HPLC Method for Determination of Rifaximin in Human Plasma Using Tandem Mass Spectrometry Detection

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The present study was aimed at developing a simple, sensitive, and specific liquid chromatography–tandem mass spectrometry method for the quantification of rifaximin in human plasma using rifaximin D6 as internal standard. Chromatographic separation was performed on Zorbax SB C18, 4.6 x 75 mm, 3.5 µm column with an isocratic mobile phase composed of 10 mM ammonium formate (pH 4.0) and acetonitrile in the ratio of (20:80 v/v), at a flow-rate of 0.3 mL/min. Rifaximin and rifaximin D6 were detected with proton adducts at m/z 786.4→754.4 and 792.5→760.5 in multiple reaction monitoring positive mode respectively. The acidified samples were subjected to liquid–liquid extraction using a mixture of methyl t-butyl ether - dichloromethane (75: 25) followed by centrifugation, nitrogen-aided evaporation and reconstitution. The method was validated over a linear concentration range of 20 - 20000 pg/mL with correlation coefficient of more than 0.9995. This method demonstrated intra and inter-day precision within 0.6 - 2.6% and 2.2 - 5.6%, and accuracy within 95.7 - 104.2% and 95.8 - 105.0% for rifaximin, respectively. Rifaximin was found to be stable throughout freeze–thawing cycles, bench top and postoperative stability studies. This method was applied successfully for the analysis of blood samples following oral administration of rifaximin (200 mg) in 17 healthy Indian male human volunteers under fasting conditions.

Key words: Bioequivalence, mass spectrometry, rifaximin

INTRODUCTION

Rifaximin (RF) is (2S, 16Z, 18E, 20S, 21S, 22R, 23R, 24R, 25S, 26S, 27S, 28E)- 5,6,21,23,25 - pentahydroxy-27-methoxy-2,4,11,16,20,22,24, 26-octamethyl-2,7-(epoxypentadeca-[1,11,13] trienimino)benzofuro[4,5-e]pyrido[1,2-a]-benzimidazole-1,15(2H)-dione,25-acetate, empirical formula C₄₃H₅₁N₃O₁₁ and molecular weight 785.89 (Figure 1). It is a semisynthetic, rifamycin-based non-systemic antibiotic owing to its poor oral bioavailability. It is used in the treatment of traveler’s diarrhea and hepatic encephalopathy [1]. Although food significantly increases the extent of absorption of rifaximin, systemic absorption of rifaximin has been found to be lower than 1% both in the fasting and fed states [2].

Currently, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has gained significance for the quantitative estimation of drugs in various biological matrices including plasma, serum, urine and ocular fluids due to its high sensitivity, selectivity, and reproducibility. A literature survey reveals that several techniques have been reported for quantification of rifaximin such as LC-MS/MS [3,4], High Performance Liquid Chromatography (HPLC) [5] and U.V spectrometry [6]. The UV method involves a tedious extraction procedure which may affect the accuracy and precision of results

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obtained. Quantification of rifaximin in rat plasma [4, 5] and pharmaceutical dosage forms by U.V [6] are published. On the other hand, only a few methods are reported for the quantification of rifaximin in human plasma using LC-MS/MS [3].

Figure 1: Structures of Rifaximin and Rifaximin-D6

The present study reports a highly sensitive, selective and reproducible analytical method for the determination of RF in plasma samples utilizing liquid chromatography coupled with electrospray tandem mass spectrometry. Deuterated rifaximin D6 (RFD6) was used as an internal standard. The method was validated according to the Food and Drug Administration (FDA) guidelines over a concentration range of 20 – 20000 pg/mL using 400 µL plasma samples. An elution time of 3.3 ± 0.2 min was achieved for both drug and internal standard. The recoveries of RF and RFD6 were 88.79 ± 2.43% and 90.94 ± 3.24% respectively. This method was successfully employed in the analysis of blood samples following oral administration of RF (200 mg) in healthy human volunteers [7].

MATERIALS AND METHODS

Instrumentation

Mass spectrometric detection was performed on an Applied Biosystems API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using Multiple Reaction Monitoring (MRM) for detection. It employed an Atmospheric Pressure Ionization (API) turbo ion spray in positive mode with Unit Resolution. For RF, the MH⁺ (m/z 786.4) was monitored as the precursor ion and m/z 754.4 was chosen as the product ion. For internal standard, the MH⁺ (m/z 792.5) was monitored as the precursor ion and m/z 760.5 as the product ion. Mass parameters were optimized as source temperature 450 °C, nebulizer gas 35 psi, heater-gas 35 psi, curtain gas 30 psi, CAD gas 4 psi (nitrogen), ion spray voltage 5500 volts, source flow rate 300 µL/min without split, entrance potential 10 V, declustering potential 40 V and collision energy 16 V and for both RF and RFD6; the collision cell exit potential was 16V for RF and 8Vfor RFD6.

Chemicals and Reagents

RF was obtained from Sigma Aldrich (Bangalore, India). RFD6 was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). All other chemicals were from SD Fine Chemicals (Mumbai, India). Blank plasma was from Navjeevan blood bank Hyderabad, India. Millipore (Milli-Q-System) water was used for experimental work.

Chromatographic conditions

Zorbx SB C₁₈, 4.6 x 75 mm, 3.5 µm was selected as the analytical column. The mobile phase composition was 10 mM ammonium formate (pH 4.0) - acetonitrile (20:80 v/v). The flow rate of the mobile phase was set at 0.3 mL/min. The column temperature was maintained at 30°C. RFD6 was used as the internal standard. RF and RFD6 were eluted within 3.3 ± 0.2 min allowing for a total runtime of 5 min.
Preparation of standard stock solutions, Analytical standards and quality control (QC) samples

Standard stock solutions of RF (100 µg/mL) and RFD6 (100 µg/mL) were prepared in methanol. The internal standard (IS) spiking solutions (20 ng/mL) were prepared in 50% acetonitrile from IS standard stock solution. Standard stock solution of RF was added to drug-free human plasma to obtain RF concentration levels of 20, 40, 200, 1000,12000, 16000, and 20000 pg/mL for analytical standards and 20, 60, 6000, and 14000 pg/mL for quality control standards. All the analytical standards were stored at -30°C until analysis. Standard stock solutions and IS spiking solutions were stored in a refrigerator (2 - 8°C). All the glassware was rinsed with 0.1% formic acid, water and methanol before dilutions. All dilutions were processed in an ice/water bath and in the absence of white light. The aqueous standards were prepared in the mobile phase and stored in a refrigerator (2 - 8°C) until analysis.

Sample preparation

Fifty µL of RFD6 (20 ng/mL) and 400 µL of plasma sample were transferred into polypropylene tubes and vortexed briefly. One hundred µL of orthophosphoric acid solution were added into each tube and vortexed, followed by 3.0 mL of the extraction solvent methyl t-butyl ether - dichloromethane (75:25) and vortexing for 20 min. The samples were centrifuged at 4000 rpm for 10 min at 20 °C. The supernatant from each sample was transferred into labeled polypropylene tubes and evaporated under nitrogen gas at 40 °C. The dried sample from each tube was reconstituted with 200 µl of mobile phase with vortexing. Finally, each sample was transferred into auto sampler vials and injected into the liquid chromatographic system for analysis.

Analysis of samples from patients

The bioanalytical method described heretofore was used to determine RF concentrations in plasma following oral administration of the drug to healthy human volunteers. These volunteers were contracted in APL Research Centre, India, and 200 mg dose (one 200mg tablet) was administered to 17 healthy volunteers. The reference product Xifaxan tablets (Salix Pharmaceuticals, Morrisville, USA) 200 mg and test product Rifaximin tablet 200 mg were used. The test and reference products were administered to the same human volunteers under fasting conditions separately with proper washing periods as per the approved protocol. The study protocol was approved by the Indian Ethical Committee (IEC) as per Indian Council of Medical Research (ICMR) guidelines (protocol number RIFA-IMTB-05EB05-2FA [VERSION00]). Blood samples were collected at pre-dose (time 0), 5 min prior to dosing, followed by further samples at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 9, 12, 16, 20, and 24.0 h. After dosing, 7 mL blood was collected each time in vacutainers containing K₂EDTA. A total of 36 samples (for test and reference) were collected and subjected to centrifugation at 3200 rpm, 10 °C for 10 min, and kept frozen at -30 °C until sample analysis.

RESULTS AND DISCUSSION

Method development and validation

The goal of the present work was to develop and validate a simple, sensitive and rapid assay method for the quantitative determination of RF from plasma samples. A simple extraction technique was utilized in the extraction of RF and RFD6 from the plasma samples. Chromatographic conditions were optimized so as to achieve the best separation and enhance the signal of RF and RFD6. The MS optimization was performed by direct infusion of solutions of both RF and RFD6 into the ESI source of the mass spectrometer. The critical parameters in the ESI source included the needle (ESI) voltage and the capillary voltage. Other parameters, such as the nebulizer and the heater gases were optimized to obtain a better spray shape, resulting in better ionization and droplet drying to form the protonated ionic RF and RFD6 (IS) molecules. A CAD product ion spectrum for RF and RFD6 yielded high-abundance fragment ions of m/z 754.4 (Figure 2) and m/z 760.5 (Figure 3), respectively. After the MRM
channels were tuned, the mobile phase was changed from more aqueous phase to organic phase with acid dopant to obtain a fast and selective LC method. A good separation and elution were achieved using 10 mM ammonium formate (pH 4.0) - acetonitrile (20:80v/v) as the mobile phase, at a flow-rate of 0.3 mL/min and injection volume of 5 µL. The analytical curves were constructed using values ranging from 20.00 to 20000.00 pg/mL of RF in human plasma. Calibration curves were obtained by weighted 1/conc\(^2\) linear regression analysis.

**Selectivity and specificity**

The analysis of RF and RFD6 using MRM function was highly selective with no interfering compounds. Chromatograms obtained from plasma spiked with RF (20.00 pg/mL) and RFD6 (20.00 ng/mL) are shown in Figure 4.

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**Figure 2:** Mass spectrum of the Rifaximin.

**Figure 3:** Mass spectrum of Rifaximin D6

**Figure 4:** MRM chromatogram of Rifaximin and Rifaximin D6 in human plasma spiked with 20 pg/mL Rifaximin and 20.0 ng/mL Rifaximin - D6
Table 1: Precision and accuracy (analysis with spiked plasma samples at three different concentrations)

<table>
<thead>
<tr>
<th>Spiked plasma concentration (pg/mL)</th>
<th>Within-run</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration measured (mean ± S.D.)</td>
<td>R.S.D.(^{a}) (%)</td>
</tr>
<tr>
<td>60.00</td>
<td>62.5±1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>6000.00</td>
<td>6137.4±69.4</td>
<td>1.1</td>
</tr>
<tr>
<td>14000.00</td>
<td>13404.8±82.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\(^{a}\) (Standard deviation/mean concentration measured) x 100.

Table 2: Stability of the samples

<table>
<thead>
<tr>
<th>Spiked plasma concentration (pg/mL)</th>
<th>Room temperature stability 24.5 h</th>
<th>Processed sample stability 59.0 h</th>
<th>Long-term stability 55 days</th>
<th>Freeze and thaw stability Cycle 3 (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration measured (mean±S.D.)</td>
<td>Concentration measured (mean±S.D.)</td>
<td>Concentration measured (mean±S.D.)</td>
<td>Concentration measured (mean±S.D.)</td>
</tr>
<tr>
<td>60.00</td>
<td>61.08±2.67</td>
<td>61.68±3.00</td>
<td>59.33±2.71</td>
<td>59.63±2.54</td>
</tr>
<tr>
<td>14000.00</td>
<td>13500±547.72</td>
<td>13500±493.96</td>
<td>13483.33±594.70</td>
<td>13466.67±527.89</td>
</tr>
</tbody>
</table>

Linearity, precision and accuracy

Calibration curves were plotted as the peak area ratio (RF/RFD6) versus (RF/RFD6) concentration. Calibration was found to be linear over the concentration range of 20.00 - 20000.00 pg/mL. The R.S.D values were less than 2.4% and the accuracy ranged from 98.90 to 100.90%. The determination coefficients (\(r^2\)) were greater than 0.9995 for the curves obtained. Precision and accuracy for this method was controlled by calculating the intra and inter-batch variations at three concentrations (60, 6000, and 14000 pg/mL) of QC samples in six replicates. The precision is expressed as R.S.D as shown in Table 1. This method demonstrated intra and inter-day precision within 0.6 to 2.6% and 2.2 to 5.6% respectively, and the accuracy was within 95.7 - 104.2% and 95.8 - 105.0% for RF (Table 1). These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Stability of samples

Quantification of the RF in plasma subjected to three freeze-thaw cycles was carried out and demonstrated the stability of the analyte. No significant degradation of the RF was observed after 59 h storage period in the autosampler tray (Table 2). In addition, the long-term stability of RF in QC samples after 55 days of storage at -30°C was also evaluated. The concentrations ranged from 93.0 to 104.0%. These results confirmed the stability of RF in human plasma for at least 55 days at -30°C as recorded in Table 2.

Recovery

The recovery following the sample preparation using liquid-liquid extraction method was calculated by comparing the peak area ratios of RF in plasma samples with the peak area ratios
of standard samples. The recovery of RF determined at three different concentrations 60, 600 and 14000 pg/mL were found to be 96.72, 91.13 and 78.52% respectively. The overall average recovery of RF and RFD6 was found to be 88.79% and 90.94%, respectively.

**Application to biological samples**

The above-validated method was used in the determination of RF in plasma samples for establishing the bioequivalence of a single 200 mg dose in 17 healthy volunteers. Typical plasma concentration-versus-time profiles are shown in Figure 5.

All the plasma concentrations of RF were in the reference curve region and remained above the 20.0 pg/mL LOQ for entire sampling period. The observed maximum plasma concentration ($C_{\text{max}}$) for reference and test were 940.88 ± 323.60 and 1277.594 ± 453.42 pg/mL, respectively. The corresponding time of maximum concentration ($T_{\text{max}}$) for reference and test were found to be 1.25 and 1.25 h, respectively. The value of area under the curve from time 0 to the last sampling time ($\text{AUC}_{0-t}$) for the reference and test were found to be 6998.43 ± 305.93 and 8652.74 ± 346.74 pg h/mL, respectively. Moreover, the area under the curve from 0 to $\infty$ ($\text{AUC}_{0-\infty}$) was 8250.37 pg h/mL for the reference and 9959.52 pg h/mL for the test sample. The elimination half-life ($t_{1/2}$) was 8.79 h for the reference drug and 8.90 h for generic drug. In addition, the mean ratio of $\text{AUC}_{0-t}/\text{AUC}_{0-\infty}$ was higher than 90% following the FDA Guidelines. The ratio T/R and 90% confidence intervals were 135.78% for $C_{\text{max}}$, 123.64% for $\text{AUC}_{0-t}$, and 120.72% for $\text{AUC}_{0-\infty}$.

Therefore, it can be concluded that the two rifaximin formulations (reference and test) analyzed were not bioequivalent in terms of rate and extent of absorption (Figure 5).

**Figure 5: Mean plasma concentrations of test and reference samples after a 200 mg single oral dose of RF.**

**CONCLUSION**

This paper reports the use of LC–MS/MS for the accurate, precise, and reliable measurement of RF concentrations in human plasma after oral administration of 200 mg to healthy volunteers. The method is fast, robust, sensitive and selective. Each sample requires less than 5 min of analysis time. The assay method is also highly specific due to the inherent selectivity of tandem mass spectrometry and offers significant advantages over other techniques previously described for measuring rifaximin in biological fluids. The validated method was successfully used for pharmacokinetic and bioequivalence studies following oral administration of RF in healthy human volunteers.

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REFERENCES


