Journal of Advanced Multidisciplinary Research Studies and Development

ISSN : 2583-6404
Sep - Oct 2025

DEVELOPMENT AND VALIDATION OF LLE –HPLC METHOD FOR THE DETERMINATION OF RIFAPENTINE IN HUMAN PLASMA

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Abstract

A simple, accurate, precise and sensitive reverse phase high performance liquid chromatography method was developed for estimation of rifapentine in human plasma by HPLC method using liquid-liquid extraction (LLE) with UV detection. This analytical method was developed by using experimental design and quantification was accomplished with the internal standard method. No interferences were 0bserved from endogenous compounds of plasma. The accuracy of rifapentine from plasma samples, measured at the three levels of the linear concentration range (1-32 µg/ml) were found between 87 and 110.8% . The intraday and interday precision and accuracy. Mobile phase containing methanol: 20mM phosphate buffer pH (6.5) in ration of 80:20 v/v was used. The flow rate was 1 ml/min with the injection volume of 20µL and effluent was monitored at 478nm. Retention time was 8.3 mins. The method was validated for several parameters like accuracy, linearity, [precision etc as per USFDA guidelines. The values of relative standard deviation and % recovery were found to be satisfactory, indicating that the proposed method is precise and accurate and hence can be used for the routine analysis of rifapentine in human plasma.

Keywords: Rifapentine, RP-HPLC, Human Plasma, Liquid-Liquid Extraction & Method Validation.

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INTRODUCTION

Rifapentine is a rifamycin antibiotic that is similar in structure and activity used in combination with other agents as therapy of tuberculosis, particularly in once r twice weekly regimens. It inhibits DNA- dependent RNA polymearase activity in suspectible cells. Specially it interacts with bacterial RNA polymearase but does not inhibit with a remarkably greater therapeutic efficacy againt *mycobacterium tuberculosis* and *mycobacterium lepraeine* experimental infection. Rifapentine has a longer half-life than rifampin and rifabutin. [1-3]

Rifapentine is chemically [(7*S*,9*E*,11*S*,12*R*,13*S*,14*R*,15*R*,16*R*,17*S*,18*S*,19*E*,21*Z*)-26-[(*E*)-(4-cyclopentylpiperazin-1-yl)iminomethyl]-2,15,17,27,29 pentahydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-6,23-dioxo-8,30-dioxa-2 azatetracyclo[23.3.1.14,7.05,28]triaconta-1(29),2,4,9,19,21,25,27-octaen-13-yl]acetate[1]

ISSN : 2583-6404
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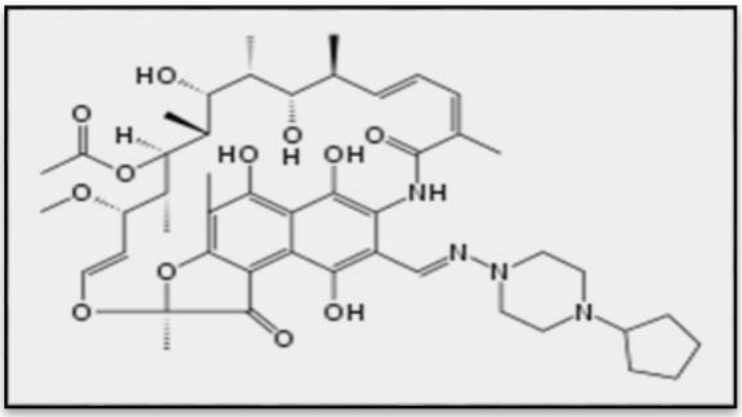

Fig1: Structure of Rifapentine

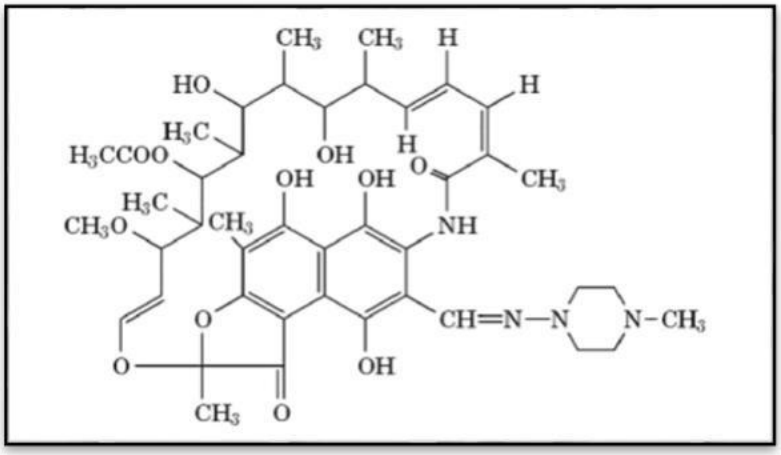

Fig 2: Structure of Rifampicin

Literature survey revealed that few chromatographic methods are reported for estimation of rifapentine from human plasma. Some of method reported include LC-MS which are most expensive methods. Thus there is a need to develop a simple , economical and rapid method for estimation of rifapentine in human plasma. In this paper we used an HPLC method with uv dectction and the results were satisfactory. Uv detection is simple and easily available method a method involve a simple liquid-liquid extraction with reproductibility which makes it suitable for bioequivalence studies. The method reported in this paper is a simple, rapid, cost effective HPLC method to quantify rifapentine with UV detection using liquid-liquid extraction and the method was validated as per US FDA guidelines.

EXPERIMENTAL

Materials and Methods

Rifapentine working standard was obtained as a gift sample from Lupin Pharma Ltd (Mumbai India). The Rifampicin internal standard was purchased. HPLC grade methanol (purity 99.9%) were procured from Molychem chemicals badlapur (Maharshtra India). The HPLC grade water was obtained by double distillation and purification through 0.45 µm filter. All the other reagents were of analytical grade obtained from Molychem chemicals badlapur (Maharshtra India).

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Chromatographic Condition

The HPLC system consisted of Agilent 1200 series with UV visible dectcor, Rheodyne manual injector fitted with 20µL loop. Separation was performed using guard column ThermoFisher C18 column and analytical column SUNQ C18 (250mml□4.6mml). The Chemstation software, version B.02.03 was used for data acquisition. The chromatographic analysis were performed at ambient room temperature. The mobile phase consisted of the mixture of solvents methanol:phosphate buffer (pH 6.5, 20Mm) in the ratio 80:20 v/v. the prepared mobile phase was filtered through 0.45µm membrance filterand ultrasonically degassed prior to use. The eleuent was monitored with UV-Visible detector at 478nm and the peak area was recorded using chromatographic data system. The flow rate was of 1ml/min was kept throughout the study.

SAMPLE PREPARATION

Preparation of Stock Solution in Dilution Solution

Stock solution of rifapentine and rifampicin internal standard (IS) were prepared in methanol as 1mg/ml working solution were prepared by diluting the stock solution with dilution solution.

Preparation of Stock Solution

For calibration curve, six different concentration (1, 2, 4, 8, 16 and 32 µg/ml) in fresh plasma were prepared by adding required volume of working solution of analyte .

Extraction of Rifapentine from Plasma

An aliquot of 1ml plasma spiked with rifapentine was taken in a stoppered test tube. Internal standard was added and mix well. To this 5 ml of ethyl acetate was added and the content of the test tube were mixed an vortex for 10 mins and centrifuged at 3000 rpm for 15 mins . 3 ml of the organic layer was separated and was evaporated to dryness. The extraction residue was reconstituted with 500µL of mobile phase and the same was injected for chromatographic analysis.

METHOD VALIDATION

The proposed RP-HPLC method was validated as per USFDA guidelines. The developed method was validated for selectivity, limit of detection and quantification, linearity, precision and accuracy, recovery, and stability. Quality control samples were used fir inter-day and intra-day precision and accuracy. Plasma calibration curves were generated by using analyte to the internal standard area ratio as the Y-axis and the analyte concentration (µg/ml) as the X-axis. Five replicates of the calibration curve were prepared to take each concentration five times. The linear regression equation (y=mx+c) was used to fit the spiked concentration and the peak area of rifapentine. Precision is reported as % RSD of the estimated concentrations and the accuracy is expressed as [(Interpolated concentration – nominal cncentation)/nominal concentration]×100

Calibration and Linearity

A calibration curve was constructed by plotting the response ratios with respect to analyte concentrations ranging from 1-32 µg/ml in human plasma. Using weighted (1/x) least squares regression analysis, linearity was assessed. A standard curve was found to be validated if the coefficient of correlation should be near 0.99 or better. The acceptability criteria for each back calculatedvalue, with the excepton of LLOQ (20%), should not be higher than 15%. Atleast 75% of standards, including the LLOQ , and 67% of the QC samples (low, medium, and high), should comply with the above mentioned limits for the standard curve to be accepted

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Extraction Recovery

The extraction recovery of analyte was determined by measuring peak areas of the drug from the prepared plasma quality control samples, 3 and 27 µg/ml plasma samples were taken at LQC (low quality control), and MQC (Medium Quality Control) samples respectively. The peak areas of extracted LQC, and MQC were compared to the absolute peak area of the unextracted drug sample having the drug concentration as 100%. To obtain good extraction efficiency the extraction recovery of rifapentine and rifampicin (internal standard) was determined using QC samples.

Selectivity

To determine the amount to which endogenous plasma components may interfere with the analyte or the internal standard, six randomly selected blank human plasma samples were passed through the extraction method. The retention time of all analytes in standard blank samples was ≤ 20% of the drug area in the extracted LLOQ samples, and the peak area at the retention time of IS was ≤ 5% of the IS area in the extracted LLOQ sample, as per the acceptance limit. It was determined that there were no significant interferences in any of the six samples.

Accuracy and Precision

Precision and accuracy were also determined from LQC (3 µg/ml), MQC (16 µg/ml) and HQC (27 µg/ml). Three replicates of each concentration were analyzed on the same day to determine the withrun accuracy and precision three replicates of each concentration were analyzed on three different days.

Stability Studies

The stability of rifapentine in plasma: a short-term sability study and a freeze- thaw study. Plasma blank samples were spiked with rifapentine at a concentration of 3µg/ml(LQC), and 27µg/ml(HQC), and each concentration was carried out three times. A short-term stability test was performed at room temperature. After 12 hours at room temperature with rifapentine spiked plasma samples, the samples were extracted and then analyzed. For the freeze- thaw stability, spiking samples were evaluated immediately after preparation and daily after three consecutive days of repeated freeze- thaw cycles.

RESULTS AND DISCUSSION

Chromatography

Representative chromatogram of blank plasma and plasma spiked with rifapentine and IS are shown in fig 3 and fig 4, respectively. Retention time for the rifapentine and internal standard were 8.3 min and 4.2 min, respectively. Good separation of rifapentinne and internal standard with very less background noise was observed. The chromatographic run time was 10 minutes for plasma sample analysis. The peak of rifapentine was well resolved without any interference from endogenous materials. Sensitivity and specificity of the method was tested and no potential metabolites of the drug or any endogenous compounds were detected at the retention time the drug. Thus the study involved HPLC- UV detection with liquid–liquid extraction. The reproducibility data and recovery clearly show the accuracy and validity of the method . Thus the method developed is simple, stable, and specific for rifapentine detection.

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Table 1: optimized chromatographic conditions

Drug	Internal standard	LLE solvent	Mobile phase	Stationary phase	Detection wavelength	Flow rate	Sample injection volume
Rifapentine	Rifampicin	Ethyl acetate	Methanol: phosphate buffer pH 6.5(80:20v/v)	SUNQ C18 (250mmlX 4.6mml)	478nm	1mL/min	20µl

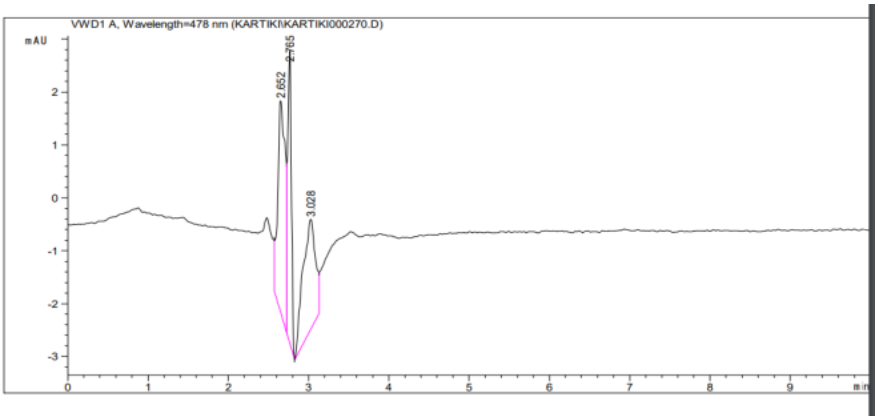

Fig 3: Representative Chromatogram of Blank Plasma

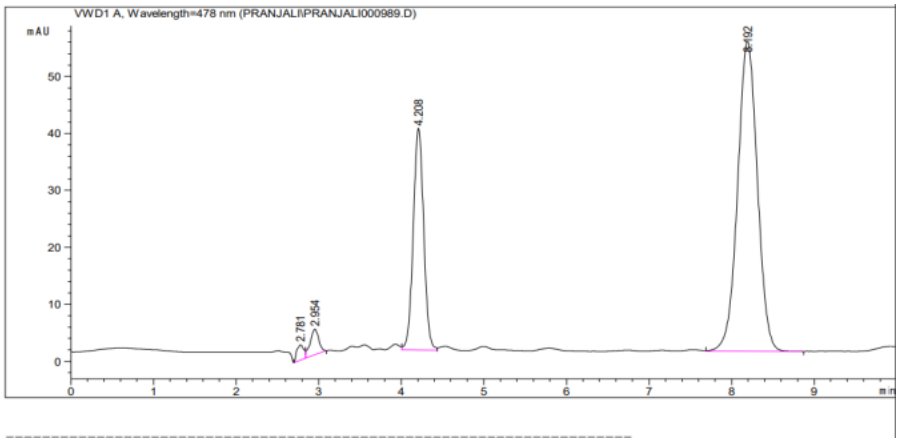

Fig 4: Representative Chromatogram of Plasma Spiked with Rifapentine and Internal standard

Linearity

The peak area ratio of rifapentine to IS in human plasma was linear with respect to the analyte concentration over the range of 1-32 µg/ml.based on the analysis of the data using linear regression with/ without intercepts and weighing factors, the calibration model was selected. The linear equation of the form y=mx+c with weighning factor 1/X could be used to obtain the best fit for the calibration curve. The calibration curve's weighted linear regression equation for the analyte was Y=0.1423 X + 0.1064, where Y represents the peak area ratio of te analyte to the IS and x represented its concentration. The correlation coefficients (r2) for rifapentine was 0.9998.

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Accuracy and Precision

The accuracy and precision of the method were assessed using spiked plasma standards at three different concentrations (3,16 and 27 µg/ml). the intra-day and inter-day variations for rifapentine were calculated using data accumulated over a period of 3 days. Intra-day accuracy and precision were evaluated by replicate analysis of a spiked plasma containing rifapentine at three different concentrations (n=3). Results are represented in table 2. This indicate the high accuracy and precision of the method.

Table 2: Accuracy and Precision of Rifapentine

Concentration added (µg/ml)	Intra-day (n=3)			Inter-day (n=3)		
	Concentration found (mean ± SD, µg/ml)	Precision (% RSD)	Accuracy (% RE)	Concentration found (mean ± SD, µg/ml)	Precision (% RSD)	Accuracy (% RE)
3	3.33 ±0.15	4.58	11	3.27 ±0.2	6.78	9.0
16	15.01 ±0.4	2.67	0.06	15.13 ±0.4	2.81	-5.43
27	23.93 ±2.21	9.46	13.37	23.68 ±1.84	7.77	-12.29

Recovery

The recovery of rifapentine from plasma were analyzed using the method mentioned in the previous section. The recovery of rifapentine was assessed at two different concentration . the extraction recovery of rifapentine at LQC and MQC was 95.83% and 93.76%, respectively. The recovery of internal standard was 92.49

Stability

The stability of rifapentine in plasma was assessed under a variety of conditions. Table 3 summarizes all stability results. After exposing samples to three freeze-thaw cycles, no significant decrease in rifapentine concentration in plasma was found. The test performed at room temperature showed that two QC samples were stable.it indicated that rifpentine was stable in human plasma under the conditions.

Table 3: Stability of Rifapentine

Stability conditions	Concentration added (µg/ml)	Concentration found (mean, µg/ml)	Precision (% RSD)	Accuracy (%RE)	% Nominal
Short term stability	3	3.16	4.82	5.33	105.33
	27	26.90	0.62	-0.37	99.6
Three freeze-thaw Cycles	3	2.88	3.94	-4	96
	27	26.19	2.97	-3	97

CONCLUSION

A simple, sensitive and reproducible HPLC method for the detection and quantification of rifapentine for *in vivo* studies had been successfully developed. Besides greater precision and sensitivity attained using this HPLC method, another advantage compared to the other costlier methods of analysis. The method is also cost effective as compared to reported LC-MS/MS

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methods. The results rifapentine the use of HPLC and the accuracy-precision data convincingly demonstrate the satisfactory performance of the proposed method of analysis which can help in therapeutic drug monitoring.

ACKNOWLEDGMENTS

The authors would like to thank the HSNC board, Dr. L. H. Hiranandani college of pharmacy for providing the facilities needed to conduct the research. I would like to acknowledge Lupin PVT LTD (India). for providing the rifapentine working standard for the study, as well as plasma blood bank(Dombivali, Maharashtra) for providing plasma sample.

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