ABSTRACT

Objective: To develop and validate simple, sensitive, accurate and selective UPLC-MS/MS method for quantification of paromomycin (PARO) in mice plasma.

Methods: Precipitation method was used for the extraction of plasma samples, an aliquot of 25 µl plasma samples was extracted using 10% perchloric acid in water. Chromatographic separation was performed using waters acquity ultra-performance liquid chromatography (UPLC) columns, BEH HILIC (50 mm × 2.1 mm, 1.7 µm) by a gradient mixture of acetonitrile and water (both containing 0.005% v/v trifluoro acetic acid) as a mobile phase at the flow rate of 0.2 ml/min. The analyte was protonated in the positive electrospray ionization (ESI) interface and detected in multiple reactions monitoring (MRM) modes using the transition m/z 308.60-455.30.

Results: The method had a short chromatographic run time of 3 min. Calibration curves were linear over wide ranges of 50.51-5019.22 ng/ml. The between and within-batch precision and accuracy of the method was determined by using 4 quality control samples, the highest % CV observed was 11.06. The mean recovery values are 78.17, 101.17 and 92.58 at low, medium and high-quality control levels; respectively.

Conclusion: It was concluded that the developed and validated UPLC-MS/MS method was rapid, sensitive, accurate, precise, linear, and specific. Therefore, this method can be used for quantification of PARO in mice plasma with various advantages over the reported methods.

Keywords: Paromomycin, Quantification, Pharmacokinetics, UPLC-MS/MS

INTRODUCTION

Paromomycin (PARO), a broad spectrum aminoglycoside antibiotic first isolated in 1956 from the fermentation of streptomyces, shows activity against both gram-positive and gram-negative bacteria as well as some protozoa and cestodes [1-2]. PARO has been approved since 2007 in India as an effective, well tolerated and affordable treatment for visceral leishmaniasis (VL). Visceral Leishmaniasis (VL) is a tropical disease caused by protozoan parasites of the genus leishmania and it is transmitted by the bite of certain species of the sand fly. Also called Kala Azar, the disease is endemic in parts of north-eastern India, sub-Saharan Africa, parts of the Mediterranean, and South America [3-6]. The disease has a worldwide distribution in Asia, East Africa, South America and the Mediterranean regions. It kills 200 000 to 300 000 people a year in the Indian subcontinent alone and is also greatly debilitating to those who survive the infection [7]. Recently it is being developed for use against multi-drug resistant strains of tuberculosis and for the treatment of intestinal cryptosporidiosis in AIDS patients [8-10].

To date, there have been many preliminary studies and published data on the various quantitative analysis of PARO, which include capillary zone electrophoresis, capillary electrophoresis, reverse-phase ion-pairing HPLC coupled with pulsed amperometric detection, LC-MS, the use of silver-coated gold nanoparticles as chromophores, and therefore, the derivatization of the amino groups is required for quantification using HPLC analyzes equipped with UV detector. This method is time-consuming tedious and needs large sample volume for quantification; so harvesting a large quantity of plasma from small animals viz mice is challenging. An HPLC method with fluorescence detection was also developed using derivatization technique (post-column) with ortho-phthalaldehyde (OPA) and 2-mercaptoethanol, this is an indirect method (unlike UPLC-MS/MS), time-consuming, required long run time and more efforts without improving sensitivity. Pre-column labelling with OPA is not used because of the low stability of the derivative. Because of the thermal instability of aminoglycosides, the analysis of aminoglycosides by gas chromatography equipped with tandem mass spectrometry (GC/MS) is difficult [12-14].

Thus considering the limitations of the various existing methods described, the present study was performed with the aim and objective to develop and validate a simple, sensitive, accurate and rapid UPLC-MS/MS method for the determination of PARO in mice plasma. Till recently there were no reports or validated methods available predominantly with high sensitivity and short run time for the determination of PARO in mice plasma samples.

MATERIALS AND METHODS

Chemicals and reagents

Paromomycin sulfate, antipyrine and KEDTA were obtained from Sigma-Aldrich (Bangalore, India). Acetonitrile, trifluoroacetic acid and perchloric acid, were obtained from Merck (Mumbai, India). All other chemicals used in this study were of analytical grade.

Animal husbandry and handling

All animal studies had the approval of the Institutional Animal Ethics Committee as per approval number FB-15-067-M-117 of SAI Life Sciences Ltd. (an AAALAC accredited facility) and were in accordance with the guidelines of the CPCSEA, Government of India. Animals were acclimatized in study rooms for at least three days prior to dosing. Mice were housed in polypropylene cages (3 animals
per cage, marked for identification) maintained in controlled environmental conditions (22±3 °C; 40-70% RH; 10-15 fresh air change cycles/h) with 12 h light and dark cycles. Animals were obtained from the In vivo Biosciences, Bangalore, India. Mice were given rodent pellet diet and UV treated filtered water ad libitum. Mice blood samples were collected from retro-orbital plexus using a sparse sampling design.

**Instrumentation**

**Stock solution, calibration standards and quality control samples**

A standard stock solution of PARO and antipyrine were prepared with a final concentration of 6.20 mg/ml and 2.40 mg/ml, respectively. These solutions were stored at 2-8 °C until use. The analytical standard for PARO in acetonitrile over a concentration range of 50.51 ng/ml to 5019.22 ng/ml by serial dilution method and same concentration range for calibration curve were also prepared in blank mice plasma. Quality control (QC) samples at four different concentration levels (50.51, 252.22, 752.88 and 1003.85 ng/ml) for PARO as LLOQ QC, low (LCQ), medium (MQC) and high (HQC), respectively were prepared in three sets independent of the calibration standards. During analysis, these QC samples were spaced after every six to seven unknown samples.

**Sample preparation**

An aliquot of 25 µl study sample or spiked calibration standard/QCs was added to individual pre-labeled microcentrifuge tubes followed by 25 µl of IS prepared in water (antipyrine, 500 ng/ml) was added except for blank mice plasma, where 25 µl of water was added. Samples were vortexed, followed by 100 µl of 10% perchloric acid in water was added and samples were vortexed for another 5 min. After centrifugation of the samples at 4000 rpm for 10 min at 4 °C, the supernatant was collected and 5 µl was injected onto the UPLC–MS/MS system.

**Chromatographic condition**

A Waters Acquity™ UPLC (USA) consisting of flow control valve, vacuum degasser operated in a gradient mode to deliver the mobile phase at a flow rate of 0.2 ml/min. The chromatographic system consisted of BEH HILIC column (50 mm x 2.1 mm, 1.7 µm) (Waters, USA) and mobile phase consists of methanol (A), 2 mmol ammonium acetate buffer (B), (pH ~3.2 adjusted with 0.1% formic acid). A gradient UPLC method with 3 min run time was employed for analysis. The mobile phase comprised 0.005% v/v trifluoroacetic acid in water and acetonitrile, the flow rate was 0.2 ml/min. Separation was achieved using BEH HILIC column (1.7 µm, 2.1 mm x 50 mm, 3/µg). Waters (USA), maintained at 45 °C employing an injection volume of 5 µl for in vivo samples.

**Mass spectrometric condition**

Mass spectrometric detection was performed on AB SCIEX triple quadrupole LC/MS/MS mass spectrometer equipped with electron spray ionization (ESI) source, the multiple reaction monitoring (MRM) modes was used for data acquisition with analyt 1.6.2 software. Peak integration and calibration were carried out by using Analyst 1.6.2 software. MS and MS/MS condition for pure standards of PARO and IS antipyrine were optimized by continuous infusion at 5 µl/min using syringe pump.

The transitions monitored were m/z 308.6 (m/z)>455.3 and 189.4>104.0 for components PARO and IS antipyrine, respectively. All analyzes were carried out in positive ion ESI with spray voltage set at 5500 V. The heated ESI temperature was set 550 °C. Nitrogen Ion Source Gas GSI and GS2 set at 30 and 60 psi, respectively. The collision assisted dissociation (CAD) was used at pressure 6 psi. Total run time for UPLC-MS/MS analysis was 3 min.

**Application to pharmacokinetic study**

The method was successfully applied to evaluate the plasma concentration versus time profile of PARO in mice plasma following intravenous administration at 15 mg/kg dose of PARO. The intravenous dose volume was 5 ml/kg and dosing was performed using sterile 26 gauge needle via the tail vein. The formulation was prepared freshly on the day of dosing. Studies were performed in healthy male BALB/c mice (30-40 g). A sparse sampling design was used (n=3 per time point).

Approximately, 60 µl of blood samples was collected (K$_2$EDTA anticoagulant, 20 µl of K$_2$EDTA solution/ml of blood, 200 mmol) at 0.25, 0.5, 1, 4, 8 and 24 h postdose. Plasma samples were separated by centrifugation of whole blood and stored below-70±10 °C until bioanalysis.

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**Fig. 1: Representative chromatograms of mice blank plasma using PARO and antipyrine LC/MS/MS method**
Pharmacokinetic parameters were calculated using non-compartmental analysis tool of WinNonlin® software (Version 6.3). The area under the concentration-time curve (AUC_{lin} and AUC_{inf}) was calculated by linear trapezoidal rule. The peak concentration (C_{max}) and time for the peak concentration (T_{max}) were observed values. The elimination rate constant value (k_{el}) was obtained by linear regression of the log-linear terminal phase of the concentration-time profile using at least 3 non-zero declining concentrations in terminal phase with a correlation coefficient of >0.8. The terminal half-life value (T_{1/2}) was calculated using the equation 0.693/k_{el}.

**RESULTS AND DISCUSSION**

**Mass spectrometry**

In order to find most, sensitive ionization mode for the components studied, ESI positive ion mode and ESI negative ion mode were tested with the various combination of mobile phase, i.e., methanol, acetonitrile and water/ammonium acetate buffer (2 mmol)/formic acid (0.1%) in positive and negative ionization mode. It was observed that the signal intensity for [M+H]^+ ions in ESI positive ion mode were 3-10-fold higher for PARO using acetonitrile: trifluoroacetic acid (0.005% v/v) with ESI negative ion mode. The protonated molecular ion of PARO and antipyrine (IS) were 308.6 were obtained for PARO. No significant solvent adduct ions or fragment ions were observed in the full scan spectra of PARO and antipyrine (IS). Thus, it was decided to utilize positive ion mode for detection and quantification of [M+H]^+ ions and representative chromatogram showed in the fig. 1 and 2.

**Liquid chromatography**

Acetonitrile rather than methanol was chosen as an organic modifier because of its better peak shape. A gradient UPLC method with a 3 min run time was employed for analysis. The mobile phase comprised of 0.005% v/v trifluoroacetic acid in water and acetonitrile and the flow rate was 0.2 ml/min. Separation was achieved using BEH HILIC column (1.7 µm, 2.1 mm x 50 mm, 3/pkg), Waters, USA maintained at 45 °C employing an injection volume of 5 µl for in vivo plasma samples.

**Optimization of UPLC–MS/MS condition**

Final MRM transitions were selected on the basis of the signal to noise ratio (S/N) ratio with on-column injection analysis. Curtain gas, collision gas induced dissociation, ion spray voltage, and temperature were set to 25.00 psi, 6 psi, 5500.00 v and 550.00 C, respectively. Positive-ion electron spray ionization mode was used and MRM transitions of 308.60/455.30 for PARO and 189.40/104.00 for antipyrine were monitored.

**Sample clean-up**

The next step was to develop the simple and efficient sample clean up devoid of matrix effect and interference from endogenous plasma components for estimation of PARO in mice plasma. Precipitation method was tried with acetonitrile; liquid–liquid extraction (LLE) using ether and different combinations of hexane and ethyl acetate (80–20%, v/v), n-hexane and isopropyl alcohol (2–5%, v/v) was also tried but none of these was found suitable to give good and consistent recovery for PARO. Finally, precipitation method was tried with 10% perchloric acid in water and found suitable to give optimum recovery for PARO and IS. Since PARO is highly polar in nature and not extracted in acetonitrile and methanol by protein precipitation method. Hence 10% perchloric acid in water was used. For determination of matrix effect, control drug-free plasma was extracted using the described method and drug was added in extracted supernatant. Matrix effect was determined by comparing the analytical response of these samples with that of standard solutions.

**Method validation**

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used as the parameter to assess the assay performance. LC–MS/MS analysis of the blank plasma samples showed no interference with the quantification of PARO and the IS (antipyrine). The specificity of the method was established with pooled and individual plasma samples from six different sources. The retention times of PARO and the IS showed less variability with a percent coefficient of variance (%CV) well within acceptable limits of 5%.

**Limit of detection (LOD) and quantification (LOQ)**

Two criteria were used to define LOQ, i.e., (1) the analytical response at LOQ must be five times the baseline noise and (2) the analytical response at LOQ can be detected with sufficient accuracy (80-120%) and precision (20%). LOD is defined as the lowest concentration of the analyte at which the signal is larger than three times the baseline noise. The measured LOD and LOQ values were 50.00 and 10.00 arbitrary units for PARO. The limit of quantification (LOQ) was set at 50.51 ng/ml. These results well met the requirements of quantifications of all analytes in plasma.

**Linearity**

The peak area ratios of analyte to IS in mice plasma were linear over the concentration range 50.51 to 5019.22 ng/ml for PARO. The
calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors (1/x, 1/x$^2$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a 1/x$^2$ weighting factor for all components. The correlation coefficients ($R^2$) for all components were above 0.9900 over the concentration range used.

**Precision and accuracy**

The intra-day precision (expressed by percent coefficient of variation i.e CV% of replicate analyses) was estimated on the four quality control levels and the within-batch precision on the ten calibration standard levels. Table 1 shows the results obtained for the within a batch and between batch precision for PARO. The precision for all these analytes under investigation was not exceeded 15% at any of the concentrations studied and well met the requirements of validation.

**Recovery**

The extraction recovery of PARO from mouse plasma was estimated at their respective low, medium and high QC levels. Plasma samples (in six replicates) containing PARO at QC concentration level was also spiked with respective internal standards. The results of absolute recoveries ranged from 78.17 to 101.17% are indicated in table 2.

**Stability**

QC samples were subjected to short term and long term storage condition (-70 °C), freeze-thaw stability, auto-sampler stability and bench-top stability studies. All stability studies were carried out at two concentration levels (low and high QC) in six replicates. The bench top stability was studied for low and high QC samples kept at room temperature (25 °C) for 6 h. Freeze-thaw stability of low and high QC samples was evaluated after 3 freeze-thaw cycles. The auto-sampler stability was studied for low and high QC samples stored at auto-sampler at 10 °C for 24 h.

The results indicated that each analyte had an acceptable stability under the conditions, as shown in table 3.

**Sample dilution**

To demonstrate the ability to dilute and analyze samples containing PARO at the concentration above the assay upper limit of quantification, a set of plasma samples was prepared to contain PARO at a concentration of 9034.60 ng/ml and placed in a-70 °C freezer overnight prior to analysis. After thawing, the certain aliquot was diluted either with 2 and 10 times mice plasma and analyzed respectively. The results of this experiment indicated that the dilution integrity of all the plasma samples was found to be less than 15% of their respective nominal concentrations.

**Advantages of present method**

The aim of present investigation was to develop and validate a simple LC-MS/MS method using gradient mode with sufficient accuracy and precision for PARO and its subsequent use in pharmacokinetic studies in mice. The present method involves simple precipitation procedure with good sensitivity and a gradient reverse-phase UPLC analysis for PARO. This method is specific for PARO with no interference with good linearity, accuracy, and precision. This method involves only 25 μl of plasma and in our LLE extraction procedure, we achieved a high level of extraction efficiency for PARO. This makes the assay highly reproducible and allows us to lower the limit of quantification. Furthermore, this one-step precipitation extraction procedure uses simple, low cost and short run time. The chromatographic conditions of this method were optimized for a short 3 min run time in UPLC-MS/MS.

**Application to pharmacokinetic study**

The method described above was successfully applied to a pharmacokinetics evaluation of PARO, in which plasma concentrations were determined up to 24 h, following intravenous administration at 15 mg/kg dose of PARO in male BALB/c mice. The

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**Table 1: Accuracy and precision from QC samples of PARO in BALB/c mice plasma**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Spiked concentration (ng/ml)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean measured concentration (ng/ml); % mean accuracy; % mean CV</td>
<td>Mean measured concentration (ng/ml); % mean accuracy; % mean CV</td>
<td></td>
</tr>
<tr>
<td>PAR</td>
<td>50.51</td>
<td>55.55; 109.99; 4.88</td>
<td>54.44; 107.79; 4.55</td>
</tr>
<tr>
<td>O</td>
<td>252.22</td>
<td>239.96; 95.17; 8.26</td>
<td>228.54; 90.61; 4.17</td>
</tr>
<tr>
<td>752.88</td>
<td>849.81; 112.87; 2.64</td>
<td>701.32; 93.15; 11.06</td>
<td></td>
</tr>
<tr>
<td>1003.85</td>
<td>1064.52; 108.04; 7.51</td>
<td>984.20; 98.04; 6.16</td>
<td></td>
</tr>
</tbody>
</table>

Data of n=6 replicates

**Table 2: Extraction recovery of PARO in BALB/c mice plasma**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (ng/ml)</th>
<th>% mean recovery</th>
<th>% mean CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARO</td>
<td>25.22</td>
<td>78.17</td>
<td>12.82</td>
</tr>
<tr>
<td></td>
<td>75.28</td>
<td>101.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1003.85</td>
<td>92.59</td>
<td></td>
</tr>
</tbody>
</table>

Data of n=6 replicates

**Table 3: Stability of PARO in BALB/c mice plasma**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Nominal concentration (ng/ml)</th>
<th>Sample condition</th>
<th>Auto-sampler stability</th>
<th>Freeze-thaw stability</th>
<th>30 d storage stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bench top stability</td>
<td>% accuracy; %CV</td>
<td>% accuracy; %CV</td>
<td>% accuracy; %CV</td>
</tr>
<tr>
<td>PARO</td>
<td>25.22</td>
<td>10.20; 5.30</td>
<td>98.36; 6.80</td>
<td>95.36; 3.65</td>
<td>107.21; 5.66</td>
</tr>
<tr>
<td></td>
<td>1003.85</td>
<td>98.50; 6.44</td>
<td>96.50; 7.12</td>
<td>105.20; 5.21</td>
<td>98.36; 8.32</td>
</tr>
</tbody>
</table>

Data of n=6 replicates; *Exposed at ambient temperature (25 °C) for 6 h; **Kept at autosampler temperature (10 °C) for 24 h; ***After three freeze-thaw cycles; ****Stored at-70 °C.
plasma concentration-time profile of PARO is shown in fig. 3 and mean pharmacokinetic parameters are presented in table 4. The compound was well distributed with a steady-state volume of distribution that was 2.62-fold higher than the total body water (0.7 l/kg). It showed a low intravenous plasma clearance with an elimination half-life of 2.60 h.

Table 4: Mean pharmacokinetic parameters following single intravenous administration of PARO in male BALB/c mice

<table>
<thead>
<tr>
<th>Analyte</th>
<th>C₀ (ng/ml)</th>
<th>AUC₀-t (h*ng/ml)</th>
<th>AUC₀-inf (h*ng/ml)</th>
<th>T₁/₂ (h)</th>
<th>CL (ml/min/kg)</th>
<th>Vss (l/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARO</td>
<td>16267.49</td>
<td>30597.46</td>
<td>30626.77</td>
<td>2.60</td>
<td>8.16</td>
<td>1.84</td>
</tr>
</tbody>
</table>

Data of n=3 replicates; AUC: area under the concentration-time curve; C₀: back extrapolated plasma concentrations; T₁/₂: half-life; CL: plasma clearance; Vss: volume of distribution at steady state.

CONCLUSION
The UPLC-MS/MS bioanalytical method for determination of PARO was developed and validated in mice plasma. The method was sensitive enough to detect low concentration of 50.51 ng/ml for PARO. Recovery of PARO from spiked control samples were >78% by using convenient and rapid precipitation method using perchloric acid in water. Intra and inter-day accuracy and precision of the validated method were within the acceptable limits of <20 at LLQ and <15% at low, medium and high concentrations. The method was successfully applied to generate the pharmacokinetic evaluation of PARO in mice plasma following intravenous administration.

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CONFLICTS OF INTERESTS
The authors declare no conflict of interest.

REFERENCES

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