Simultaneous Quantitation of Rifampicin and Piperine in Rat Plasma by a Selective and Sensitive Liquid Chromatography-Tandem Mass Spectrometric Method and Its Pharmacokinetic Application

Suresh PS, Rajnish Kumar, Anitha Police, Mohd Zainuddin and Ramesh Mullangi

Drug Metabolism and Pharmacokinetics, Jubilant Biosys Ltd, Bangalore, India

*Corresponding author: mullangi_ramesh@jubilantinnovation.com (R.M)

Abstract

A highly sensitive, specific and rapid assay has been developed and validated for the simultaneous estimation of rifampicin (RIF) and piperine (PIP) in rat plasma with liquid chromatography coupled to tandem mass spectrometry with an electrospray ionization interface in the positive-ion mode. The assay procedure involves liquid-liquid extraction of RIF, PIP and internal standard (IS) from 50 µL rat plasma using acetonitrile:methanol (1:1, v/v). RIF and PIP were separated using 10 mM ammonium acetate (pH-4.2) and acetonitrile (10:90, v/v) at a flow rate of 0.5 mL/min on an Atlantis dC18 column. The total run time was 2.5 min and the elution of RIF, PIP and IS occurred at 1.17, 1.79 and 1.51 min, respectively. The MS/MS ion transitions monitored were m/z 823 → 791 for 286 → 115 and 180 → 110 for RIF, PIP and IS, respectively. The calibration curves were linear (r²>0.998) over the concentration range of 1.09-1091 and 1.05-1050 ng/mL for RIF and PIP, respectively. The inter- and intra-day precisions were in the range of 9.76-11.1 and 4.05-14.2% for RIF; 6.99-12.1 and 3.19-13.6% for PIP, respectively. Both the compounds were found to be stable in battery of stability studies. This novel validated assay was applied to derive the pharmacokinetic parameters for RIF and PIP post-dosing of RIF and PIP orally to Sprague-Dawley rats.

Keywords: Rifampicin; Piperine; Rat plasma; Method validation; LC-MS/MS; Pharmacokinetics

Introduction

Rifampicin (Figure 1) is a macrolyclic antibiotic, which kills Mycobacterium tuberculosis [1]. RIF will inhibit the synthesis of bacterial DNA-dependent RNA synthesis by blocking RNA transcription [2]. RIF is widely used in combination with pyrazinamide, isoniazid, ethambutol and streptomycin for the treatment of tuberculosis. After an oral administration, it is rapidly absorbed from the gastrointestinal tract and attains peak plasma concentration (6-8 µg/mL) between 2-4 h. The elimination half-life is ~3-4 h around 70% of the administered drug is excreted through feces.

Piperine (Figure 1) is an alkaloid obtained from Piper nigrum. Piperine is an inhibitor of CYP450 enzymes thus it will reduce the metabolism of active compounds [3] and increase the concentration of active compounds in the body fluids. Piperine is reported as a bioenhancer for many of therapeutic drugs in the market [4-7]. Apart from CYP inhibition, it will inhibit human P-glycoprotein (P-gp), which is a transporter responsible for efflux mechanism, transport of xenobiotics and metabolites [8-10]. Several LC-MS/MS methods have been reported in the literature for the quantification of RIF individually [11-13] or along with other drugs [14-21] in biological samples. Similarly few LC-MS/MS methods were reported for quantification of PIP individually [22] or along with other drugs [23-25]. Risorine is the fixed-dose combination of RIF (200 mg) and PIP (10 mg) along with isoniazid (300 mg) for the treatment of tuberculosis.
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in humans being marketed in India by Cadila Pharmaceuticals. Risorine uses lower doses of RIF (200 mg instead of 450 mg) but maintains same efficacy due to presence of PIP. Addition of PIP inhibits auto induction of RIF and maintains same level of RIF throughout the therapy [26].

To the best of our knowledge there are no published methods for the simultaneous quantification of RIF and PIP by LC-MS/MS. The objective of this study was to develop and validate a novel LC-MS/MS method for simultaneous quantification of RIF and PIP in rat plasma. This validated method was applied to quantitate plasma levels of RIF and PIP in a rat pharmacokinetic study.

Experimental

Materials and reagents

Rifampicin and phenacetin (internal standard, IS) were obtained from Sigma-Aldrich (St. Louis MO). Piperine was isolated from black pepper seeds. Purity was found to be more than >99.8% for all the compounds. Structures of analytes and IS are shown in Figure 1. L-Ascorbic acid was purchased from Sigma-Aldrich (St. Louis MO). Acetonitrile (HPLC and MS grade), water (HPLC and MS grade) and methanol (HPLC and MS grade) were obtained from J.T. Baker (Phillipsburg NJ, USA). Acetic acid (MS grade) and ammonium acetate (MS grade) were obtained from Sigma-Aldrich (St. Louis MO). Control rat plasma was obtained from Animal House, Jubilant Biosys (Bangalore, India).

HPLC operating conditions

A Shimadzu VP (Shimadzu, Kyoto, Japan) SIL series LC system equipped with degasser (G1379A), quaternary pump (10ADvp), column oven (CTO-10A) along with auto-sampler (SIL-HTC) along with system controller (SCL-10Avp) was used to inject 2 µL aliquots of the processed samples on a Atlantis dC18 column (50 × 4.6 mm, 3 µm; Waters, Milford, MA, USA), which was kept at ambient temperature (30 ± 1°C). The isocratic mobile phase, a mixture of acetonitrile and 10 mM ammonium acetate (pH 4.2; adjusted by adding glacial acetic acid) mixture (90:10, v/v) was filtered through a 0.22 µm Durapore GVWP filter (Cat No: GVWP04700, Millipore, USA) and then degassed ultrasonically for 15 min was delivered at a flow rate of 0.5 mL/min into the mass spectrometer electrospray ionization chamber.

Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analytes and IS using an AB Sciex (Foster City, CA, USA) API 5500 mass spectrometer, equipped with a TurboionSpray™ interface at 400°C. The common parameters viz., curtain gas, GS1 gas and GS2 gas were set at 40, 55 and 55 L/min, respectively, whereas the CAD gas was set at 7.0 L/min. The compounds parameters viz., declustering potential (DP), collision energy (CE), enhance potential (EP) and collision exit potential (CXP) for RIF, PIP and IS were 40, 23, 10, 34 V; 70, 60, 10, 12 V and 40, 29, 10, 14 V respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z 823 precursor ion to the m/z 791 product ion for RIF, m/z 286 precursor ion to the m/z 115 product ion for PIP and m/z 180 precursor ion to the m/z 110 product ion for IS. Quadrupoles Q1 and Q3 were set on low resolution. The analytical data were processed by Analyst software (version 1.6.1).

Preparation of stock and standard solutions

Primary stock solutions of RIF and PIP for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions were prepared in methanol (200 µg/mL). The IS stock solution was prepared in methanol (200 µg/mL). The stock solutions RIF and PIP and IS were stored at 4°C, which were found to be stable for one month (data not shown) and successively diluted with methanol to prepare working solutions to prepare calibration curve (CC). Another set of working stock solutions of RIF and PIP were made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored approximately at 4°C for a week (data not shown). Appropriate dilutions of stock solution was made in methanol to produce working stock solutions of 10908, 5454, 2727, 1091, 545, 272, 109, 54.5 and 10.9 ng/mL for RIF and 10500, 5250, 2625, 1050, 525, 105, 52.5 and 10.5 ng/mL for PIP, respectively. Working stocks were used to prepare plasma calibration standards. A working IS solution (50 ng/mL) was prepared in methanol. Calibration samples were prepared by spiking 45 µL of pooled blank rat plasma with the appropriate working solution of the RIF and PIP (5 µL) and IS (10 µL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking pooled blank rat plasma in bulk with appropriate concentrations viz., 1.09, 3.27, 491 and 818 ng/mL for RIF and 1.05, 3.15, 473 and 788 ng/mL for PIP and 50 µL aliquots were distributed into different tubes. All the samples were stored at -80 ± 10°C till further analysis.

Sample preparation

To an aliquot of 50 µL plasma sample, IS solution (10 µL of 50 ng/mL) was added and mixed for 15 sec on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 0.5 mL of acetonitrile/methanol (1:1, v/v), the mixture was vortexed for 2 min; followed by centrifugation for 5 min at 14000 rpm on a Centrifuge 5430R (Eppendorff, Germany) at 5°C. The organic layer (0.4 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap® Zymark® Kopkinton, MA, USA). The residue was reconstituted in 200 µL of the mobile phase and 2 µL was injected onto LC-MS/MS system.

Validation procedures

The method was validated for selectivity, recovery, matrix effect, linearity, accuracy, precision, and stability according to the FDA guidelines for the validation of bioanalytical methods [27].

Specificity and selectivity: The selectivity of the method against endogenous interferences was verified by the analysis of blank
and spiked LLOQ samples prepared using six different sources of non-pooled, analyte-free plasma to determine the extent to which endogenous plasma components might contribute to the interference at the retention time of analytes and the IS. If interference exits, the signal should not exceed 20% of LLOQ peak area and 5% of IS peak area [27].

Recovery: The efficiency of RIF, PIP and IS extraction from rat plasma was determined by comparing the responses of the analytes extracted from replicate QC samples (n=6) with the response of analytes from neat standards at equivalent concentrations by simple liquid liquid extraction process. Recovery was determined at QC low, QC medium and QC high concentrations viz., 3.27, 491 and 818 ng/mL for RIF and 3.15, 473 and 788 ng/mL for PIP, where as the recovery of the IS was determined at a single concentration of 50 ng/mL.

Matrix effect: Post column infusion method [28] was used to evaluate matrix effect. Briefly, an infusion pump delivers a constant amount of analyte RIF, PIP or IS in to LC system outlet entering to mass spectrometer inlet. Mass spectrometer was operated in MRM mode to follow the analyte signal. Rat blank plasma extract was injected on LC column under same chromatographic condition. Since the analyte was infused at constant rate, a steady ion response was obtained as a function of time. Any endogenous compound that elutes from the column and causes a variation in ESI (electro spray ionization) response of the infused analyte was seen as a suppression or enhancement in the response of the infused analyte.

Calibration curve: Calibration curves were acquired by plotting the peak area ratio of RIF/PIP:IS against the nominal concentration of calibration standards. The concentrations used for each analytes were 1.09, 5.45, 10.9, 54.5, 109, 273, 545 and 1091 ng/mL for RIF and 1.05, 5.25, 10.5, 52.5, 105, 263, 525 and 1050 ng/mL for PIP. The results were fitted to linear regression analysis with the use of 1/x^2 weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better for both analytes. The acceptance criteria for each back-calculated standard concentration were 15% deviation from the nominal value except at LLOQ, which was set at 20% [27].

Precision and accuracy: The intra-assay precision and accuracy were estimated by analyzing six replicates containing four different QC levels i.e., 1.09, 3.27, 491 and 818 ng/mL for RIF and 1.05, 3.15, 473 and 788 ng/mL for PIP. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within 85-115% from the nominal values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, where it should be within 80-120% for accuracy and less than 20% of RSD [27].

Stability experiments: The stability of RIF, PIP and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24 h (in the auto sampler at 4°C) after the initial injection. The peak areas of the analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. Stability of RIF and PIP in the biomatrix during 6 h (benchtop) was determined at ambient temperature (25 ± 1°C) at two concentrations viz., 3.27 and 818 ng/mL for RIF and 3.15 and 788 ng/mL for PIP in quadruplicates. Freezer stability of RIF and PIP in rat plasma was assessed by analyzing the LQC and HQC samples stored at -80 ± 10°C for at least 30 days. The stability of RIF and PIP in rat plasma following three freeze/thaw cycles was assessed using QC samples spiked with RIF and PIP. The samples were stored at -80 ± 10°C between freeze/thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 1 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., 85-115% of nominal value) and precision (i.e. 15% RSD) as compared to freshly spiked (0 h) samples [27].

Incurred samples reanalysis (ISR): The recent EMA and FDA guidelines have emphasized on the necessity of ensuring incurred sample reproducibility [27,29]. EMA 2011 guidelines on bioanalytical method validation provided the rational and procedure for conduct of incurred sample reanalysis (ISR). As per the guidance, 10% of the samples should be reanalysed in case the number of samples is <1000 [29]. Furthermore, it is advised to obtain samples around C_{max} and in the elimination phase. As per the guidance, the difference in concentrations between the initial value and the ISR should be less than ±20% of their means for at least 67% of the repeats. Large differences between results may indicate analytical issues and should be investigated.

Pharmacokinetic study
All the experiments were approved by Institutional Animal Ethical Committee. Male Sprague-Dawley rats (n=4, 225-240 g) were procured from Bioneeds, Bangalore, India. The animals were housed in Jubilant Biosys animal care facility in a temperature and humidity controlled room with a 12:12 h light:dark cycles, had free access to food (Provinim, Bangalore, India) and water for one week before using for experimental purpose. During (~12 h) fasting period animals had free access to water. Animals received RIF and PIP (10 mg/Kg) by oral route in the form of a solution (prepared using 0.5% DMSO, 50% polyethylene glycol 400 and 49.5% normal saline).
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Following dosing, blood samples (sparse sampling; ~100 µL) were collected from the retro-orbital plexus into microfuge tubes containing Na₂EDTA (as an anticoagulant) at 0.25, 0.5, 1, 2, 4, 8, 10 and 24 h post-dosing. Animals were provided with standard diet 2 h after dosing. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 g for 5 min and stored at -80 ± 10°C until bioanalysis. An aliquot of 50 µL of thawed plasma samples were spiked with IS and processed as mentioned Sample preparation section. Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run.

The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples accuracy greater than 85-115% of the nominal concentration (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration-time data of RIF and PIP was analyzed by non-compartmental method using Phoenix Version 1.3 (Pharsight Corporation, Mountain View, CA).

Results

Liquid chromatography

The chromatographic conditions, in particular the mobile phase composition and column selection were aimed at getting adequate response and good peak shape for analytes and IS along with good resolution. We have used several LC conditionson different stationary phase reversed phase columns viz., Chromolith RP18e column (100 × 4.6 mm; Merck), Atlantis dC18column (50 × 4.6 mm, 3 µm; Waters, Milford, MA, USA), Xterra Phenyl (3.9 ×150 mm, 5 µm; Waters, Milford, MA, USA) and Phenomenex Gemini (Phenomenex, 2 × 50 mm, 5 µm). Among the selected columns the required resolution and symmetric peak shapes were achieved on Atlantis dC18 column. In order to achieve shorter run time, the mobile phase composition was optimized to acetonitrile: 10 mM ammonium acetate pH 4.2 (90:10, v/v) at a flow rate of 0.5 mL/min to elute the analytes and IS within 2.5 min. The peak shape was also improved by the addition of 10 mM ammonium acetate at pH 4.9 with glacial acetic acid. The retention times of RIF, PIP and IS were approximately 1.17, 1.79 and 1.51 min, respectively.

Mass spectrometry

In order to optimize ESI conditions for RIF, PIP and IS, quadrupole full scans were carried out in both negative and positive ion detection modes, however signal intensity and stability were much better in positive mode, therefore ESI-positive mode was used for the quantification. During a direct infusion experiment, the mass spectra for RIF, PIP and IS revealed peaks at m/z 823, 286 and 180, respectively as protonated molecular ions, in Q1 MS full mass spectra. The product ion mass spectrum for RIF, PIP shows the formation of characteristic product ions at m/z 791, 115 and 110. Following detailed optimization of mass spectrometry conditions (provided in Section 2.3) MRM reaction pair m/z 823 precursor ion to the m/z 791 was used for quantification of RIF and m/z 286 precursor ion to the m/z 115 was used for quantification of PIP, respectively. For IS m/z 180 precursor ion to the m/z 110 was used for quantification purpose.

Matrix effect

Post column infusion method, defined by Bonfiglio et al., (1999) [28] was used to evaluate matrix effect. Figure 2 represents matrix effect chromatograms overlaid by a control matrix standard chromatogram to indicate the elution profile for RIF, PIP and IS over the analyte and IS infusion matrix effect baseline. No significant signal suppression was observed for RIF, PIP and IS, respectively.

Specificity and selectivity

Figures 3 and 4 shows a typical overlaid chromatogram for the (a) rat blank plasma (free of analytes and IS), (b) rat blank plasma spiked with IS (c) rat blank plasma spiked with RIF and
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PIP at LLOQ and IS (d) an in vivo plasma sample showing RIF and PIP [obtained at 1.0 h after oral administration of RIF and PIP] and IS. No interfering peaks from endogenous compounds are observed at the retention times of analyte and IS in rat blank plasma. The retention time of RIF, PIP and IS were 1.17, 1.79 and 1.79 min, respectively. The total chromatographic run time was 2.5 min.

Recovery

A simple liquid-liquid extraction with acetonitrile/methanol (1:1, v/v) proved to be robust and provided cleanest samples. The results of the comparison of neat standards versus plasma-extracted standards were estimated for each analyte at 3.27, 491 and 818 ng/mL for RIF and 3.15, 473 and 788 ng/mL for PIP, and the absolute mean recoveries were 90.2 ± 11.7, 95.4 ± 4.43 and 91.7 ± 8.53%; 63.1 ± 10.0, 58.0 ± 1.98 and 60.8 ± 3.67% for RIF and PIP, respectively. The absolute recovery of IS at 50 ng/mL was 85.1 ± 6.28%.

Calibration curve

The plasma calibration curve was constructed using eight calibration standards (viz., 1.09-1091 ng/mL for RIF and 1.05-1050 ng/mL for PIP) for each analyte. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighting factor ($1/X^2$). The average regression (n=4) was found to be ≥ 0.998. The lowest concentration with the RSD <20% was taken as LLOQ and was found to be 1.09 ng/mL for RIF and 1.05 ng/mL for PIP. The % accuracy observed for the mean of back-calculated concentrations for four calibration curves for RIF and PIP was within 88.7-106 and 94.1-114, respectively; while the precision(% CV) values ranged from 0.70-11.5 and 0.09-11.9, respectively.

Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 1. The assay values on both the occasions (intra- and inter-day) and in both the matrices were found to be within the accepted variable limits.

Stability

The predicted concentrations were 3.27 and 818 ng/mL for RIF and 3.15 and 788 ng/mL for PIP. Samples deviated within ±15% of the nominal concentrations in a battery of stability tests viz.,

![Figure 3: Typical MRM chromatograms of RIF (left panel) and IS (right panel) in (a) rat blank plasma (b) rat blank plasma spiked with IS (c) rat blank plasma spiked with RIF at LLOQ (1.09 ng/mL) and IS (d) a 1.0 h in vivo plasma sample (18068 ng/mL) showing RIF peak obtained following oral dosing to rat along with IS.](image)

![Figure 4: Typical MRM chromatograms of PIP (left panel) and IS (right panel) in (a) rat blank plasma (b) rat blank plasma spiked with IS (c) rat blank plasma spiked with PIP at LLOQ (1.05 ng/mL) and IS (d) a 1.0 h in vivo plasma sample (109 ng/mL) showing PIP peak obtained following oral dosing to rat along with IS.](image)
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Table 1: Intra and inter-day precision of determination of RIF/PIP in rat plasma.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Run</th>
<th>Measured concentration (ng/mL)</th>
<th>Mean</th>
<th>S.D</th>
<th>R.S.D</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intraday variation (Six replicates at each concentration)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1</td>
<td>1.09/1.05</td>
<td>1.03/1.13</td>
<td>0.11/0.14</td>
<td>11.2/12.0</td>
<td>97.5/108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.09/2.10</td>
<td>0.97/1.09</td>
<td>0.08/0.15</td>
<td>7.76/13.5</td>
<td>89.3/98.5</td>
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<td></td>
<td></td>
<td>3.08/3.10</td>
<td>1.10/1.08</td>
<td>0.14/0.15</td>
<td>12.5/13.6</td>
<td>101/103</td>
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<tr>
<td></td>
<td></td>
<td>4.09/4.10</td>
<td>0.98/1.06</td>
<td>0.01/0.09</td>
<td>1.16/8.31</td>
<td>89.5/101</td>
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<td></td>
<td>2</td>
<td>1.09/1.05</td>
<td>3.30/3.12</td>
<td>0.21/0.19</td>
<td>6.25/6.16</td>
<td>101/99.0</td>
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<td>2.09/2.10</td>
<td>2.97/2.89</td>
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<td>2.92/2.98</td>
<td>0.31/0.19</td>
<td>10.4/6.34</td>
<td>96.6/94.5</td>
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<td>3.08/6.12</td>
<td>93.7/95.3</td>
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<td>3</td>
<td>1.09/1.05</td>
<td>510/505</td>
<td>64.7/23.5</td>
<td>12.7/4.65</td>
<td>104/107</td>
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<td>99.0/97.4</td>
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<td>97.8/99.5</td>
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<td>761/761</td>
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<td>93.0/96.6</td>
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<td></td>
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<td>4.09/4.10</td>
<td>887/809</td>
<td>65.8/31.9</td>
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<td>108/103</td>
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<td>Inter day variation (Twenty four replicates at each concentration)</td>
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<td>1.02/1.10</td>
<td>0.11/0.13</td>
<td>10.7/12.1</td>
<td>90.2/103</td>
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<td>3.08/3.00</td>
<td>0.30/0.21</td>
<td>9.83/6.99</td>
<td>96.0/95.2</td>
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<td></td>
<td>3</td>
<td>491/473</td>
<td>499/479</td>
<td>55.1/35.6</td>
<td>11.1/7.43</td>
<td>100/101</td>
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<td>4</td>
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<td>849/799</td>
<td>82.8/61.8</td>
<td>9.76/7.74</td>
<td>104/101</td>
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</tbody>
</table>

R.S.D: Relative standard deviation (S.D × 100/Mean)

Table 2: Summary of stability data of RIF/PIP in rat plasma.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Stability</th>
<th>Measured concentration (ng/mL)</th>
<th>Mean</th>
<th>S.D</th>
<th>R.S.D</th>
<th>Accuracy (%)</th>
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<tr>
<td></td>
<td>Six replicates at each concentration</td>
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<tr>
<td></td>
<td>0 h</td>
<td>3.27/3.15</td>
<td>3.30/3.12</td>
<td>0.21/0.19</td>
<td>6.25/6.16</td>
<td>101/99.0</td>
</tr>
<tr>
<td></td>
<td>6 h (bench-top)</td>
<td>3.58/3.17</td>
<td>0.42/0.11</td>
<td>11.7/3.48</td>
<td>109/102</td>
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<tr>
<td></td>
<td>30 h (in-injector)</td>
<td>3.52/3.11</td>
<td>0.36/0.25</td>
<td>10.3/7.99</td>
<td>107/99.7</td>
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<td></td>
<td>3rd freeze-thaw</td>
<td>2.99/3.04</td>
<td>0.07/0.32</td>
<td>2.35/10.5</td>
<td>90.6/97.3</td>
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<td></td>
<td>30 day at -80°C</td>
<td>2.97/2.92</td>
<td>0.11/0.32</td>
<td>3.60/11.0</td>
<td>90.0/93.7</td>
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<tr>
<td></td>
<td>0 h</td>
<td>818/788</td>
<td>866/842</td>
<td>43.1/47.6</td>
<td>4.98/5.66</td>
<td>106/107</td>
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<td></td>
<td>6 h (bench-top)</td>
<td>859/820</td>
<td>88.5/49.4</td>
<td>10.3/6.02</td>
<td>99.2/97.4</td>
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<td>30 h (in-injector)</td>
<td>843/787</td>
<td>85.6/42.2</td>
<td>10.2/5.37</td>
<td>97.4/93.5</td>
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<td>3rd freeze-thaw</td>
<td>818/755</td>
<td>86.7/34.1</td>
<td>10.6/4.52</td>
<td>94.5/89.7</td>
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<tr>
<td></td>
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<td>832/751</td>
<td>85.4/49.6</td>
<td>10.3/6.60</td>
<td>96.1/89.3</td>
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</tr>
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</table>

R.S.D: Relative standard deviation (S.D × 100/Mean)
Simultaneous Quantitation of Rifampicin and Piperine in Rat Plasma by a Selective and Sensitive Liquid Chromatography-Tandem Mass Spectrometric Method and Its Pharmacokinetic Application

in-injector (30 h), bench-top (6 h), repeated three freeze/thaw cycles and at -80 ± 10°C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

**Dilution effect**

The dilution integrity was confirmed for QC samples that exceeded the upper limit of standard calibration curve. The results have shown that the precision and accuracy for two sets of six replicates of diluted samples were within acceptance range (data not shown).

**Incurred samples reanalysis**

All the 16 samples selected for ISR met the acceptance criteria. The back calculated accuracy values ranged between 82.0 to 106% from the initial assay results.

**Application of the method**

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmakokinetics of RIF and PIP in rat following oral dosing. RIF and PIP were quantifiable till 24 h following oral dosing. Plasma concentration versus time profiles for oral route was shown in Figure 5. Following oral administration of RIF maximum concentration ($C_{\text{max}}$) in plasma (1907 ± 188 ng/mL) was achieved at 4.00 ± 0.00h ($T_{\text{max}}$). The AUC$_{0-\infty}$ (area under the plasma concentration–time curve from time zero to last measurable concentration time point) was found to be 19821 ± 6156 ng*h/mL. The terminal half-life was found to be 2.89± 0.0h. Following oral administration of PIP $C_{\text{max}}$ (3281 ± 344 ng/mL) attained at 0.44 ± 0.13 h ($T_{\text{max}}$). The AUC$_{0-\infty}$ was 6101 ± 1168 ng*h/mL and terminal half-life was found to be 4.10 ± 1.56 h.

**Conclusion**

In summary, we have developed and validated a highly sensitive, specific and reproducible LC-MS/MS assay to quantify RIF and PIP simultaneously in rat plasma as per the regulatory guidelines. The method showed suitability for pharmacokinetic studies in rat. The simplicity and ruggedness of the assay and sample turnover rate of 2.5 min per sample make it an attractive procedure for routine bioanalysis of RIF and PIP. We believe that the current method with little or no modifications can be extended to analyze RIF and PIP in patient’s samples who are on treatment for tuberculosis with Risorine.

**References**


Figure 5: Plasma concentration vs time profiles of RIF and PIP (10 mg/Kg) after single dose oral administration to male Sprague-Dawley rats.
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