CHAPTER 4

IN-VIVO

PHARMACOKINETIC STUDIES
4.1. Intravenous pharmacokinetics, oral bioavailability, dose proportionality and in-situ permeability of anti-malarial lumefantrine in rats

4.1.1. Introduction

According to the World Health Organization (WHO), there were in 2008 an estimated 247 million malaria cases among more than 3 billion people at risk, causing nearly one million deaths (even much more according to other estimates), mostly of children under 5 years and pregnant women (1). The burden of malaria disease continues to increase as the countries in which it is endemic face the risk of widespread resistance of the parasite to conventional anti-malarial drugs and increasing resistance of the vector to insecticide. Artemether/lumefantrine (AL; Coartem®) is an artemisinin-based combination therapy (ACT) that offers PCR-corrected 28-day cure rates of >95% (2-9), if given in a six-dose regimen. AL meets the WHO pre-qualification criteria for efficacy, safety and quality and is the only ACT that has been approved by ICH stringent regulatory authorities (10).

Despite the potency of artemether, 100-100,000 residual parasites remain when the drug is used alone for a three-day treatment course, and as a result up to 10% of patients experience recrudescence (11, 12). It was recognized that combination treatment, which eliminated the final parasites, would be advantageous. Lumefantrine, the other active constituent of AL, acts over a longer period to eliminate the residual 100-100,000 parasites that remain after artemether is cleared from the body and thus minimizes the risk of recrudescence. Artemether and lumefantrine have different modes of action and act at different points in the parasite life cycle (13, 14). Artemisinin derivatives, such as artemether, have multiple mechanisms of action, including interference with parasite transport proteins, disruption of parasite mitochondrial function, modulation of host immune function and inhibition of angiogenesis (15); Whereas, lumefantrine prevents the detoxification of haem, such that toxic haem and free radicals induce parasite death (13). Additionally, the differing pharmacokinetics of the two agents offers an advantage for combination therapy. Furthermore,
in-vitro, artemether and lumefantrine have shown synergistic action against *Plasmodium falciparum* under in-vitro conditions (16).

The anti-malarial agent lumefantrine, which was originally synthesized by the Academy of Military Medical Sciences in Beijing (17), was identified by researchers at the Academy as a promising agent for combination with artemisinin. Lumefantrine, 2-(dibutylamino)-1-((9E)-2,7-dichloro-9-((4-chlorophenyl) methylidene) fluoren-4-yl) ethanol is an arylamino alcohol (14). Its molecular weight is 528.939 g/mol. It is a lipophillic compound with low intrinsic clearance and erratic oral variability and therapeutic levels are more reliably achieved by co-administration with a fatty meal (14,18-22). Lumefantrine eliminates very slowly with a terminal half-life of 2-3 days in healthy volunteers and 4-6 days in patients with falciparum malaria (14, 18, 23-24). Its plasma protein binding is almost 100% (25). Lumefantrine is predominantly, metabolized by cytochrome P450 3A4 (CYP3A4), to desbutyl lumefantrine (DLF). The in-vitro antiparasitic effect of desbutyl- lumefantrine is 5 to 8 fold higher than lumefantrine (26).

Recently, Wong *et al* (27) reported that DLF has potential as an anti-malarial drug in its own right. Its in-vitro potency relative to that of the parent compound (lumefantrine), its synergy with dihydroartemisinin and the positive relationship between day 7 plasma concentrations and adequate clinical and parasitological response (ACPR) suggest that it could be a useful alternative to lumefantrine as a part of artemisinin-based combination therapy (ACT).

The limitation of artemether-lumefantrine combination is the side effects associated with artemether, i.e. hearing impairment, its high cost, and its variable absorption and the strong food effect on the pharmacokinetics of lumefantrine. Development of newer anti-malarial combinations require detailed preclinical pharmacokinetic assessment of combination partner drugs separately as well as in combination for better understanding of their efficacy, toxicity and safety profile before going in to clinical studies. Preclinical pharmacokinetic information is also very useful in dose/dosage regimen selection of combination partner drugs for clinical assessment. For the development of newer anti-malarial combination(s) and selection of better partner drugs, it is long felt need to understand the detailed preclinical
pharmacokinetics of existing combination drugs (viz. lumefantrine, artemether etc.) in preclinical experimental animal species.

All the studies reporting pharmacokinetics of lumefantrine dealt with clinical data. Despite the widespread clinical use of lumefantrine, there is no study reporting the detailed preclinical pharmacokinetics. However, the preclinical pharmacokinetics of artemether in rats has been reported very recently (28). The focus of present study is to report bioavailability, pharmacokinetics, dose linearity and permeability of lumefantrine. The presence of preclinical pharmacokinetic data in public domain will be of immense help in making informed decisions while selecting the better partner drug(s) for newer combination(s).

4.1.2. Methods

4.1.2.1. Chemicals and reagents

Lumefantrine, desbutyl-lumefantrine and halofantrine (IS) were a generous gift from Ipca Laboratories Ltd. (Mumbai, India). Phenol red and HPLC grade acetonitrile were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India). HPLC grade n-hexane was obtained from E Merck Limited (Mumbai, India). HPLC grade methanol was purchased from Thomas Baker Pvt. Limited (Mumbai, India). Ammonium acetate, ethanol and glacial acetic acid (GAA) AR were purchased from E Merck Limited (Mumbai, India). Potassium dihydrogen orthophosphate was purchased from New India Chemical Enterprises (Cochin, India). Polyethylene glycol (PEG400) and Carboxy methyl cellulose (CMC) were purchased from Sigma Aldrich Ltd (St Louis, USA). Dimethylformamide was purchased from Thomas Baker (chemicals) Pvt. Limited (Mumbai, India). Urethane was purchased from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). Ultra pure water was obtained from a Sartorius Arium 611 system. Heparin sodium injection I.P. (1000 IU/mL, Biologicals E. Limited, Hyderabad, India) was purchased from local pharmacy. Blank, drug free plasma samples were collected from adult, healthy male Sprague–Dawley (SD) rats at the Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinized blood (25 IU/mL) at 2000×g for
10 min at 20 °C. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

4.1.2.2. Animals

Young, adult male SD rats, weighing 200-220 g, were procured from the National Laboratory Animal Center, CDRI (Lucknow, India). Rats were housed in well ventilated cages at room temperature (24±2°C) and 40-60 % relative humidity while on a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of three days prior to the experiment. Approval from the Institutional Animal Ethics Committee was sought and the study protocols were approved before the commencement of the studies.

4.1.2.3. In-situ permeability studies

Single-pass intestinal perfusion studies in rats were performed using established methods adapted from the literature (29). Briefly, male SD rats were fasted overnight for 12 to 16 h with free access to water and anaesthetized using an intra-peritoneal injection of urethane (1g/kg) and placed on a heated pad to keep normal body temperature. Upon verification of the loss of pain reflex, a midline longitudinal abdominal incision was made, and the lumen of the jejunum (10 cm) was flushed with 10 ml of saline pre-warmed to 37 °C. The proximal end of the lumen was catheterized with an inlet polypropylene tube, which was connected to a perfusion pump. The distal end of the jejunum was also catheterized with an outlet polypropylene tube to collect intestinal effluent. Care was taken to handle the small intestine gently and to minimize the surgery in order to maintain an intact blood supply. The entire excised area was covered with an absorbable cotton pad soaked in warmed normal saline. After allowing 30 min to reach steady-state outlet concentrations, outlet perfusate samples were collected every 15 min for 120 min perfusion period. Phenol red was used as a marker of osmosis/zero permeability. At the end, the length of segment was measured without stretching and finally the animal was euthanized. Samples were stored at −20 °C until analysis.

4.1.2.4. HPLC analysis of In-situ permeability samples

The concentration of lumefantrine and phenol red in permeability samples was determined by high-performance liquid chromatography (HPLC) coupled with PDA detector. Chromatographic separation was performed on a Supelco Discovery C18 column (4.6 × 150
mm, 5.0 μm). Mobile phases were duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min and then were pumped in gradient mode. The detail of the gradient program is given in Table 4.1.1. The lumefantrine and phenol red were detected at the wavelength of 235 and 420nm, respectively.

Table- 4.1.1. HPLC gradient used for the determination of lumefantrine and phenol red in in-situ permeability samples

<table>
<thead>
<tr>
<th>Time (Minute)</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 4</td>
<td>65</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>4 – 12</td>
<td>30</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>12 – 17</td>
<td>65</td>
<td>35</td>
<td>1</td>
</tr>
</tbody>
</table>

Solvent A: KH₂PO₄ buffer (10 mM, pH-3)
Solvent B: Methanol

4.1.2.5. Permeability data analysis

The single pass intestinal perfusion is based on reaching steady state with respect to the diffusion of compound across intestine. Steady state is confirmed by plotting the ratio of the outlet to inlet concentrations (corrected for water transport) versus time. The outlet concentrations were corrected by multiplying the inlet concentration with \((\text{phenol red})_{in} / (\text{phenol red})_{out}\). Permeability calculations across rat jejunum \((P_{eff})\) were performed from intestinal perfusate samples collected over 30–120 min (steady state).

\((\text{phenol red})_{in}\) and \((\text{phenol red})_{out}\) are the inlet and outlet concentrations of the water flux marker phenol red. The effective permeability coefficient \((P_{eff})\) and drug absorption rate constant \((K_a)\) were calculated using the following equations:

\[
P_{eff} = \frac{-Q_{in} \ln \left( \frac{C_{out}}{C_{in}} \right)}{2 \pi r l}
\]  

(1)
where, $C_{out}$ is the corrected concentration of the permeant in the exiting perfusate; $C_{in}$ is the concentration of the permeant in entering perfusate; $Q_{in}$ is the flow rate of entering perfusate (0.2 mL/min); $r$ is the inner radius of the intestine, which is 0.18 cm (30); and $l$ is the length of the intestine.

### 4.1.2.6. Pharmacokinetic studies

#### 4.1.2.6.1. Dose proportional oral pharmacokinetic studies.

Male SD rats weighing 200–220 g were fasted overnight (12-14 h) before dosing and had free access to water throughout the experimental period. Lumefantrine in 0.25% CMC suspension was administered orally at a dose of 10, 20 & 40 mg/kg to groups of five rats at each dose level. Animals were provided with standard diet 3 h after dosing. The rats were anaesthetized using ether and blood samples (approximately 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.5, 2, 5, 8, 24, 30, 48, 54, 72 and 120 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min on Sigma 1-15K (Frankfurt, Germany) and stored frozen at $-70 \pm 10^\circ$C until bioanalysis.

#### 4.1.2.6.2. Intravenous pharmacokinetic study.

Another group of male SD rats (N=4) weighing 200–220 g, were used in this part of the study. The intravenous formulation was prepared in DMF–PEG 400–ethanol–water (5: 2.5: 1: 1.5 v/v) and finally filtered through 0.22 μm filter before administration. The solution of lumefantrine was administered to rats via a lateral tail vein as a bolus dose of 0.5 mg/kg. Animals had free access to food and water throughout the experimentation period. Blood samples (approximately 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.08, 0.5, 2, 4, 6, 25, 30, 48, 54, 72, 96 and 120 h post-dosing and plasma was harvested by centrifuging the blood at 13000 rpm for 10 min and stored frozen at $-70 \pm 10^\circ$C until bioanalysis.
4.1.2.7. Sample preparation

A simple liquid–liquid extraction method was followed for extraction of lumefantrine and desbutyl-lumefantrine from rat plasma. To 100µL of plasma in a tube, 10µL of IS solution (halofantrine at 1µg/mL in methanol), 50µL of GAA, 50µL of phosphate buffer (50mM, pH 3) were added and mixed for 15 s on a cyclomixer (Spinix Tarsons, Kolkata, India). Next a 2mL aliquot of extraction solvent, n-hexane was added. The mixture was then vortexed for 3 min, followed by centrifugation for 5 min at 2000×g at 20°C on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6mL) was separated and evaporated to dryness under vacuum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200µL of the mobile phase and 10µL of this solution was subjected to LC-MS/MS analysis.

4.1.2.8. LC-MS/MS analysis of lumefantrine and desbutyl-lumefantrine in study samples

Plasma concentrations of lumefantrine and desbutyl-lumefantrine were determined using partially validated LC-MS/MS method that was accurate, precise, specific, sensitive and reproducible. Analyses were carried out using a HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin-Elmer instruments, Norwalk, USA) on a XBridge RP18 column (4.6 × 50 mm, 5.0 µm). The system was run in isocratic mode with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.01M ammonium acetate (pH 4.5) in the ratio of 95:5 (v/v) at a flow rate of 0.65 mL/min. Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring transition of m/z 529 precursor ion (M+H)+ to the m/z 511.3 product ion for lumefantrine, m/z 472.1 precursor ion (M+H)+ to the m/z 454.1 product ion for desbutyl lumefantrine and m/z 502 precursor ion (M+H)+ to the m/z 511.3 product ion for IS. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada). The retention times for lumefantrine, desbutyl-lumefantrine and IS were 4.81, 2.61 and 2.30 min, respectively. The lower limit of quantification of the method was 2
ng/mL and linearity in the calibration curve standards were demonstrated up to an upper limit of 500 ng/mL. Prior to the analysis of samples, three concentrations (nominal concentrations of 8, 180 and 400 ng/mL) of quality control (QC) samples were prepared in rat plasma. Along with the study samples, QC samples (N=4, at each concentration level) were distributed among the unknown samples in the analytical run.

### 4.1.2.9. Pharmacokinetic analysis

Plasma data were subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). The observed maximum plasma concentration ($C_{\text{max}}$) and the time to reach the maximum plasma concentration ($T_{\text{max}}$) were obtained by visual inspection of the experimental data. The area under the plasma concentration time curve (AUC$_{0-t}$) was calculated using linear trapezoidal method. The total area under the plasma concentration–time curve from time zero to time infinity (AUC$_{0-\infty}$) was calculated as the sum of AUC$_{0-t}$ and $C_{\text{last}}$/kel, where, $C_{\text{last}}$ represents the last quantifiable concentration and Kel represents the terminal phase rate constant. The apparent elimination half-life ($t_{\text{1/2}}$) was calculated as $0.693$/kel and the kel was estimated by linear regression of the plasma concentrations in the log-linear terminal phase. Clearance (CL) following i.v. dosing was calculated as Dose/AUC$_{0-\infty}$. The apparent volume of distribution (Vd) was given by the quotient between CL and elimination rate constant Kel following administration of the intravenous bolus dose.

The absolute bioavailability (%F) of lumefantrine was calculated using the relationship,

\[
%F = \frac{(AUC_{(0-\infty)} \text{ oral} \times \text{ Dose (i.v.)})}{(AUC_{(0-\infty)} \text{ i.v.} \times \text{ Dose (oral))} \times 100
\]

### 4.1.3. Results and discussion

#### 4.1.3.1. Analytical results

The rat plasma samples generated following administration of lumefantrine were analyzed by the partially validated method along with QC samples. Linearity, specificity & selectivity, recovery, matrix effect and accuracy & precision were measured and used as the parameter to assess the assay performance. The peak area ratios of analytes to internal
standard in rat plasma were linear over the concentration range 2-500 ng/ml for both the analytes. The choice of the regression methods was determined. Both lumefantrine and desbutyl-lumefantrine data fit well with a linear regression model, and weighting of $1/\text{concentration}^2$. The correlation coefficients of the standard curves for lumefantrine and desbutyl-lumefantrine, ranging from 2 to 500 ng/ml, were all >0.996. LC-MS/MS analysis of the blank plasma samples showed no interference with the quantification of lumefantrine, desbutyl lumefantrine and IS (Figure-4.1.1.). The extraction recovery of analytes, was determined by comparing the peak areas of extracted plasma (pre-spiked) standard QC samples (N=6) to those of the post-spiked standards at equivalent concentrations (31). The effect of rat plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (N=6) with the response of analytes from neat standard samples at equivalent concentrations (31). The recovery and matrix effect testing was performed at three concentrations QC low, QC medium and QC high concentrations viz., 8, 180, and 400 ng/mL for analytes, whereas the recovery and matrix effect of the IS were determined at a single concentration of 50 ng/mL. The extraction recoveries of the lumefantrine and desbutyl-lumefantrine ranged from 70.45 to 80.12%, and the extraction recovery of the internal standard was 73.31%. The ion suppression or enhancement by plasma was less than 12% for the analytes and IS which demonstrated that the matrix effects do not cause quantitation bias.
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Fig 4.1.1.: Typical multiple reaction monitoring chromatograms of lumefantrine and desbutyl lumefantrine in rat plasma (A) Rat blank plasma, (B) drug free plasma spiked with lumefantrine and desbutyl-lumefantrine at LLOQ (2 ng/mL) and halofantrine (IS)

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 2 ng/mL (lower limit of quantitation, LLOQ), 8 (QC low), 180 ng/mL (QC medium) and 400 ng/mL (QC high). The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The intra- and inter-day assay precision ranged from 3.74 to 7.63% and 5.79 to 7.32% (R.S.D. %), respectively, and intra- and inter-day assay accuracy were between 95.28 to 105.46% and 96.51 to 105.09%, respectively for both the analytes (Table 4.1.2. and 4.1.3.). The mean predicted concentrations of QC samples (distributed among the unknown samples) were between 89.98–107.56% of the nominal values.
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### Table 4.1.2. Intra and Inter-day assay precision & accuracy for lumefantrine in rat plasma

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Observed concentration (ng/mL, mean±S.D.)</th>
<th>Precision&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Accuracy&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (N=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.03 ± 0.11</td>
<td>5.60</td>
<td>101.25</td>
</tr>
<tr>
<td>8</td>
<td>7.84 ± 0.31</td>
<td>3.94</td>
<td>97.78</td>
</tr>
<tr>
<td>180</td>
<td>186 ± 9.06</td>
<td>4.87</td>
<td>103.33</td>
</tr>
<tr>
<td>400</td>
<td>421.83 ± 15.77</td>
<td>3.74</td>
<td>105.46</td>
</tr>
<tr>
<td>Inter-day (N=18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.07 ± 0.13</td>
<td>6.51</td>
<td>103.62</td>
</tr>
<tr>
<td>8</td>
<td>7.90 ± 0.46</td>
<td>5.79</td>
<td>98.51</td>
</tr>
<tr>
<td>180</td>
<td>173.72 ± 11.39</td>
<td>6.55</td>
<td>96.51</td>
</tr>
<tr>
<td>400</td>
<td>405.25 ± 29.67</td>
<td>7.32</td>
<td>101.31</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>b</sup> Calculated as (mean determined concentration/nominal concentration) × 100.

### Table 4.1.3. Intra and Inter-day assay precision & accuracy for desbutyl-lumefantrine in rat plasma

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Observed concentration (ng/mL, mean±S.D.)</th>
<th>Precision&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Accuracy&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (N=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.10 ± 0.16</td>
<td>7.63</td>
<td>104.75</td>
</tr>
<tr>
<td>8</td>
<td>7.90 ± 0.42</td>
<td>5.33</td>
<td>98.79</td>
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<tr>
<td>180</td>
<td>171.50 ± 9.35</td>
<td>5.45</td>
<td>95.28</td>
</tr>
<tr>
<td>400</td>
<td>421.50 ± 20.31</td>
<td>4.82</td>
<td>105.38</td>
</tr>
<tr>
<td>Inter-day (N=18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.10 ± 0.14</td>
<td>6.56</td>
<td>105.09</td>
</tr>
<tr>
<td>8</td>
<td>7.77 ± 0.47</td>
<td>6.03</td>
<td>97.17</td>
</tr>
<tr>
<td>180</td>
<td>178 ± 12.67</td>
<td>7.12</td>
<td>98.89</td>
</tr>
<tr>
<td>400</td>
<td>404.35 ± 27.33</td>
<td>6.76</td>
<td>101.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as % R.S.D. (S.D./mean) × 100.

<sup>b</sup> Calculated as (mean determined concentration/nominal concentration) × 100.
4.1.3.2. *In-situ* permeability study

*In-situ* perfusion of intestinal segments of rodents (rats or rabbits) is frequently used to study the permeability and absorption kinetics of drugs. The $P_{eff}$ values of lumefantrine was determined as the average of six 15 min sampling periods starting from 30 min after the initiation of perfusion, when steady-state had been achieved (Figure- 4.1.2.). Phenol red was used as non-absorbable marker for correction of water flux. During in-house permeability study of amongst USFDA approved high permeability markers, metoprolol showed minimum permeability in rat jejunum ($1.88 \times 10^{-5}$ cm/s). $P_{eff}$ value of lumefantrine was found to be $4.37 \times 10^{-5}$ cm/s which is greater than metoprolol permeability. Therefore, lumefantrine can be classified under high permeability class of BCS (biopharmaceutical classification system).

![Representative plot of the concentration ratio of the outlet to inlet concentrations vs. time for lumefantrine in single pass intestinal perfusion in rats (N=4)](image)

**Fig 4.1.2.** Representative plot of the concentration ratio of the outlet to inlet concentrations vs. time for lumefantrine in single pass intestinal perfusion in rats (N=4)

4.1.3.3. Pharmacokinetic study

The plasma concentrations of lumefantrine were measurable up to 120 hr after oral and intravenous. Figure-4.1.3. depicts the mean plasma concentration-time profiles of lumefantrine following single oral and intravenous administration to male SD rats. The mean oral and intravenous pharmacokinetic parameters for lumefantrine are summarized in Table 4.1.4. The variability in plasma concentrations between-animals were observed for lumefantrine after
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oral administration. However, the low between-animal variability in plasma concentrations after intravenous doses suggests absorption to be critical for between-animal variability in drug exposure. This is also seen in clinical use with substantial inter-individual variability in the pharmacokinetics of lumefantrine after oral administration (14, 18).

**Fig 4.1.3.** Plasma concentration versus time profiles of lumefantrine after oral and intravenous administration in rats (N=5). All concentrations are on the logarithmic scale

The $T_{\text{max}}$ of lumefantrine after oral administration was found to be in the range of 2-8 h. The reason for longer $T_{\text{max}}$ seems to be the low aqueous solubility of lumefantrine since, lumefantrine displayed high permeability in the *in-situ* permeability study. Similarly in humans the $T_{\text{max}}$ of lumefantrine occurs later, at approximately six hours post-dosing in healthy volunteers and 3-4 hours in malaria patients (14,18).

For nominal doses increasing in a 1:2:4 proportion, the $C_{\text{max}}$ and $AUC_{0-\infty}$ values increased in the proportions of 1:0.63:1.53 and 1:0.83:1.81, respectively. Both $C_{\text{max}}$ and $AUC_{0-\infty}$ values of lumefantrine were not increased proportionally with increment of dose, which could be due to dissolution-limited absorption at higher doses due to low solubility of lumefantrine.
The plasma concentrations following intravenous administration of lumefantrine dropped to 45% in approximately 0.05 h. Following intravenous administration, the $t_{1/2}$ was found to be 30.92 ($\pm 4.81$) h. AUC$_{0-\infty}$, clearance (CL) and volume of distribution (Vd) of lumefantrine following administration of 0.5 mg/kg i.v. were 9529.47 ($\pm 1283.18$) ng.h/mL, 0.03 ($\pm 0.02$) L/h/kg and 2.40 ($\pm 0.67$) L/kg, respectively.

The Vd value (2.40 L/kg) of lumefantrine is greater than the total blood volume (0.054 L/kg) indicating extensive extravascular distribution. Furthermore, the mean hepatic blood flow in rats is approximately 3.22 L/h/kg (32). Using the haematocrit in rat of 0.48 (32), this yields a mean hepatic plasma flow of 1.74 L/h/kg. The CL value for lumefantrine (0.03 L/h/kg) represents less than 2% of the hepatic plasma flow (1.74 L/h/kg), indicating that lumefantrine is low extraction compound. Absolute oral bioavailability (% F) of lumefantrine across the tested doses ranged between 4.80% and 11.56%. The bioavailability was decreased at higher doses. This non-linear relationship between dose and bioavailability is well described for other highly lipophilic drugs, e.g. halofantrine (33). The variable bioavailability of lumefantrine between individual doses was also observed in humans (18). The bioavailability of a drug determines the amount reaching the systemic circulation and it in turn determines the pharmacological effects. Hence, preclinical pharmacokinetic data will be of immense help for deciding the partner drug’s dose and concentration(s) required for therapeutic efficacy in order to keep the drug’s concentration at or above cidal concentration in order to prevent/delay the drug resistance at sub-cidal level.

The plasma concentrations of desbutyl-lumefantrine were measurable up to 120 h after oral and up to 96 hr after intravenous administration. Figure- 4.1.4. depicts the mean plasma concentration-time profiles of desbutyl-lumefantrine following single oral and intravenous administration of lumefantrine to male SD rats. The mean oral and intravenous pharmacokinetic parameters for desbutyl-lumefantrine are summarized in Table 4.1. The desbutyl-lumefantrine was detected from 2 hr time point, except at 40 mg/kg where it was detected from first time point i.e. 0.05 hr. For nominal doses increasing in a 1:2:4 proportion, the $C_{\text{max}}$ and AUC$_{0-t}$ values increased in the proportions of 1:1.45:2.57 and 1:1.08:1.87, respectively. Following the intravenous administration of lumefantrine, the $C_{\text{max}}$ and AUC$_{0-t}$ value of desbutyl-lumefantrine was found to be 7.91 ($\pm 1.89$) ng/mL and 375.75 ($\pm 74.26$)
ng.h/mL, respectively. In conclusion, we successfully derived the pharmacokinetic parameters of lumefantrine and its metabolite desbutyl-lumefantrine in rats for the first time. Lumefantrine displayed similar pharmacokinetics in the rat as in humans (14, 18), with multiphasic disposition, low clearance, and a large volume of distribution resulting in a long terminal elimination half-life.

**Fig 4.1.4.** Plasma concentration versus time profiles of desbutyl-lumefantrine after oral and intravenous administration of lumefantrine in rats (N=4). All concentrations are on the logarithmic scale

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intravenous</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0-t (hr*ng/mL)</td>
<td>9189.76 ± 1372.42</td>
<td>21294.02 ± 3235.75</td>
<td>17683.7 ± 3168.23</td>
<td>38248.94 ± 7792.61</td>
</tr>
<tr>
<td>AUC0-∞ (hr*ng/mL)</td>
<td>9529.47 ± 1283.18</td>
<td>22025.48 ± 3448.87</td>
<td>18281.07 ± 3062.84</td>
<td>39958.71 ± 8362.60</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>1890 ± 330.61</td>
<td>1488 ± 311.47</td>
<td>938.75 ± 370.64</td>
<td>2280 ± 522.32</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>-</td>
<td>6.8 ± 2.40</td>
<td>4.25 ± 2.87</td>
<td>5 ± 2.45</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>2.40 ± 0.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>0.03 ± 0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t1/2 (hr)</td>
<td>30.92 ± 4.81</td>
<td>36.08 ± 8.52</td>
<td>25.7 ± 1.85</td>
<td>38.23 ± 4.51</td>
</tr>
<tr>
<td>% F</td>
<td>-</td>
<td>11.56</td>
<td>4.80</td>
<td>5.24</td>
</tr>
</tbody>
</table>

**Table 4.1.4.** Pharmacokinetic parameters of lumefantrine after oral and intravenous administration in rats
Chapter 4: *In-vivo* Pharmacokinetic Studies

**Table 4.1.5.** Pharmacokinetic parameters of desbutyl-lumefantrine after oral and intravenous administration of lumefantrine in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intravenous</th>
<th>Per-oral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg/kg</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt;</td>
<td>375.75 ± 74.26</td>
<td>828.18 ± 281.31</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>7.91 ± 1.89</td>
<td>13.54 ± 4.27</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>13.50 ± 13.28</td>
<td>21.40 ± 9.53</td>
</tr>
</tbody>
</table>

**4.1.4. Conclusion**

The peak plasma concentration (C<sub>max</sub>) and area under the plasma concentration–time curve from time zero to time infinity (AUC<sub>0-∞</sub>) values for lumefantrine were not increased proportionally to the administered dose. For nominal doses increasing in a 1:2:4 proportion, the C<sub>max</sub> and AUC<sub>0-∞</sub> values increased in the proportions of 1:0.6:1.5 and 1:0.8:1.8, respectively. For lumefantrine nominal doses increasing in a 1:2:4 proportion, the C<sub>max</sub> and the area under the plasma concentration time curve (AUC<sub>0-t</sub>) values for desbutyl-lumefantrine increased in the proportions of 1:1.45:2.57 and 1:1.08:1.87, respectively. After intravenous administration the clearance (Cl) and volume of distribution (V<sub>d</sub>) of lumefantrine in rats were 0.03 (±0.02) L/h/kg and 2.40 (±0.67) L/kg, respectively. Absolute oral bioavailability of lumefantrine across the tested doses ranged between 4.97% and 11.98%. Lumefantrine showed high permeability (4.37 x 10^-5 cm/s) in permeability study. Therefore, lumefantrine can be classified as class II drug under biopharmaceutical classification system (BCS) due to it's high permeability and low solubility.
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### 4.2. Gender Differences in Pharmacokinetics of lumefantrine and its metabolite desbutyl-lumefantrine in Rats

#### 4.2.1. Introduction

Studying gender specific pharmacokinetics has gained momentum in recent past. The increased interest is mainly due to the increasing data on gender variation in drug efficacy and toxicity profiles which depend mainly on pharmacokinetic and metabolic pattern. The differences in pharmacokinetic properties in sex-based studies are conceived to twig from physiological variations between male and female (34, 35).

Malaria is still posing challenge and threat due to recent failure of therapies because of development of resistant strains of pathogens. Furthermore, the resistant developed in vector to insecticide becoming a threat to the major part of the world (36). In its efforts to combat malaria, the Academy of Military Medical Sciences, Beijing synthesized a racemic fluorine derivative called lumefantrine (originally called Benflumetol). Lumefantrine is available in many markets of the world mainly as combination with artemether commercially known as Coartem®/Riamet® for the treatment of Plasmodium falciparum malaria. Lumefantrine absorption starts after a lag-time of up to two hours. The elimination of lumefantrine is very slow with a terminal half-life of 2-3 days in healthy volunteers and 4-6 days in patients with falciparum malaria (37, 38, 39, 40, 41). Lumefantrine has been reported to mainly bio-transformed by cytochrome P450 isozyme 3A4 in to desbutyl-lumefantrine (DLF) in human liver microsomes (42, 43). The *in-vitro* antiparasitic effect of DLF is 5 to 8 fold higher than lumefantrine (44). Recently wong et al. (45) reported that DLF has a stronger role than the parent compound in suppressing recrudescence and/or preventing reinfection. Therefore, the formation of DLF may influence the efficacy of antimalarial therapy.

Further, gender specific expression of CYP3A is already well established (35, 46). Therefore it could be expected that the pharmacokinetics of lumefantrine and formation of its metabolite (DLF) could be different in male compared with those in female. Therefore, the purpose of this study is to report the gender differences in the pharmacokinetics of lumefantrine and its metabolite (DLF) after oral and intravenous administration of lumefantrine in rats.
4.2.2. Materials and Methods

4.2.2.1. Chemicals and reagents

Lumefantrine, DLF and halofantrine (IS) were a generous gift from IPCA Laboratories Ltd. (Mumbai, India). Ultra pure water was obtained from a Sartorius Arium 611 system. Other chemicals were of reagent grade or HPLC grade.

4.2.2.2. Pharmacokinetic studies

4.2.2.2.1. Oral pharmacokinetic study

Male and female Sprague-Dawley rats (N=5) weighing 200–220 g were fasted overnight (12-14 h) before dosing and had free access to water throughout the experimental period. Lumefantrine was administered orally at a dose of 20 mg/kg in 0.25% CMC suspension. Animals were provided with standard diet 3 h after dosing. The rats were anaesthetized using ether and blood samples (approximately 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.5, 2, 5, 8, 24, 30, 48, 54, 72 and 120 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min on Sigma 1-15K (Frankfurt, Germany) and stored frozen at $-70 \pm 10^\circ$C until bioanalysis. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

4.2.2.2.2. Intravenous pharmacokinetic study

Another group of male and female Sprague-Dawley (N=4) weighing 200–220 g, were used in this part of the study. The intravenous formulation was prepared in DMF–PEG 400–ethanol–water (5: 2.5: 1: 1.5 v/v) and finally filtered through 0.22 μm filter before administration. The solution of lumefantrine was administered to rats via a lateral tail vein as a bolus dose of 0.5 mg/kg. Animals had free access to food and water throughout the experimentation period. Blood samples (approximately 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.08, 0.5, 2, 4, 6, 25, 30, 48, 54, 72, 96 and 120 h post-dosing. Other procedures were similar to those in the oral study.

4.2.2.3. LC-MS/MS analysis of pharmacokinetic study samples

Plasma concentrations of lumefantrine and DLF were determined using partially validated LC-MS/MS method that was accurate, precise, specific, sensitive and reproducible. A simple liquid–liquid extraction method was followed for extraction of lumefantrine and desbutyl-lumefantrine from rat plasma. To 100μL of plasma in a tube, 10μL of IS solution (halofantrine
at 1µg/mL in methanol), 50µL of GAA, 50µL of phosphate buffer (50mM, pH 3) were added and mixed for 15 s on a cyclomixer. Next a 2mL aliquot of extraction solvent, n-hexane was added. The mixture was then vortexed for 3 min, followed by centrifugation for 5 min at 2000×g at 20°C. The organic layer (1.6mL) was separated and evaporated to dryness under vacuum in speedvac concentrator. The residue was reconstituted in 200µL of the mobile phase and 10µL of this solution was subjected to LC-MS/MS analysis.

Analyses were carried out using a HPLC system on a XBridge RP18 column (4.6 × 50 mm, 5.0 µm). The system was run in isocratic mode with mobile phase consisting of acetonitrile:methanol (50:50, v/v) and 0.01M ammonium acetate (pH 5.5) in the ratio of 90:10 (v/v) at a flow rate of 0.5 mL/min. Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring transition of m/z 529 precursor ion (M+H)+ to the m/z 511.3 product ion for lumefantrine, m/z 472.1 precursor ion (M+H)+ to the m/z 454.1 product ion for DLF and m/z 502 precursor ion (M+H)+ to the m/z 511.3 product ion for IS. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada). The lower limit of quantification of the method was 2 ng/mL and linearity in the calibration curve standards were demonstrated up to an upper limit of 500 ng/mL. Along with the plasma samples, QC samples were distributed among unknown samples in the analytical run.

4.2.2.4. Pharmacokinetic analysis

Plasma data were subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). The observed maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained by visual inspection of the experimental data. The area under the plasma concentration time curve (AUC_{0-t}) was calculated using linear trapezoidal method. The total area under the plasma concentration–time curve from time zero to time infinity (AUC_{0-∞}) was calculated as the sum of AUC_{0-t} and C_{last}/k_{el}, where, C_{last} represents the last quantifiable concentration and K_{el} represents the terminal phase rate constant. The apparent elimination half-life (t_{1/2}) was calculated as 0.693/kel and the k_{el} was estimated by
linear regression of the plasma concentrations in the log-linear terminal phase. Clearance (CL) following i.v. dosing was calculated as Dose/AUC\(_{0-\infty}\). The apparent volume of distribution (Vd) was given by the quotient between CL and elimination rate constant kel following administration of the intravenous bolus dose. The absolute bioavailability (%F) of lumefantrine was calculated using the relationship,

\[
%F = \frac{\text{AUC}_{(0-\infty)} \text{ oral} \times \text{Dose (i.v.)}}{\text{AUC}_{(0-\infty)} \text{ i.v.} \times \text{Dose (oral)}} \times 100
\]

4.2.2.5. Statistical analysis

A value of p<0.05 was considered to be statistically significant using an unpaired t-test. All data are expressed as mean ± standard deviation.

4.2.3. Results and Discussion

There is increasing evidence of sex difference in pharmacokinetics of drugs due to differences in basic physiology of male and female (41, 35). It is, therefore, essential to understand sex differences in drug disposition, as they may affect drug safety and effectiveness. In the present study we are reporting gender difference in pharmacokinetics of lumefantrine and its metabolite desbutyl lumefantrine.

The rat plasma samples generated following administration of lumefantrine were analyzed by the partially validated method along with QC samples. The mean predicted concentrations of QC samples (distributed among the unknown samples) were between 91.86–97.23% of the nominal values. The mean plasma concentration versus time profiles of lumefantrine and DLF after oral (20 mg/kg) and intravenous (0.5 mg/kg) administration of lumefantrine to male and female rats are shown in Figure 4.2.1, and comparison of pharmacokinetic parameters of lumefantrine and DLF are presented in Table 4.2.1. The AUC\(_{0-\infty}\) of lumefantrine was found significantly higher in female rats after oral as well as intravenous administration in comparison to male rats. The higher AUC\(_{0-\infty}\) of lumefantrine in female may be supported by its low clearance in comparison to male upon oral as well as intravenous administration. Due to the slower clearance of lumefantrine in female rats, the elimination half-life of lumefantrine was found to be higher in female rats (39.33±7.60 hours) as compared to male rats (30.92±4.81 hours).

AUC\(_{0-t}\) of DLF was found to be 6.67 times and 3.16 times higher in male rats than female rats upon intravenous and oral administration, respectively and the difference observed
was found to be statistically significant. The greater AUC0-t of DLF in male rats could be due to higher metabolism (higher clearance value) of lumefantrine in male rats in comparison to female rats. The Cmax was found to be 2.38 times higher, which is statistically significant, in male rats in comparison to female rats upon oral administration.

Our study suggests significant gender difference in the pharmacokinetics of lumefantrine and its major active metabolite desbutyl lumefantrine. Recent literature (45) suggests the significant role of desbutyl lumefantrine in the efficacy of lumefantrine containing antimalarial therapies. Hence, the therapeutic outcome/efficacy may get affected due to difference in the rate and extent of formation of desbutyl lumefantrine. However, further studies in humans are required to substantiate these findings.
Figure 4.2.1. Plasma concentration versus time profiles of lumefantrine (A) and desbutyl-lumefantrine (B) after oral and intravenous administration of lumefantrine in rats. All concentrations are on the logarithmic scale.
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Table 4.2.1. Mean (± standard deviation) Pharmacokinetic parameters of lumefantrine and desbutyl-lumefantrine after oral and intravenous administration of lumefantrine in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intravenous (0.5 mg/kg)</th>
<th>Per-oral (20 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (ng/mL)</td>
<td>Female (ng/mL)</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| AUC$_{0-\infty}$ (hr*ng/mL) | 9529.47 ± 1283.1*       | 22271.07 ± 3720.18* | 18281.07 ± 3062.84      | 25777.20 ± 5592.88*
| C$_{\text{max}}$ (ng/mL) | -                      | -                   | 938.75 ± 370.64         | 868.75 ± 241.71     |
| T$_{\text{max}}$ (hr) | -                      | -                   | 4.25 ± 2.87             | 5 ± 2.45            |
| Vd (L/kg)           | 2.40 ± 0.67             | 1.18 ± 0.14*        | 1.66 ± 0.36             | 1.06 ± 0.16*        |
| CL (L/h/kg)         | 0.033 ± 0.02            | 0.023 ± 0.004       | 0.040 ± 0.00            | 0.022 ± 0.00*       |
| t$_{1/2}$ (hr)      | 30.92 ± 4.81            | 39.33 ± 7.60        | -                      | -                   |
| % F                 | -                      | -                   | 4.8                    | 2.89                |
| Desbutyl-lumefantrine |                         |                     |                         |                     |
| AUC$_{0-\infty}$ (hr*ng/mL) | 375.75 ± 74.26         | 56.35 ± 40.56*      | 897.39 ± 151.38         | 284.13 ± 31.98*     |
| C$_{\text{max}}$ (ng/mL) | 7.91 ± 1.89            | 7.10 ± 1.23         | 19.66 ± 3.72            | 8.27 ± 0.88*        |
| T$_{\text{max}}$ (hr) | 13.50 ± 13.28           | 0.08 ± 0.00*        | 6.20 ± 1.64             | 8 ± 0.00            |

*Statistically significant at p < 0.05 when compared with the male (Intravenous, 0.5 mg/kg).

§Statistically significant at p < 0.05 when compared with the male (Per-oral, 20 mg/kg).
4.2.4. Conclusion

After intravenous and oral administration of lumefantrine the area under the curve (AUC) of lumefantrine was significantly higher in female rats than that in male rats. Whereas the AUC of DLF was significantly lower in female rats in comparison to male rats. This lower AUC of DLF in female rats could have been due to reduced metabolism of lumefantrine in female rats. The bioavailability (% F) of lumefantrine was 1.66 times higher in male rats than that in female rats.
4.3. Role of P-gp in Absorption of Lumefantrine

4.3.1. Introduction

Intestinal drug efflux by P-glycoprotein (P-gp), the multidrug resistance transporter, is widely recognized as a major determinant for low or variable oral absorption and bioavailability (47, 48). P-gp is a member of the ATP-binding cassette transporter superfamily (ABCB1) and is located on the apical membrane of intestinal enterocytes where it can actively efflux drugs from the cell back into the intestinal lumen (49). Cytochrome P450 3A4 (CYP3A4) is the major oxidative drug metabolizing enzyme found in the intestine and is localized to the endoplasmic reticulum of the enterocytes (50, 51). There is remarkable overlap between both the substrates and inhibitors of CYP3A4 and P-gp (52). The extensive overlap in the substrate specificities, tissue localization, and coinducibility of P-gp and CYP3A4 has led to the hypothesis that these two proteins work together to protect the body from absorption of harmful xenobiotics, including drugs (53). Due to the spatial localization of P-gp and CYP3A4 in the intestine, P-gp controls the access of the drug to the metabolizing enzyme and results in increased metabolism from prolonged exposure to the enzyme through repeated cycles of absorption and efflux (47, 48).

Lumefantrine was reported to be a substrate of CYP 3A4 and gets metabolized to Desbutyl lumefantrine. Absorption of lumefantrine, a highly lipophilic compound, starts after a lag-time of up to 2 h, with peak plasma concentration about 6–8 h after dosing. It is having very low oral bioavailability (about 10%). Being a highly lipophilic drug (having log P value of 8.34), and very poor water solubility lumefantrine belongs to BCS class II. Being a BCS class II drug and a substrate of CYP 3A4, lumefantrine may be a substrate of P-gp (54), as both CYP3A4 and P-gp are colocalised in the intestine and share most of their substrates and inhibitors. We therefore investigated whether lumefantrine is a P-gp substrate and whether P-gp-mediated efflux explains the apparently low bioavailability of lumefantrine by *in-situ* as well as *in-vivo* methods in male SD rats by administering with or without Verapamil (P-gp inhibitor).
4.3.2. Methods

4.3.2.1. Chemicals and reagents

Propranolol, digoxin and verapamil were purchased from Sigma (St. Louis, USA). Lumefantrine and halofantrine (I.S) were generous gift from IPCA laboratories ltd (Mumbai, India). Phenol red, methanol and acetonitrile of HPLC grade were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd (Mumbai, India). Ammonium acetate, acetic acid, ammonia, carboxy methyl cellulose (CMC) and PEG 400 were purchased from Sigma Aldrich Ltd (St Louis, USA). Sodium dihydrogen ortho-phosphate (NaH$_2$PO$_4$·2H$_2$O) and anhydrous di-sodium hydrogen ortho-phosphate (Na$_2$HPO$_4$) were purchased from Glaxo Laboratories Limited (Mumbai, India). Sodium chloride (NaCl) was purchased from Ranbaxy Laboratories Limited (Punjab, India). Ethanol was purchased from Merck (Germany). Triple distilled water was used during the entire experiment. Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd. (New Delhi, India). All other chemicals were of AR grade.

4.3.2.2. Animals and legal prerequisite

Young, adult male Sprague-Dawley (SD) rats, weighing 220±25 g, were procured from the National Laboratory Animal Center, C.D.R.I. (Lucknow, India). Rats were housed in well ventilated cages at room temperature (24±2°C) and 40-60 % relative humidity while on a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of 3 days prior to the experiment. Approval from the Local Animal Ethics Committee was sought and the study protocols were approved before the commencement of the studies.

4.3.3. In-situ Single Pass Intestinal Perfusion study

4.3.3.1. Experimental

Single-pass intestinal perfusion studies in rats were performed using established methods adapted from the literature (55, 56). Briefly, male SD rats were fasted overnight for 12 to 16 h with free access to water and anaesthetized using an intra-peritoneal injection of urethane (1.5g /kg) and placed on a heated pad to keep normal body temperature. Upon
verification of the loss of pain reflex, a midline longitudinal abdominal incision was made, and the lumen of the jejunum (10 cm) was flushed with 10 ml of saline pre-warmed to 37 °C. The proximal end of the lumen was catheterized with an inlet polypropylene tube, which was connected to a perfusion pump (Perista Pump: Atto, Tokyo, Japan). The distal end of the jejunum was also catheterized with an outlet polypropylene tube to collect intestinal effluent. Care was taken to handle the small intestine gently and to minimize the surgery in order to maintain an intact blood supply. The entire excised area was covered with an absorbable cotton pad soaked in normal saline. Perfusion buffer containing lumefantrine (20µM), Propranolol (20 µM, USFDA high permeable marker) and phenol red (10µM) was infused, with or without the presence of well-known P-gp inhibitor verapamil (200µM). Phenol red was used as a non-absorbable marker for measuring water flux and to correct for changes in the water flux across the incised ileal segment. After allowing 30 min to reach steady-state outlet concentrations, outlet perfusate samples were collected every 15 min for 120 min perfusion period. At the end, the length of segment was measured without stretching and finally the animal was euthanatized with overdose of ether anesthesia. Samples were stored at −20°C until analysis.

Similar procedure was followed for digoxin (a well known P-gp substrate) with or without verapamil to check the suitability of SPIP model for studying the role of P-gp.

4.3.3.2. HPLC analysis of SPIP study samples

Single pass intestinal perfusion samples for lumefantrine with or without verapamil were analyzed using HPLC method. The liquid chromatographic system consisted of a Shimadzu LC-10 AT VP pump, SIL-10 AD VP autosampler, DGU-14A degasser, SPD- M 10A VP photo diode array detector and CTO-10A-VP column oven (Shimadzu Corporation, Kyoto, Japan). All the parameters of HPLC were controlled by Class VP software. Technical details about the HPLC analysis are listed in Table 4.3.1. The system was run in gradient mode with mobile phase consisting of methanol and 0.01M ammonium acetate (pH 5.0) at a flow rate of 0.5 mL/min. Mobile phase was duly filtered through 0.22 µm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min and used for chromatographic separation. The samples collected from intestine were centrifuged at 2000g for 5 min and directly injected to HPLC. 50µL aliquots of the processed samples were injected on a Waters
Symmetry Shield C18 column (4.6mm×50mm, 5.0µm). Lumefantrine, propranolol, phenol red and verapamil were detected at the wavelength of 300, 220, 420 and 220 nm, respectively.

**Table 4.3.1.:** Gradient program used for the permeability samples analysis

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>Mobile Phase composition</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>Methanol and amm. acetate buffer 52:48 % (v/v)</td>
<td>1.5ml/min</td>
</tr>
<tr>
<td>3-9</td>
<td>Methanol and amm. acetate buffer 95:5 % (v/v)</td>
<td>1.5ml/min</td>
</tr>
<tr>
<td>9-15</td>
<td>Methanol and amm. acetate buffer 52:48 % (v/v)</td>
<td>1.5ml/min</td>
</tr>
</tbody>
</table>

Permeability samples for digoxin, with or without verapamil were analyzed by using HPLC with Photodiode array (PDA) detection. 50µL aliquots of the processed samples were injected on a Waters Symmetry Shield C18 column (4.6mm×50mm, 5.0µm). The system was run in isocratic mode with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.01M ammonium acetate (pH 5.0) in the ratio of 50:50 (v/v) at a flow rate of 0.5 mL/min. Both digoxin and verapamil were detected at 220nm wavelength.

**4.3.3.3. Permeability Data Analysis**

The single pass intestinal perfusion is based on reaching steady state with respect to the diffusion of compound across intestine. Steady state is confirmed by plotting the ratio of the outlet to inlet concentrations (corrected for water transport) versus time. The outlet concentrations were corrected by multiplying the outlet concentration with (phenol red)\textsubscript{out}/(phenol red)\textsubscript{in}.

Permeability calculations across rat jejunum ($P_{\text{eff}}$) were performed from intestinal perfusate samples collected over 30–120 min (steady state). The intestinal net water flux (NWF, µl/h/cm) was calculated according to Eq. (1):

$$NWF = \left(1 - \frac{\text{[Ph.red]}_{\text{out}}}{\text{[Ph.red]}_{\text{in}}}\right) \frac{Q_{\text{in}}}{l}$$

(1)

Where, (Ph.red)\textsubscript{in} and (Ph.red)\textsubscript{out} are the inlet and outlet concentrations of the water flux marker phenol red. A negative net water flux indicates loss of fluid from the mucosal side.
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(lumen) to the serosal side (blood). A positive net water flux indicates secretion of fluid into the segment. The drug absorption rate constant (Ka) and the effective permeability coefficient (\( P_{\text{eff}} \)) were calculated using the following equations:

\[
P_{\text{eff}} = \frac{-Q_{\text{in}} \ln \left( \frac{C_{\text{out}}}{C_{\text{in}}} \right)}{2\pi rl}
\]

\[
K_a = \left[ 1 - \frac{C_{\text{out}}}{C_{\text{in}}} \right] \times \frac{Q}{\pi r^2 l}
\]

Where, \( C_{\text{out}} \) is the corrected concentration of the permeate in the exiting perfusate; \( C_{\text{in}} \) is the concentration of the permeate in entering perfusate; \( Q_{\text{in}} \) is the flow rate of entering perfusate (0.2 mL/min); \( r \) is the inner radius of the intestine, which is 0.18 cm (10); and \( l \) is the length of the intestine. All the results have shown as mean ± S.D.

4.3.4. *In-vivo* pharmacokinetic study

4.3.4.1. Systemic Bioavailability of lumefantrine in rats

The animals were fasted overnight with free access to water before drug administration. Intravenous formulation (solution) of lumefantrine was prepared in DMF-PEG 400-ethanol-water (5:2.5:1:1.5, parts) and finally filtered through 0.22µm filter before administration. The oral formulation (suspension) of lumefantrine was prepared in 0.25% Sodium CMC suspension. Rats were randomized into two groups (n=5, per group): to receive lumefantrine at 10 mg/kg by oral gavage and lumefantrine 0.5 mg/kg by I.V. bolus injection through the tail vein. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at time intervals of 0.5, 2, 5, 8, 24, 30, 54, 72 and 120 h post dosing. Plasma was harvested by centrifuging the blood at 2000×g for 5min at 20°C and stored frozen at −20°C until analysis.

4.3.4.2. Effect of Co-administered Verapamil on Plasma Pharmacokinetics of Lumefantrine in Rats

We examined the effects of co-administered verapamil (P-gp inhibitor) at 10 mg/kg dose on the plasma pharmacokinetics of lumefantrine in healthy male Sprague-Dawley rats.
The dose of verapamil was selected based on previously reported literature (57). Verapamil formulation (solution) was prepared by dissolving in distilled water. Rats were randomized to receive the following different treatments (n = 5 per group): lumefantrine at 10 mg/kg by oral gavage plus water (control vehicle), and lumefantrine at 10 mg/kg in combination with verapamil at 10 mg/kg by oral gavage. The P-gp inhibitor, verapamil was administered 10 min before lumefantrine dosing. Blood samples were collected at time intervals of 0.5, 2, 5, 8, 24, 30, 54, 72 and 120 h post dosing, into heparinized microfuge tubes. The concentrations of lumefantrine in plasma samples were determined by LC-MS/MS.

4.3.4.3. Sample preparation

A simple liquid–liquid extraction method was followed for extraction of lumefantrine from rat plasma. To 100µL of plasma in a tube, 10µL of IS solution (halofantrine at 1µg/mL in methanol), 50µL of GAA, 50µL of phosphate buffer (50mM, pH 3.0) were added and mixed for 15 s on a cyclomixer (Spinix Tarsons, Kolkata, India). Next a 2mL aliquot of extraction solvent, n-hexane was added. The mixture was then vortexed for 3 min, followed by centrifugation for 5 min at 2000×g at 20°C on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6mL) was separated and evaporated to dryness under vacuum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200µL of the mobile phase and 10µL of this solution was subjected to LC-MS/MS analysis.

4.3.4.4. LC-MS/MS analysis of Lumefantrine pharmacokinetic samples

LC-MS/MS analyses of pharmacokinetic study samples were done by using the validated method published from our lab (58). Briefly chromatographic conditions were as follows. The HPLC (Perkin Elmer instruments, Norwalk, USA) system consists of Series 200 pumps and auto-sampler with temperature controlled Peltier-tray. 10µL aliquots of the processed samples were injected on a Symmetry Shield C18 column (2.1mm×30mm, 3.5µm). The system was run in isocratic mode with mobile phase consisting of acetonitrile: methanol (50:50, v/v) and 0.01M ammonium acetate (pH 5.5) in the ratio of 90:10 (v/v) at a flow rate of 0.5 mL/min. The Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 529 precursor ion (M+H)+ to the m/z 511.3 product ion.
for lumefantrine and m/z 502 precursor ion (M+H)^+ to the m/z 142.2 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada). The lower limit of quantitation of the method was 2 ng/ml and linearity in calibration curve standards were demonstrated up to an upper limit of 500 ng/ml. Along with the study samples, quality control samples were distributed among the unknown samples in the analytical run.

4.3.4.5. Pharmacokinetic Calculations

Plasma data were subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). The observed maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained by visual inspection of the experimental data. The area under the plasma concentration time curve (AUC_{0-t}) was calculated using linear trapezoidal method. The total area under the plasma concentration–time curve from time zero to time infinity (AUC_{0-\infty}) was calculated as the sum of AUC_{0-t} and C_{last}/k_{el}, where, C_{last} represents the last quantifiable concentration and K_{el} represents the terminal phase rate constant. The apparent elimination half-life (t_{1/2}) was calculated as 0.693/k_{el} and the k_{el} was estimated by linear regression of the plasma concentrations in the log-linear terminal phase. Clearance (CL) following I.V. dosing was calculated as Dose/AUC_{0-\infty}. The apparent volume of distribution (V_d) was given by the quotient between CL and elimination rate constant k_{el} following administration of the I.V. bolus dose.

The absolute bioavailability (%F) of lumefantrine was calculated using the relationship,

\%
F = (AUC_{(0-\infty)} \text{ oral x Dose (i.v.,)}/ AUC_{(0-\infty)} \text{ i.v., x Dose (oral)} \times 100

4.3.5. Results and Discussion

4.3.5.1. In-situ Single Pass Intestinal Perfusion study of Rat jejunum

Preliminary tests revealed that there is no significant absorption of lumefantrine, digoxin, verapamil and permeability markers in the tubing used for perfusion experiment and all the analytes were observed to be stable in blank perfusion buffer collected from intestine for about 6 h at room temperature.
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The effective permeability ($P_{\text{eff}}$) of lumefantrine was determined by measuring the disappearance of lumefantrine from perfusate. The $P_{\text{eff}}$ values of lumefantrine was determined as the average of six 15 min sampling periods starting from 30 min after the initiation of perfusion, when steady state had been achieved (figure 4.3.1.). The permeability values of lumefantrine, propranolol and digoxin are shown in Table 4.3.2. The permeability coefficient, $P_{\text{eff}}$ of propranolol without and with verapamil (at 200µM) co-administration were found to be similar, which indicates that there is no change in integrity of intestinal membrane. The lumefantrine $P_{\text{eff}}$ increases significantly ($p<0.05$, 2.3 folds) by co-administration of verapamil (P-gp selective inhibitor). This clearly indicates that the P-gp efflux transporter is playing role in the absorption of lumefantrine.

The *in-situ* system suitability for studying the role of P-gp, was tested using standard P-gp substrate digoxin at 20 µM with or without verapamil at 200 µM. The significant increase in the permeability ($p<0.05$, 6.8 folds) of digoxin upon co-administration with verapamil, demonstrates the suitability of SPIP model for the studying the role of P-gp in absorption.

To further address the role of Pgp/MDR1 in the *in-vivo* disposition of lumefantrine, we examined the effects of co-administered verapamil on the systemic bioavailability and plasma pharmacokinetics of lumefantrine.

### Table 4.3.2. Permeability values across isolated perfused rat jejunum. Data are the mean ± S.D. (n=5)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>$P_{\text{eff}}$ ($\times 10^{-5}$) cm/s</th>
<th>$K_a$ ($\times 10^{-3}$) min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumefantrine</td>
<td>20µM</td>
<td>3.56±0.51</td>
<td>22.31±3.02</td>
</tr>
<tr>
<td></td>
<td>+ Verapamil 200µM</td>
<td>8.06±1.35*</td>
<td>47.44±7.44</td>
</tr>
<tr>
<td>Propranolol</td>
<td>20µM</td>
<td>6.40±1.17</td>
<td>34.96±8.45</td>
</tr>
<tr>
<td></td>
<td>+ Verapamil 200µM</td>
<td>6.10±1.14</td>
<td>37.02±6.10</td>
</tr>
<tr>
<td>Digoxin</td>
<td>20µM</td>
<td>0.19±0.03</td>
<td>1.33±0.21</td>
</tr>
<tr>
<td></td>
<td>+ Verapamil 200µM</td>
<td>1.30±0.32*</td>
<td>8.56±2.09</td>
</tr>
</tbody>
</table>

* $p<0.05$, significantly different in comparison to respective control group
Fig. 4.3.1.: Representative plot of the concentration ratio of the outlet and inlet concentrations vs. time for lumefantrine in single-pass intestinal-perfusion in rat. (Error bars represent S.D., n=5)

Fig. 4.3.2.: Effect of verapamil on lumefantrine and digoxin effective permeability (P_{eff}). (Error bars represent S.D., n=5)

4.3.5.2. In-vivo pharmacokinetic study

4.3.5.2.1. Oral Bioavailability of Lumefantrine in Rats

The representative plasma concentration-time profiles of lumefantrine after oral (10 mg/kg) and I.V. (0.5 mg/kg) administration in rats are shown in Fig. 4.3.3. and the pharmacokinetic parameters of lumefantrine are listed in Table 4.3.3. After oral administration
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of lumefantrine, the $C_{\text{max}}$, $T_{\text{max}}$, $t_{1/2}$ and $\text{AUC}_{0-120h}$ of lumefantrine were 1230±78.5 ng/ml, 2.75±1.5 h, 50.41±8.79 h and 14134.09±2517.71 h*ng/ml, respectively.

In addition, after I.V. bolus injection of lumefantrine at 0.5 mg/kg, the $\text{AUC}_{0-120h}$, $t_{1/2}$, CL, and $V_d$ were 9189.76 ± 1372.42 h*ng/ml, 30.92 ± 4.81 h, 0.03 ± 0.02 L/h/kg and 2.40 ± 0.67 L/kg, respectively. The bioavailability (%F) of lumefantrine was found to be 8.27% upon oral administration at 10 mg/kg.

4.3.5.2.2. Effect of Co-administered Verapamil on the Plasma Pharmacokinetics of Lumefantrine in Rats

The plasma concentration-time profiles of lumefantrine alone or in combination with verapamil at 10 mg/kg are shown in Fig.4.3.4, and the pharmacokinetic parameters are shown in Table 4.3.3. The co-administration of verapamil at 10 mg/kg caused a significant increase in plasma $C_{\text{max}}$ from 1230±78.5 (lumefantrine alone) to 2005±587.68 ng/mL and $\text{AUC}_{0-120h}$ from 14134.09±2517.71 (lumefantrine alone) to 25711.35±2759.15 h*ng/ml, respectively, compared with the control rats receiving lumefantrine alone. The oral bioavailability of lumefantrine was correspondingly increased from 8.27% to 14.02% in verapamil treated rats. The clearance was observed to be same in both the groups (Table 4.3.3.), which indicates that increased bioavailability of lumefantrine is due to the inhibition of intestinal P-gp, there is no effect on systemic disposition.
Table 4.3.3.: The pharmacokinetics parameters (n=5) of lumefantrine after oral administration (10 mg/kg), with or without verapamil (10 mg/kg), and lumefantrine I.V. bolus injection (0.5 mg/kg). Data are the mean ±S.D. (n=5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lumefantrine control (Oral, 10mg/kg)</th>
<th>Lumefantrine oral (10mg/kg) with Verapamil (10mg/kg)</th>
<th>Lumefantrine I.V. (0.5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (0-t) (hr*ng/ml)</td>
<td>14134.09±2517.71</td>
<td>25711.35±2759.15*</td>
<td>9189.76 ± 1372.42</td>
</tr>
<tr>
<td>AUC (0-\infty) (hr*ng/mL)</td>
<td>15769.25±2754.50</td>
<td>26719.68±2687.18*</td>
<td>9529.47± 1283.18</td>
</tr>
<tr>
<td>T(_{max}) (hr)</td>
<td>2.75±1.5</td>
<td>2.75±1.5</td>
<td>----</td>
</tr>
<tr>
<td>C(_{max}) (ng/ml)</td>
<td>1230±78.5</td>
<td>2005±587.679*</td>
<td>----</td>
</tr>
<tr>
<td>T(_{1/2}) (hr)</td>
<td>50.41±8.79</td>
<td>37.72±4.98*</td>
<td>30.92 ± 4.81</td>
</tr>
<tr>
<td>V(_d) (L/kg)</td>
<td>----</td>
<td>----</td>
<td>2.40 ± 0.67</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>0.0525</td>
<td>0.0526±0.002</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>F (%)</td>
<td>8.27</td>
<td>14.02*</td>
<td>----</td>
</tr>
</tbody>
</table>

* p<0.05, significantly different in comparison to lumefantrine control group

Fig 4.3.3.: The representative plasma concentration-time profiles of lumefantrine after oral administration at 10 mg/kg and intravenous bolus injection at 0.5 mg/kg (n=5 per group)
Fig4.3.4.: The representative plasma concentration-time profiles after oral administration of lumefantrine at 10 mg/kg alone and with coadministration of verapamil at 10mg/kg (n=5 per group)

4.3.6. Conclusion

The present study demonstrated the role of P-gp in the absorption of lumefantrine based on in-situ and in-vivo studies. Further research addressing the role of P-gp/MDR1 and other transporters in the disposition and in-vivo distribution of lumefantrine has to be investigated.
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4.4. References

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27. Wong RP, Salman S, IllettKF, Siba PM, Mueller I, Davis TM: **Desbutyl-lumefantrine is a metabolite of lumefantrine with potent in-vitro antimalarial activity that may influence artemether-lumefantrine treatment outcome. Antimicrob Agents Chemother 2011, 55:**1194-1198


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