/ml of ascorbic acid at -80°C, for at least 5 months.

The CV for intra-day variability ranged between 5.5–7.89% while the inter-day CV ranged between 7.35 to 9.93%. The percent accuracy was in the range of 89-102% for intra-day assays and 91-104% for inter-day assay (Table 1). The derived pharmacokinetic parameters for rifampicin in TB patients are given (Table 2). Representative HPLC chromatograms of rifampicin with blank plasma spiked with internal standard at 6.25 µg/ml, 12.5 µg/ml, LLOQ (0.781 µg/ml) and patient samples are provided in figures 2a, 2b, 2c and 3a, 3b, respectively.

Behaviour of mean values of pharmacokinetic profile of Rifampicin 450 and 600 mg are shown in Figure 3c.

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Figure: 1
Calibration curve of rifampicin

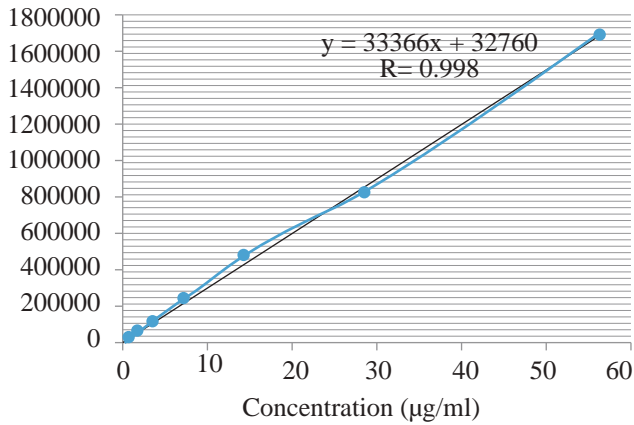$y = 33366x + 32760$
$R = 0.998$

Figure: 2c
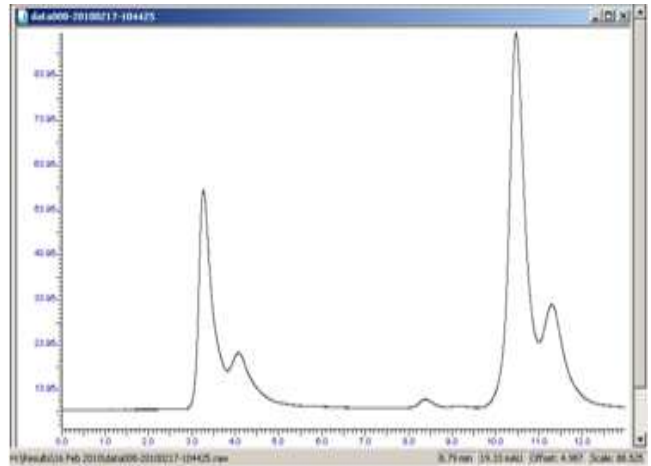Representative HPLC chromatogram of rifampicin at LLOQ (0.781 µg/ml)

Figure: 2a
Chromatogram obtained from blank plasma spiked with internal standard and external standard of 6.25 µg/ml of rifampicin
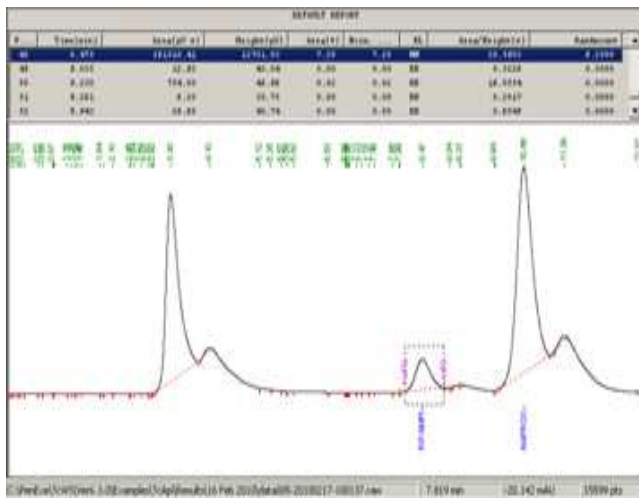

Figure: 3a
Chromatogram obtained from a TB patient plasma spiked with internal standard
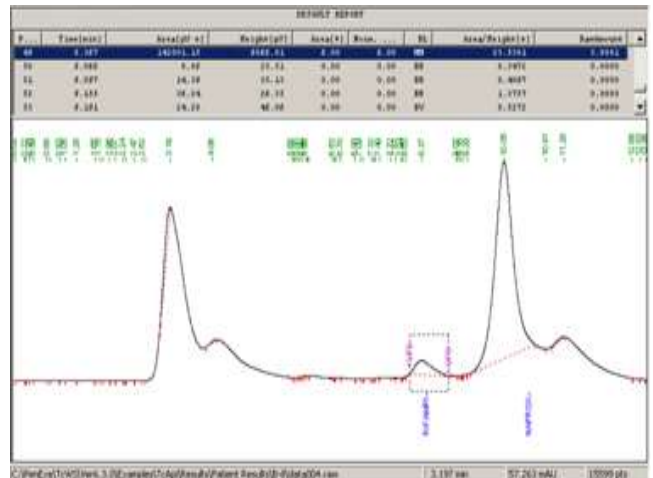

Figure: 2b
Chromatogram obtained from blank plasma spiked with internal standard and external standard of 12.5 µg/ml of rifampicin
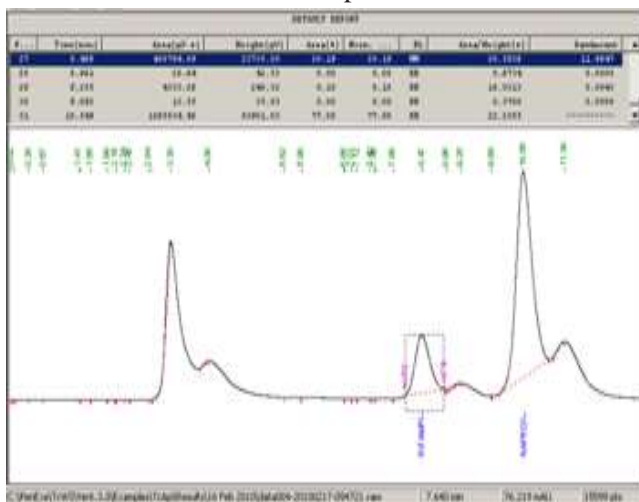

Figure: 3b
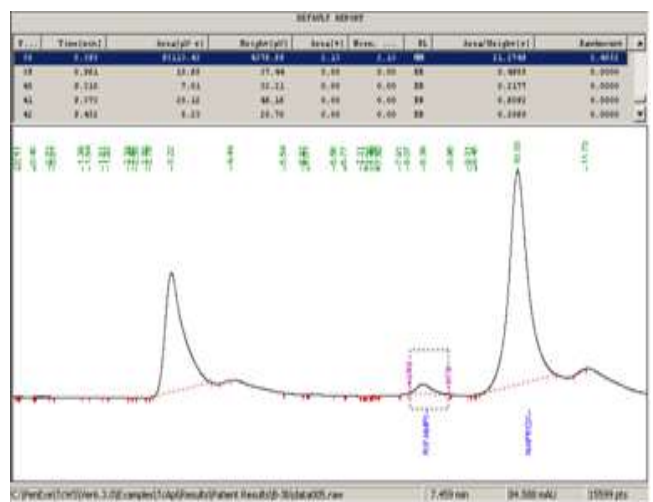Chromatogram obtained from a TB patient plasma spiked with internal stand

Figure: 3c
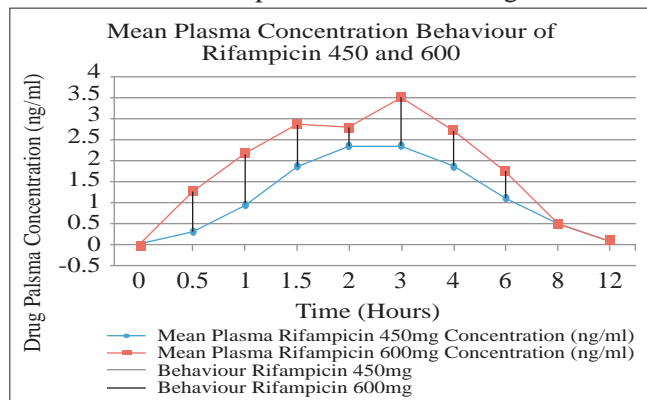Behaviour of mean values of pharmacokinetic profile
of Rifampicin 450 and 600 mg

Table: 1
Intra and inter-day precision and accuracy of rifampicin
assay

Assay Validation Procedures	Conc Added (µg/ml)	Conc Found (µg/ml) Mean ± SD	Coefficient of Variation (CV %)	Accuracy %
LLOQ	0.781	0.724 ± 0.050	6.80	92.73
	3.125	3.194 ± 0.252	7.89	102.21
Intra-assay	12.5	11.12 ± 0.722	6.49	88.96
Reproducibility	25	24.93 ± 1.370	5.5	99.74
	3.125	3.28 ± 0.326	9.93	104.96
Inter-assay	12	11.38 ± 0.860	7.56	91.04
Reproducibility	25	25.06 ± 1.841	7.35	100.23

Table: 2
Pharmacokinetic Parameters of Rifampicin in 20 TB
patients after standard doses of rifampicin

Pharmacokinetic Parameters	Mean	± SD	Min	Max
AUC0-8 [h.mg/l]	17.43	± 6.97	6.08	34.17
AUC0-12 [h.mg/l]	16.83	± 6.63	6.01	31.53
CL [l/h]	35.93	± 14.81	17.56	74.04
Vd [l]	72.29	± 39.43	15.73	145.2
k [/h]	0.647	± 0.392	0.171	1.591
t ½ [h]	1.54	± 0.97	0.44	4.06
Tmax [h]	2.2	± 0.66	1	3
Cmax [µg/ml]	3.77	± 1.23	1.79	6.62

Min = Minimum; Max = Maximum
*Adapted from previous published work by the same author 14.

DISCUSSION:

A number of analytical methods have been reported in literature for determination of rifampicin in plasma. However this modified analytical method showed reasonable specificity, sensitivity, linearity, precision and accuracy in the entire range of clinically significant concentrations in the plasma. This modification and validation of method may be beneficial for therapeutic monitoring of RMP in TB patients with less expenses and easy reproducibility as internal standard naproxen used was cost-effective and easily available. In fact, there are many compounds selected for internal standard in different validated analytical methods reported in literature e.g, papaverine chloride, acetonitrile, acetanilide, carbamazepine[6,7,8.]However the associated solvents and chromatographic conditions requirement is very expensive in all these methods[9,10,11,12,13.]Some of these reported methods require LC-MS setup which is not even available at most research centers in Pakistan. So in a country with high TB prevalence the use of a validated and reliable method with use of internal standard naproxen instead of very expensive rifapentine may even improve the value of this chromatographic method and therapeutic drug monitoring in slow responders or non-responders and in quality evaluation of widely available FDCs and single dose formulations. The pharmacokinetic profile of our study patients has been published previously, according to which the mean maximum plasma concentration (Cmax) of rifampicin was 3.77 ± 1.23 µg/ml at standard doses. Our previous study also reported a widespread inter individual variability in plasma levels of rifampicin at two hours with a coefficient of variation 32.63%. Among 20 patients, seven patients exhibited peak plasma concentration of rifampicin between 4 and 8 µg/ml. whereas 13 patients had peak plasma levels below 4 µg/ml [14] The peak therapeutic range of rifampicin at 2 hours for optimum anti-mycobacterial effect is 8–24 µg/ml. Rifampicin concentration below 4ìg/ml is sub-therapeutic and associated with a risk of emergence of drug resistance.[11] A number of studies with tuberculosis patients, treated with 10 mg/kg RMP daily have revealed suboptimal peak plasma levels most probably due to lack of compliance, auto-induction of its own metabolism, dosage formulation and interaction with other drugs, mal-absorption syndromes, low albumin levels.[16,17,18,19] The compliance of patients to treatment was ensured in this study using DOTS in hospitalized patients and drug interactions were also ruled out when recruiting the patients for study.

In pharmacokinetic profile of our patients, the more noticeable decrease in the area under curve might be due to reduced bioavailability of rifampicin from the FDC. Our study patients were receiving a FDC product of RMP and INH in addition to pyrazinamide and ethambutol. Rifampicin in fixed dose combinations shows considerable variation in rate and extent of absorption as compared to single dose formulations. Dissolution and disintegration properties of oral formulations of rifampicin, delayed absorption of formulations of infer this significant drop in bioavailability of rifampicin.[20,21,22,23,24,25]

CONCLUSION:

In conclusion, this study has demonstrated modification and validation of a HPLC method, which can be effectively used for therapeutic monitoring of RMP in TB patients. Further studies are desperately needed to evaluate the bioavailability of RMP from local FDC preparations used in TB patients in context of its highest sterilizing potential and alarming increase in the incidence

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of MDR-TB in Pakistan. The quality control problems are needed to be seriously addressed in local drug preparations especially FDCs, widely used among TB patients in Pakistan.

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