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

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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 rifampicin. Rifampicin or rifampin (in USA) (RIF) (Figure 1) is a semi-synthetic derivative of a complex macrocyclic antibiotic called rifamycin. It is used extensively in the treatment of tuberculosis together with isoniazid, ethambutol and pyrazinamide. 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. 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 and metabolized in the liver to an active metabolite, deacetyl rifampicin and undergoes enterohepatic recycling.

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. Several analytical methods are already available for the determination of RIF in biological fluids and pharmaceutical dosage forms, including methods based on HPTLC, HPLC, SPE or SBSE, UPLC, LC–MS/MS and MALDI–TOF. 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.

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.

Chromatographic System

The chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10Avp, 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.

![Fig. 1: Chemical Structure of Rifampicin](image1)

![Fig. 2: Chemical Structure of Hydrochlorothiazide](image2)
The selectivity of the method was evaluated by analyzing six selectivity guidelines. Calibration range, accuracy and precision, limit of quantitation, and recovery were determined using a 1:1 mixture of RIF and an internal standard. The internal standard was added to the plasma samples to prevent possible interference from endogenous substances. Calibration curves were generated using a linear least squares regression analysis. The accuracy and precision of the method were evaluated by analyzing QC samples at low, medium, and high concentrations. The recoveries were calculated using a 1:1 mixture of RIF and an internal standard. The recoveries were determined using a weighted linear regression analysis.

Sample Preparation
Aqueous stock solutions were prepared initially. Aqueous stock solution, 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 KEDTA human plasma, and mixed thoroughly for two minutes to achieve the desired concentration. The calibration standard concentrations were 0, 0.3, 0.6, 1.23, 2.45, 5.64, 10.70, 22.05, and 25.49 µg/ml. Each of these standard solutions was distributed into disposable polypropylene microcentrifuge 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 prelabeled 2.0 ml polypropylene centrifuge tubes. Internal standard solution, 1.2 ml, was then added to 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, 1.0 ml, was then 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 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 (LOQ), %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 KEDTA 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/concentration² 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 (LOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and 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 500 µg/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/concentration² 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 ± 0.1 gave maximum response for the analyte at 335 nm. The run time of analysis was increased when a longer reverse phase column (250x4.6 mm id) was used. The resolution between 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 C18 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 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 resolution of the analyte.
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. 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 Hydrochlorthiazide as Internal Standard at LLOQ level (C) Zero Blank Containing Hydrochlorthiazide as Internal Standard](image_url)
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\(\sqrt{\text{concentration}}\) quadratic regressions, with the \(R^2\) 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\(^4\).

![Fig. 4: Calibration Curve of Rifampicin](image)

Table 1: System Suitability Study

<table>
<thead>
<tr>
<th>Nominal Concentration ((\mu\text{g/mL}))</th>
<th>0.330</th>
<th>0.930</th>
<th>9.130</th>
<th>18.130</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY 1</td>
<td>Mean</td>
<td>S.D.</td>
<td>% CV</td>
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<td></td>
<td>0.351</td>
<td>0.010</td>
<td>1.49</td>
<td></td>
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<tr>
<td></td>
<td>0.925</td>
<td>0.018</td>
<td>1.95</td>
<td></td>
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<tr>
<td></td>
<td>8.998</td>
<td>0.027</td>
<td>0.30</td>
<td></td>
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<tr>
<td></td>
<td>17.985</td>
<td>0.632</td>
<td>3.51</td>
<td></td>
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<tr>
<td></td>
<td>0.009</td>
<td>0.017</td>
<td>0.48</td>
<td></td>
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<tr>
<td></td>
<td>0.932</td>
<td>0.45</td>
<td>2.89</td>
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<tr>
<td></td>
<td>9.419</td>
<td>0.532</td>
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<td></td>
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<tr>
<td></td>
<td>18.384</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>0.942</td>
<td>0.48</td>
<td></td>
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<tr>
<td></td>
<td>9.377</td>
<td>0.742</td>
<td></td>
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<td></td>
<td>18.754</td>
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<tr>
<td></td>
<td>0.927</td>
<td>0.71</td>
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<tr>
<td></td>
<td>0.067</td>
<td>3.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 (Table 3). 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%\(^6\).
Table 3: Results of regression analysis of the linearity data

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD (n = 6)</th>
</tr>
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<tr>
<td>Slope</td>
<td>0.2228 ± 0.002</td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.0413 ± 0.012</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.9971 ± 0.0008</td>
</tr>
</tbody>
</table>

Carryover test

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

<table>
<thead>
<tr>
<th></th>
<th>Nominal Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.930 (LQC)</td>
</tr>
<tr>
<td></td>
<td>18.130 (HQC)</td>
</tr>
<tr>
<td>Short-term stability (4 Days)</td>
<td>104.48</td>
</tr>
<tr>
<td>Mean</td>
<td>2.49</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.41</td>
</tr>
<tr>
<td>% CV</td>
<td>2.59</td>
</tr>
<tr>
<td>Long-term stability (10 Days)</td>
<td>103.67</td>
</tr>
<tr>
<td>Mean</td>
<td>2.176</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.10</td>
</tr>
<tr>
<td>% CV</td>
<td>2.41</td>
</tr>
<tr>
<td>Freeze – Thaw stability (3 Cycles)</td>
<td>101.76</td>
</tr>
<tr>
<td>Mean</td>
<td>4.181</td>
</tr>
<tr>
<td>S.D.</td>
<td>4.11</td>
</tr>
<tr>
<td>% CV</td>
<td>3.68</td>
</tr>
</tbody>
</table>

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 ± 2.17 and 93.2 ± 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 µg/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 µl). 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.

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