

## DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF RIFAMPICIN IN HUMAN PLASMA

T. SRIRAM SIDDHARTHA<sup>1</sup>, B. PRASANTHI<sup>1</sup>, TATA SANTOSH<sup>2</sup>, J. VIJAYA RATNA<sup>1\*</sup>

<sup>1</sup>Department of Pharmaceutical Technology, A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530003, India,

<sup>2</sup>Pharmaceutical Research & Development Laboratory, Corpuscle Research Solutions, Visakhapatnam 530017.

Email: vijaya.ratna@gmail.com

Received: 06 Sep, 2012, Revised and Accepted: 11 Oct, 2012

### ABSTRACT

A rapid, specific and accurate high performance liquid chromatographic method for the determination of rifampicin (RIF) by UV detection in human plasma was developed and validated. Rifampicin was extracted from plasma utilizing liquid-liquid extraction process using a 70:30 % v/v mixture of t-butyl methyl and dichloromethane. Hydrochlorothiazide (HCTZ) was used as internal standard. Both RIF and HCTZ were eluted under isocratic mode using a 150 X 4.6 mm i.d, 5 µm Phenomenex ODS 2 C18 column. A mixture of 40:60 % v/v acetonitrile and 10mM potassium dihydrogen phosphate (pH adjusted to 3.2) was used as mobile phase at a flow rate of 1.0 ml/min. The retention times for RIF and HCTZ were 6.80 and 2.56 minutes, respectively. The method showed good linearity in the range of 0.31 – 25.48 µg/ml. The recovery of rifampicin was 90.07 % with a CV of 3.26 % and recovery of internal standard was 91.24 % with a CV of 2.31 %. A rapid, sensitive, simple and cost effective method for the estimation of rifampicin in human plasma was developed.

**Keywords:** Rifampicin, Tuberculosis, HPLC.

### INTRODUCTION

Treatment of tuberculosis is now very complex because of the emergence of multi drug resistant bacteria, which are resistant to first-line anti-tuberculosis drugs, isoniazid and rifampin.<sup>1</sup> Rifampicin or rifampin (in USA) (RIF) (Figure 1) is a semi-synthetic derivative of a complex macrocyclic antibiotic called rifamycin<sup>2</sup>. It is used extensively in the treatment of tuberculosis together with isoniazid, ethambutol and pyrazinamide.<sup>3</sup> Rifampicin is a bactericidal drug for *Mycobacterium tuberculosis*. It has a plasma half-life of 3–4 hours, and peak concentrations of 8–12 µg/ml are reached within 1–2 hours of the recommended daily dose of 600 mg in adults<sup>4</sup>. Rifampicin is quickly absorbed from the gastrointestinal tract with peak serum concentrations of 6–8 µg/ml occurring 1.5–2.0 hours after administration. Rifampicin is 75% bound to plasma proteins<sup>5</sup> and metabolized in the liver to an active metabolite, deacetyl rifampicin and undergoes enterohepatic recycling<sup>6</sup>.

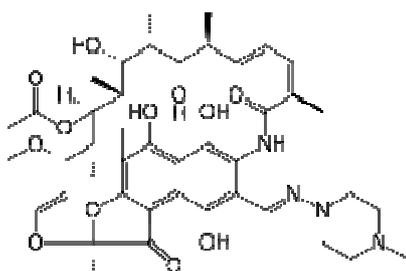


Fig. 1: Chemical Structure of Rifampicin

Measurement of plasma rifampicin concentrations may be required to determine the optimum drug dose for individuals, especially in patients with impaired metabolism of the drug, such as those with liver disease<sup>7</sup>. Several analytical methods are already available for the determination of RIF in biological fluids and pharmaceutical dosage forms, including methods based on HPTLC<sup>8</sup>, HPLC<sup>9-17</sup>, HPLC following SPE<sup>18</sup> or SBSE<sup>19</sup>, UPLC<sup>20</sup>, LC-MS/MS<sup>21-24</sup> and MALDI-TOF<sup>25</sup>. Mass Spectroscopy methods for the determination of rifampicin in human plasma have been described using electro spray ionization (ESI). Since rifampicin can be considered as a macromolecule with unusual molecular structure, the ESI interface leads to the formation of an unstable product ion. Additionally, rifampicin has a strong chromophore showing reddish brown color at a wavelength of 337

nm. This chromophore not only allows for successful determination in human plasma by UV detection but also offers approximately the same sensitivity offered by LC-MS/MS detection. One of the reported methods for the quantitative analysis of rifampicin using HPLC-MS interface has a lower limit of quantitation (LLOQ) of 0.1µg/ml. Many of the other methods involve time-consuming sample preparation and extraction and a very lengthy elution time. In the current study we achieved an acceptable LLOQ of 0.3 µg/ml using HPLC-UV interface. This limit of quantification achieved by using HPLC-UV interface can be directly applied to the successful estimation of rifampicin in a bioequivalence study. Although LC-MS/MS is a versatile tool, the development of HPLC based separation method, makes it more economical and simpler both in terms of maintenance and data interpretation. In the current study, the objective is to develop a method with a low LLOQ using HPLC-UV interface which can be directly applied to the successful estimation of rifampicin in a bioequivalence study and to validate the developed method according to FDA guidelines<sup>26</sup>.

### MATERIALS AND METHODS

#### Solvents and Chemicals

Rifampicin (purity 98.00 % w/w) was used as received from Lupin Laboratories Ltd. Hydrochlorothiazide (used as internal standard, purity 99.0 % w/w) (Fig 2) is purchased from Sigma Aldrich Inc. HPLC grade acetonitrile and ascorbic acid (as stabilizing agent) were purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

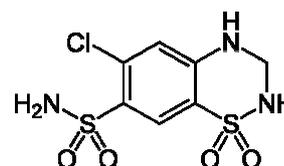


Fig. 2: Chemical Structure of Hydrochlorothiazide

#### Chromatographic System

The chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system were controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software.

The detector was set at a wavelength of 337 nm. Chromatographic separations were accomplished using a Phenomenex C<sub>18</sub>, 5 µm, 150 mm×4.6 mm column. The mobile phase was composed of a mixture of 40 parts of acetonitrile and 60 parts of 10mM phosphate buffer of pH 5.2, adjusted with phosphoric acid. The mixture was filtered through 0.22 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and was then degassed by flushing with nitrogen for five minutes. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consisted of a mixture of 50: 50 % v/v acetonitrile: HPLC grade water.

#### Preparation of Standard Solutions

A stock solution of rifampicin was prepared in diluent solution (50% v/v methanol in water) such that the final concentration was approximately 2 mg/ml. Stock solution of hydrochlorothiazide (approx 1 mg/ml) was prepared in HPLC grade methanol. To prevent oxidative degradation of rifampicin, a 1M solution of ascorbic acid, prepared in HPLC grade water, was added to the plasma spiked samples, to stabilize them. The solutions were stored at 4°C and they were stable for two weeks.

#### Sample Preparation

Aqueous stock dilutions were prepared initially. Aqueous stock dilution, 0.5 ml each, was transferred into a 10 ml volumetric flask, to which 0.8 ml of 1M ascorbic acid solution was added and mixed. The final volume was made up with screened drug-free K<sub>2</sub>EDTA human plasma and mixed thoroughly for two minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0 (no rifampicin added), 0.3, 0.6, 1.23, 2.45, 5.64, 10.78, 22.05 and 25.48µg/ml. Each of these standard solutions was distributed into disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volumes of 0.7 ml and the tubes were stored at -70°C, pending analysis.

The extraction of the plasma samples involved liquid-liquid extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 µl was then transferred to pre-labeled 2.0 ml polypropylene centrifuge tubes. Internal standard dilution, 25 µl, (500µg/ml) was then added and mixed. Extraction solvent, 1.2 ml, was then added to extract the drug and internal standard. The samples were then kept on a reciprocating shaker and allowed to mix for 20 minutes. Samples were then centrifuged at 5000 rpm for five minutes at 4 °C. Supernatant solution, 1 ml, was then transferred into prelabelled polypropylene tubes and was allowed to evaporate to dryness under nitrogen at a constant temperature of 40 °C. The dried residue was then dissolved in 200 µl of mobile phase and transferred into shell vials containing vial inserts for analysis. Samples, 20 µl by volume, were then injected into the system for analysis. The auto sampler temperature was maintained at 4 °C throughout the analysis. The column temperature oven was maintained at ambient temperature.

#### Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, %recovery, matrix effects, freeze-thaw, and autosampler stability. The initial assay was fully validated for RIF analysis in human plasma according to FDA guidelines.

#### Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K<sub>2</sub>EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

#### Calibration curve

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of RIF in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate.

Calibration curves for RIF were represented by the plots of the peak-area ratio (RIF/HCTZ) versus the nominal concentration of the RIF in calibration standards. The regression line was generated using  $1/\text{concentration}^2$  factor as the mathematical model of best fit. RIF concentrations in QC samples, recovery samples, stability samples and experimental plasma pharmacokinetic samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

#### Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

#### Stability Studies

Autosampler, and freeze-thaw stability of RIF was determined at low, medium and high QC concentrations. To determine the impact of freeze-thaw cycles on RIF concentration, samples were allowed to undergo 3 freeze (-50 °C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the RIF concentrations were analyzed in triplicates and compared to the control sample that had been stored at -50 °C. Autosampler stability of extracted samples was determined by comparing RIF concentration in freshly prepared samples and samples kept in autosampler at 4°C for 24 hours.

#### Recovery

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of RIF as in the QC samples. This should highlight any loss in signal due to the extraction process. HCTZ recovery was determined for a single concentration of 500µg/ml.

#### Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Version 1.23 SP 1 software. Standard curves for quantification of RIF were constructed using a  $1/\text{concentration}^2$  weighted linear regression of the peak area ratio versus RIF concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

## RESULTS AND DISCUSSION

#### Method Development

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of rifampicin in human plasma. Initial experiments were performed using potassium dihydrogen phosphate in the pH range of 3.0 to pH 5.0. The response was checked at the detector using a connector (without the column). A pH value of  $3.2 \pm 0.1$  gave maximum response for the analyte at 335nm. The run time of analysis was higher when a longer reverse phase column (250x4.6 mm id) was used. The resolution between the peaks was decreased and peaks were not of acceptable shape, when the experiment was performed using a shorter column (50x4.6 mm id). However, good resolution, less tailing and high theoretical plates were obtained with phenomenex C<sub>18</sub> 150x4.6 cm 5 µm column.

The mobile consists of 40:60 v/v acetonitrile and 10 mM potassium dihydrogen phosphate (pH 3.2). The flow rate of the method was 1.0 ml/min. The column temperature was maintained at 25 °C. At the reported flow rate, peak shape was excellent, however, increasing or decreasing the flow rate increased the tailing factor and resulted in poor peak shape and in decreased resolution between the drug and the internal standard. There was no interference in the drug and the internal standard, from the extracted blank. The peak shape and symmetry were found to be good when the mobile phase composition of 40:60 v/v was used with better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused rifampicin to elute very early leading to poor

relative retention time between rifampicin and internal standard. Mobile phase with organic portion lesser than 40 % led to very late elution of rifampicin. The peaks were also broad and had unacceptable asymmetry factor.

Extraction methods were initially attempted using protein precipitation technique. Organic solvents such as acetonitrile and/or methanol were used as reagents for protein precipitation<sup>27</sup>. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma: organic solvents. The recovery of the RIF was poor while that of the internal standard was relatively unchanged as compared with liquid-liquid extraction.

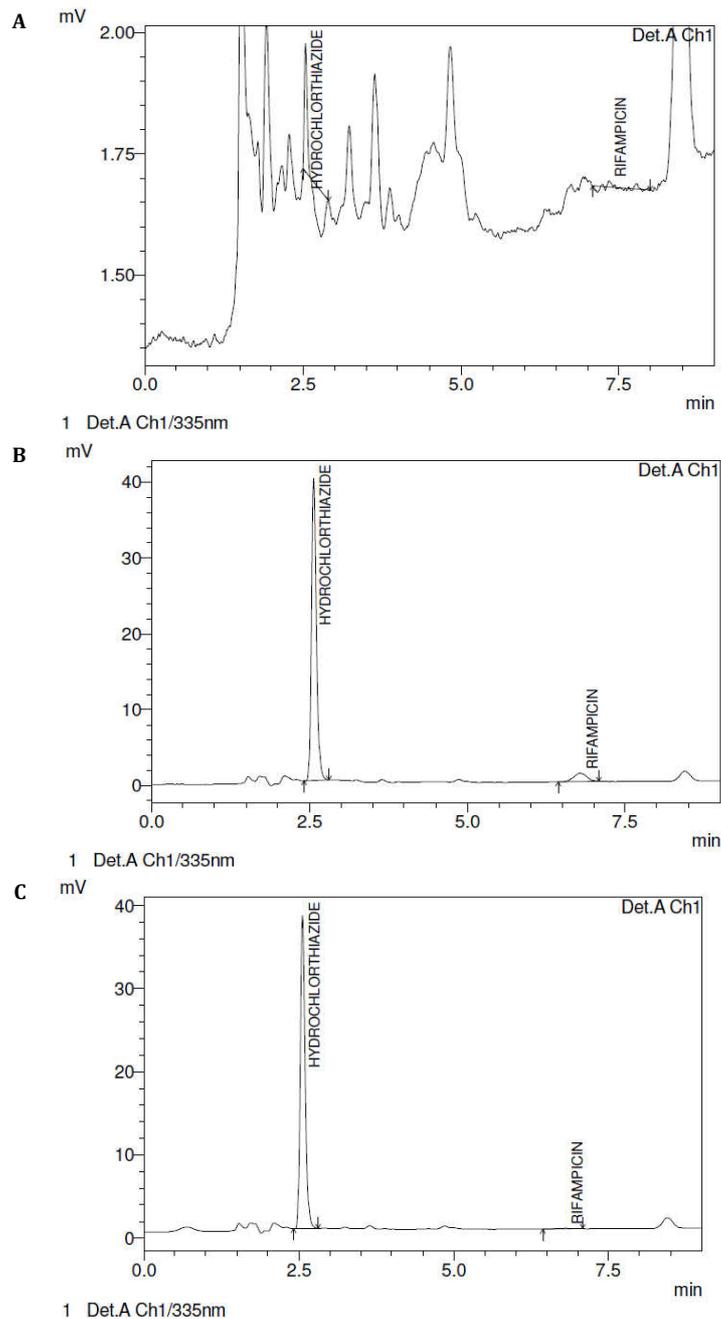
Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, the final analysis was carried out using liquid-liquid extraction (LLE). Although SPE methods render a neat sample for final analysis, polar interferences

do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique.

Various solvents such as ethyl acetate, diethyl ether, 100 % t-butyl methyl ether and combinations of t-butyl methyl ether and dichloromethane were used for extraction. The recovery of rifampicin and internal standard was poor when ethyl acetate or diethyl ether was used individually. The highest recovery from the plasma samples was obtained with a 70: 30 % v/v of t-butyl methyl ether: dichloromethane.

#### Detection and chromatography

Figure 3 shows the typical chromatograms of a blank human plasma sample (A), a spiked plasma sample with RIF (300.0 ng/mL, LLOQ) and HCTZ (500.0 ng/mL) (B), a zero blank sample containing only the internal standard indicating the specificity of the method. The retention times for RIF and HCTZ were 6.80 and 2.56 min, respectively.



**Fig. 3: Chromatograms of (A) Extracted Blank Sample (B) Rifampicin Containing Hydrochlorothiazide as Internal Standard at LLOQ level (C) Zero Blank Containing Hydrochlorothiazide as Internal Standard**

**Method validation**

**Selectivity**

The method was found to have high selectivity for the analyte; since no interfering peaks from endogenous compounds were observed at the retention time for RIF, in any one of the six independent blank plasma extracts evaluated (Table 1).

**Calibration curves**

Calibration curves for RIF in human plasma were calculated by weighted<sup>1</sup>/concentration<sup>2</sup> quadratic regressions, with the r<sup>2</sup> values of > 0.99 for all curves generated during the validation. The calibration curve accuracy for plasma is presented in Figure 4, demonstrating that measured concentration is within ± 15% of the actual concentration

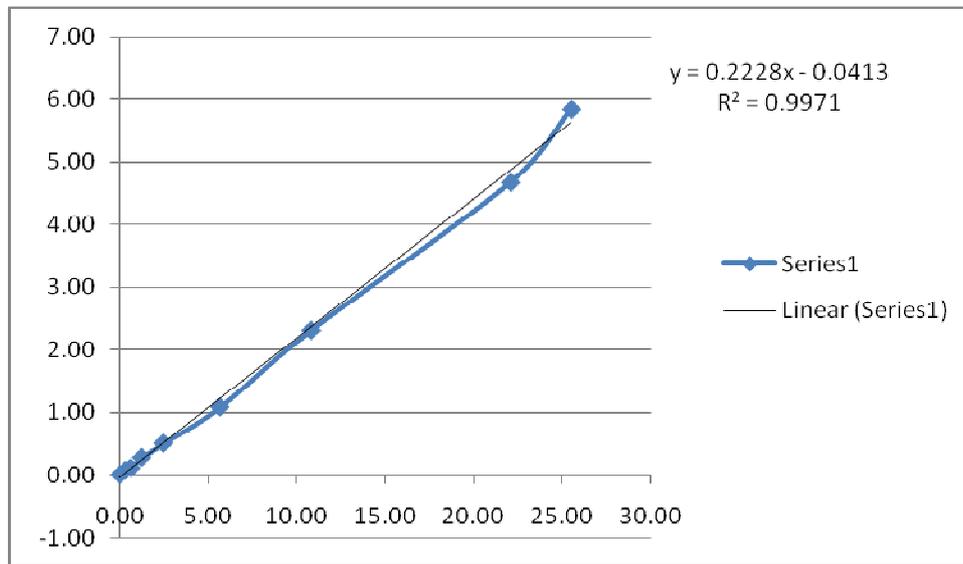
point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios.

**Accuracy and precision**

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in Table 2. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines<sup>24</sup>.

**Table 1: System Suitability Study**

	Rifampicin (5 µg/mL)		Internal Standard (25 µg/mL)	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
Mean (n = 6)	6.70	253385.5	2.56	727220.7
S. D.	0.01	3335.07	0.02	7425.45
% CV	0.149	1.32	0.781	1.02



**Fig. 4: Calibration Curve of Rifampicin**

**Table 2: Intra and Inter day accuracy and precision of HPLC assay**

	Nominal Concentration (µg/mL)			
	0.330	0.930	9.130	18.130
<b>DAY 1</b>				
Mean	0.351	0.925	8.998	17.985
S.D.	0.010	0.018	0.027	0.632
% CV	2.85	1.95	0.30	3.51
<b>DAY 2</b>				
Mean	0.327	0.932	9.419	18.384
S.D.	0.009	0.017	0.045	0.532
% CV	2.75	1.82	0.48	2.89
<b>DAY 3</b>				
Mean	0.345	0.942	9.377	18.754
S.D.	0.015	0.027	0.067	0.742
% CV	4.35	2.87	0.71	3.96

Each mean value is the result of triplicate analysis

**Limit of detection and limit of quantification**

Limit of detection, LOD, is defined as the lowest concentration that produces a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 100ng/ml. The LLOQ has been accepted as the lowest points on the standard curve with a relative

standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (250ng/ml) met the criteria for the LLOQ (Table3). The method was found to be sensitive for the determination of RIF in human plasma samples. The Upper limit of quantification (ULOQ) has been accepted as the highest point on the standard curve with a relative standard deviation of less than 15%<sup>26</sup>.

**Table 3: Results of regression analysis of the linearity data**

	Mean $\pm$ SD (n = 6)
Slope	0.2228 $\pm$ 0.002
Intercept	-0.0413 $\pm$ 0.012
Correlation coefficient (r <sup>2</sup> )	0.9971 $\pm$ 0.0008

**Carryover test**

A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octadecyl-based chromatographic

packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

**Stability studies**

The results of autosampler and freeze-thaw stability are presented in Table 4. Determination of RIF stability following three freeze-thaw cycles showed that for all QC samples there was a minor change in the RIF concentration.

**Table 4: Short Term, long term and Freeze Thaw stability of Rifampicin**

	Nominal Concentration ( $\mu$ g/mL)	
	0.930 (LQC)	18.130 (HQC)
<b>Short-term stability (4 Days)</b>		
Mean	104.48	108.80
S.D.	2.549	2.671
% CV	2.41	2.45
<b>Long-term stability (10 Days)</b>		
Mean	103.67	103.89
S.D.	2.176	3.541
% CV	2.10	3.41
<b>Freeze - Thaw stability (3 Cycles)</b>		
Mean	101.76	99.86
S.D.	4.181	3.671
% CV	4.11	3.68

Each mean value is the result of triplicate analysis

**Recovery**

Percentage recovery of RIF was measured by dividing the AUC values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of RIF from plasma spiked samples of RIF, in terms of LQC and HQC was respectively, 92.5  $\pm$  2.17 and 93.2  $\pm$  3.04. The overall recovery of RIF was 90.07% with a coefficient of variation of 3.26 % (n = 6). Internal standard recovery at 500  $\mu$ g/ml of HCTZ was 91.24 % with a coefficient of variation of 2.34%.

**CONCLUSION**

A HPLC method was developed and validated for the determination of RIF in human plasma. The extraction process was a single-step liquid-liquid extraction (LLE) procedure employing the use of 70:30 % v/v of t-butyl methyl ether and dichloromethane. LLE method is usually devoid of polar interferences, thus rendering the sample clean for final analysis. The noise is usually absent or at minimum, as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500  $\mu$ l). In conclusion, method validation following FDA guideline, indicated that the developed method has high sensitivity with an LLOQ of 300 ng/ml, high recovery (>90%), reliability, specificity and excellent efficiency with a total running time of 9.0 minutes per sample, which is important for large batches of samples. The sensitive, simple and rapid HPLC method is suitable for pharmacokinetic, bioavailability or bioequivalence studies of RIF in human subjects. This method may be successfully utilized to analyze RIF concentrations in human plasma.

**ACKNOWLEDGEMENT**

The authors are grateful to the Department of Science and Technology, New Delhi, India for granting research fellowship under DST-PURSE Programme, to carry out this work. The authors would like to express their gratitude to M/s Lupin Pharma Pvt. Ltd. for supplying the gift sample of rifampicin.

**REFERENCES**

1. Fivy Kurniwati, Syed Azar Syed Sulaiman, Syed Wasif Gillani; Study on drug-resistant tuberculosis and tuberculosis treatment on patients with drug resistant tuberculosis in chest

- clinic outpatient department, International Journal of Pharmacy and Pharmaceutical Sciences 2012; 4(2): 733-737.
2. S.d. Seth, Vimelesh Seth; Antituberculosis drugs and drugs for mycobacterium avium complex, Textbook of Pharmacology, 2008; 3<sup>rd</sup> edition: Chapter 77: Page X.77. Elsevier
3. Enoche Flornce Oga; Spectrophotometric determination of isoniazid in pure and pharmaceutical formulations using vanillin, International Journal of Pharmacy and Pharmaceutical Sciences 2010; 2: 55-58.
4. Peloquin C.A., Jaresko G.S., Yong C.L., Keung A.C., Bulpitt A.E. and Jelliffe R.W; Population pharmacokinetic modeling of isoniazid, rifampicin and pyrazinamide, Antimicrobial Agents and Chemotherapy, 1997; 41: 2670-79.
5. Conte JE Jr, Lin E, Zurlinden E; Liquid chromatographic determination of Rifampicin in human plasma, bronchoalveolar lavage fluid, and alveolar cells, Journal of Chromatographic Science 2000; 38(2):72-6.
6. Peters JH, Gordon GR, Murray Jr, Ichikawa W, Gilber RH, Welch TM; Measurement of Rifampicin in plasma: chemical vs bacteriologic assay, Proceedings of the Western Pharmacology Society, 1977; 20(-HD-): 211-5.
7. J B McConnell, H Smith, M Davis, and R Williams; Plasma rifampicin assay by an improved solvent extraction technique, British Journal of Clinical Pharmacology, Nov 1979; 8 (5): 507.
8. Udupa N, Goyal P, Agarwal S, Venkatesh S, Pandey S; Simultaneous HPTLC determination of rifampicin and isoniazid in rat plasma, Indian Journal of Pharmaceutical Sciences, 2003; 65: 414-416.
9. Calleja I, Blanco-Prieto M.J., Ruz N, Renedo M.J., Dios-Vieitez M.C; High performance liquid-chromatographic determination of rifampicin in plasma and tissues, Journal of Chromatography A, 2004; 1031: 289-294.
10. Khuahawar M.Y., Rind F.M.A; Liquid chromatographic determination of isoniazid, pyrazinamide and rifampicin from pharmaceutical preparations and blood. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2002; 766: 357-363.
11. Calleri E., De Lorenzi E., Furlanetto S., Massolini G., Caccialanza G; Validation of a RP-LC method for the simultaneous determination of isoniazid, pyrazinamide and rifampicin in a pharmaceutical formulation, Journal of Pharmaceutical and Biomedical Analysis, 2002; 29: 1089-1096.

12. Mohan B., Sharda N., Singh S; Evaluation of the recently reported USP gradient HPLC method for analysis of anti-tuberculosis drugs for its ability to resolve degradation products of rifampicin, *Journal of Pharmaceutical and Biomedical Analysis*, 2003; 31: 607-612.
13. Walubo A., Smith P., Folb P.J; Comprehensive assay for pyrazinamide, rifampicin and isoniazid with its hydrazine metabolites in human plasma by column liquid chromatography, *Journal of Chromatography B: Biomedical Sciences and Applications*, 1994; 658: 391-396.
14. Woo J., Wong C.L., Teoh R., Chan K; Liquid chromatographic assay for the simultaneous determination of pyrazinamide and rifampicin in serum samples from patients with tuberculosis meningitis; *Journal of Chromatography B: Biomedical Science and Applications* 1987; 420: 73-80.
15. Ishii M. Ogata H; Determination of rifampicin and its main metabolites in human plasma by high-performance liquid chromatography, *Journal of Chromatography B: Biomedical Sciences and Applications*, 1988; 426: 412-416.
16. Seifart H.I., Kruger P.B., Parkin D.P., Van Jaarsveld P.P., Donald P.R; Therapeutic monitoring of antituberculosis drugs by direct in-line extraction on a high performance liquid chromatography system, *Journal of Chromatography B: Biomedical Sciences and Applications*, 1993; 619: 285-290.
17. Ratti B., Parenti R.R., Toselli A., Zerilli L.F; Quantitative assay of Rifampicin and its main metabolite 25-desacetyl-rifampicin in human plasma by reversed-phase high-performance liquid chromatography, *Journal of Chromatography B: Biomedical Sciences and Applications*, 1981; 225: 526-531.
18. Smith P.J., Dyk J.V., Fredericks A; Determination of rifampicin, isoniazid and pyrazinamide by high performance liquid chromatography after their simultaneous extraction from plasma, *International Journal of Tuberculosis and Lung Diseases*, 1999; 3: S325-S328.
19. Balbao M.S., Bertucci C., Bergamaschi M.M., Queiroz R.H.C., Malfara W.R., Dreossi S.A.C; Rifampicin determination in plasma by stir bar-sorptive extraction and liquid chromatography, *Journal of Chromatography B: Biomedical Sciences and Applications*, 2010; 5: 1078-1083.
20. Gikas E., Bazoti F.N., Fanourgiakis P., Perivolioti E., Roussidis A., Skoutelis A; Simultaneous quantification of daptomycin and rifampicin in plasma by ultra performance liquid chromatography: application to a pharmacokinetic study, *Journal of Chromatography B: Biomedical Sciences and Applications*, 2010; 51: 901-906.
21. Hartkoorn R.C., Khoo S., Back D.J., Tjia J.F., Waitt C.J., Chaponda MA; Rapid and sensitive HPLC-MS method for the detection of plasma and cellular rifampicin, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 2007; 857: 76-82.
22. Korfmacher W.A., Bloom J., Churchwell M.I., Getek T.A., Hansen Jr E.B., Holder C.L., Characterization of three rifamycins via electro spray mass spectrometry and HPLC - thermo spray mass spectrometry, *Journal of Chromatographic Sciences*, 1993; 31: 498-501.
23. Baietto L., Avolio A., De Rosa F.G., Garazzino S., Michelazzo M., Ventimiglia G., Development and validation of a simultaneous extraction procedure for HPLC-MS quantification of daptomycin, amikacin, gentamicin, and rifampicin in human plasma, *Analytical and Bioanalytical Chemistry*, 2010; 396: 791-798.
24. De Velde F., Alffenaar J.W.C., Wessels A.M.A., Greijdanus B., Uges D.R.A., Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 2009; 877: 1771-1777.
25. Notari S., Mancone C., Sergi M., Gullotta F., Bevilacqua N., Tempestilli M., , Determination of antituberculosis drug concentration in human plasma by MALDI-TOF/TOF, *IUBMB Life*, 2010; 62: 387-393.
26. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research, 2001.
27. Cara Polson, Pratibha Sarkar, Bev Inledon, Vanaja Raguvaran, Russell Grant; Optimization of Protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry, *Journal of Chromatography B*, 2003; 785: 263 - 275.