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1. Bioanalytical Method Development and Validation:

One of the prerequisites for successfully carrying out pre-clinical as well as clinical pharmacokinetic studies is the availability of reliable, reproducible, accurate and precise bioanalytical methods. Thus we have developed and validated LC-MS/MS methods for our compounds of interest. The methods were validated as per the Food and Drug Administration (FDA) Guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The following methods have been developed and validated:

- LC-ESI-MS/MS method for the simultaneous quantification of Lumefantrine and its metabolite Desbutyl-Lumefantrine in rat plasma
- LC-ESI-MS/MS method for the simultaneous quantification of Lumefantrine and 99-411 in rat plasma
- LC-ESI-MS/MS method for the simultaneous quantification of Lumefantrine and 97-78 in rat plasma
- LC-ESI-MS/MS method for the simultaneous quantification of Piperaquine and 99-411 in rat plasma
- LC-ESI-MS/MS method for the simultaneous quantification of Piperaquine and 97-78 in rat plasma

All the developed methods found to have acceptable in terms of sensitivity, precision, accuracy, selectivity and stability as per the US-FDA guidelines. The recoveries for analytes and internal standard were found to be consistent and reproducible, with minimum interference and ion suppression. All the validated methods were successfully applied for pharmacokinetic and interaction studies.
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2. *In-vivo* Pharmacokinetic Studies:

- Intravenous pharmacokinetics, oral bioavailability, dose proportionality and *in-situ* permeability of anti-malarial lumefantrine in rats

Lumefantrine is an antimalarial drug. A single dose of 10, 20 or 40 mg/kg of this drug was given orally to male rats (N=5 per dose level) to evaluate dose proportionality. In another study, a single intravenous bolus dose of lumefantrine at 0.5 mg/kg was given to rats (N=4) through the lateral tail vein in order to obtain the absolute oral bioavailability and clearance parameters. Blood samples were drawn at predetermined intervals and the concentration of lumefantrine and its metabolite desbutyl-lumefantrine in plasma was determined by partially validated LC-MS/MS method. Plasma concentration versus time data were generated following oral and intravenous dosing and pharmacokinetic analysis was performed using non-compartmental analysis. The peak plasma concentration (Cmax) and area under the plasma concentration–time curve from time zero to time infinity (AUC0-∞) values for lumefantrine were not increased proportionally to the administered dose. For nominal doses increasing in a 1:2:4 proportion, the Cmax and AUC0-∞ 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 Cmax and the area under the plasma concentration time curve (AUC0-t) 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 (Vd) 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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- **Gender Differences in Pharmacokinetics of lumefantrine and its metabolite desbutyl-lumefantrine in Rats**

  Lumefantrine has been reported to mainly bio-transformed by cytochrome P450 isozyme 3A4 in to desbutyl-lumefantrine (DLF) in human liver microsomes. Since, CYP3A4 expressed in sex specific manner, it could be expected that the pharmacokinetics of lumefantrine would be changed in male rats compared with those in female rats. Gender differences in lumefantrine and its active metabolite DLF pharmacokinetics was evaluated after intravenous (0.5 mg/kg) and oral (20 mg/kg) administration of lumefantrine to male and female Sprague–Dawley rats. The quantitative bioanalysis was carried out by the partially validated liquid chromatography tandem mass spectrometry method. After intravenous and oral administration of lumefantrine the area under the curve (AUC) of lumefantrine was significantly higher in female rats in comparison to 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 female rats than that in male rats.

- **Role of P-gp in the Absorption of Lumefantrine**

  The determination of permeability of antimalarial drug (lumefantrine) was done using validated single pass intestinal perfusion model. Lumefantrine was found to be high permeable compound as its $P_{eff}$ was found to be $3.56\pm0.51*10^{-5}$ cm/sec, which was close to the US-FDA approved high permeability compounds.

  From the literature, it is well known that lumefantrine has very poor water solubility and from our findings it is a high permeable compound. Thus according to BCS classification system, it could be classified into BCS class II. According to modified BCS classification system, BCS class II compounds could be the substrate of efflux transporters. Furthermore, lumefantrine is a substrate of CYP 3A4. Both CYP3A4 and P-gp are co-localised in the
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intestine and share most of their substrates and inhibitors. Based on these evidences we hypothesized that lumefantrine could be a substrate of P-gp, which may explains its reason for low bioavailability. To check our hypothesis, we carried out _in-situ_ and _in-vivo_ studies and it was found that P-gp is playing significant role in limiting the oral absorption of lumefantrine.

3. _In-vitro_ Studies

- _In-vitro_ Protein Binding Study of Lumefantrine by charcoal adsorption assay

The percent protein binding of lumefantrine was found to be 92.21 ± 1.68%. High plasma protein binding of lumefantrine, may lead to low clearance and long elimination $t_{1/2}$ and $V_d$.

- _In-vitro_ Metabolic Stability of Lumefantrine

The metabolic stability of lumefantrine was determined using male rat liver microsomes in an _in-vitro_ study design. The metabolic stability of lumefantrine in male rat liver microsomes was determined in order to assess the potential of this compound to accumulate in the body due to lacking or negligible metabolic degradation. Approximately, 80 % of the intact lumefantrine remained in incubation mixture at the end of incubation (120 min).

- **Simulated gastric fluid (SGF)/Simulated Intestinal Fluid (SIF) Stability Study of Lumefantrine**

The K values reported indicate that the drug is stable in both the SGF and SIF. Further, Lumefantrine is more stable in the intestinal pH than gastric pH (probably upon oral administration). The stability study in SGF showed that lumefantrine is slightly unstable in acidic pH, which may be the reason for its very low bioavailability in various species following oral administration. SGF and SIF stability studies are very short and simple studies but at the same time provide valuable preliminary information about the presystemic degradation GIT.

- **Red Blood Cell (RBC) uptake study of Lumefantrine**

Since whole blood and not plasma or serum flows through capillaries in the body, it would appear that whole blood and not plasma or serum is the most appropriate fluid for calculating pharmacokinetic parameters. In agreement with this rationale, the routine determination of the
whole blood to plasma ratio was recently proposed for drugs under development [8, 9]. Though moderate in normal, the uptake can be more in diseased stages, as most of the antimalarial drugs are known to have higher RBC uptake in diseased conditions.

4. Interaction studies:
The simplest reason for combining antimalarials is to increase efficacy. Additionally, drug combinations can shorten duration of treatment, hence increasing compliance, and decrease the risk of resistant parasites arising through mutation during therapy. Antimalarial combination chemotherapy is widely advocated and WHO, in particular, encourages the use of artemisinin-containing regimens. Multidrug resistance in parasites has forced the use of combination antimalarial regimens. The need for effective treatments has thrown together antimalarial combinations that have often worked better than monotherapies, though sometimes only temporarily. However, we are still far from discovery of an ideal regimen. Until better regimens become available there are enormous demands on policy makers to choose between existing combinations, many of which have not been sufficiently studied for informed judgments to be made. The available marketed antimalarial combinations combine the rapid schizontocidal activity of an artemisinin derivative (artesunate, artemether or dihydroartemisinin) with a longer half-life partner drugs viz. mefloquine, lumefantrine, halofantrine and piperaquine. The major problems associated with currently available semisynthetic artemisinin derivatives are low oral bioavailability, time dependent pharmacokinetics (major cause of recrudescence), toxicity and high cost (due to poor yield of the artemisinin extraction process) etc.

In the present study, we are reporting the \textit{in-vivo} pharmacokinetic compatibility assessment of trioxane candidate antimalarials (99-411 and 97-78) with longer half-life partner drugs viz. lumefantrine and piperaquine.

\begin{itemize}
  \item [\textbf{Interaction study of Lumefantrine with 99-411 and vice versa}]
  
  In rats, 99-411 co-administration at a dose of 70 mg/kg had no significant effect on pharmacokinetics of LUME. The presence of LUME at a dose of 10 mg/kg significantly
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(p<0.05) increased the AUC\(_{0-\infty}\) (493.06 \%) and C\(_{\text{max}}\) (490.09 \%) of orally administered 99-411. Consequently, the relative bioavailability (RB\%) of 99-411 in the presence of LUME is remarkably increased (493.05 \%) compared to the 99-411 alone. The T\(_{\text{max}}\) was not significantly altered by LUME. 99-411 clearance (Cl/F) was decreased by 82.52 \% and Vd/F decreased by 93.32 \% with 99-411 co-administration; this resulted in a significant decrease in plasma t\(_{1/2}\) (2.13 h versus 4.85 h; P<0.05) since decrease in Vd/F was 2 fold higher than decrease in CL/F. If the results of this study are further confirmed by clinical trials, Lumefantrine can be used clinically in combination with 99-411.

- Interaction study of Lumefantrine with 97-78 and vice versa

In rats, 97-78 co-administration at a dose of 70 mg/kg significantly (P<0.05) decreased the AUC\(_{0-\infty}\) (63.26 \%) of orally administered LUME. Consequently, the relative bioavailability (RB\%) of LUME in the presence of 97-78 is remarkably decreased (64.42 \%) compared to the LUME alone. The T\(_{\text{max}}\) and C\(_{\text{max}}\) were not significantly altered by 97-78. LUME clearance (Cl/F) was increased by 64.84 \% and Vd/F increased by 62.89 \% with 97-78 co-administration. As both apparent CL/F and apparent Vd/F were increased almost proportionally, there was no significant effect on the t\(_{1/2}\) of LUME in presence of 97-78 (47.81 h versus 46.94 h). LUME co-administration at a dose of 10 mg/kg significantly decreased the T\(_{\text{max}}\) of 97-78 (2.2 h versus 0.5 h; p<0.05). LUME had no significant effect on other PK parameters of 97-78.

- Interaction study of Piperaquine with 99-411 and vice versa.

In rats, 99-411 co-administration at a dose of 70 mg/kg significantly (p<0.05) decreased the AUC\(_{0-\infty}\) (61.39 \%) and C\(_{\text{max}}\) (61.67 \%) of orally administered PPQ. Consequently, the relative bioavailability (RB\%) of PPQ in the presence of 99-411 is remarkably decreased (61.39 \%) compared to the PPQ alone. PPQ clearance (Cl/F) was increased by 41.94 \% and Vd/F increased by 194.49 \% with 99-411 co-administration; this resulted in a significant increase in plasma t\(_{1/2}\) (41.67 h versus 21.44 h; p<0.05) since increase in Vd/F was 4.6 fold higher than increase in CL/F.

PPQ co-administration at a dose of 50 mg/kg significantly (p<0.05) increased the AUC\(_{0-\infty}\) (196.11 \%) and C\(_{\text{max}}\) (202.28 \%) of orally administered 99-411. Consequently, the relative
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bioavailability (RB%) of 99-411 in the presence of PPQ is remarkably increased (196.14 %) compared to the 99-411 alone. The $T_{max}$ was significantly reduced by PPQ (5.25 h versus 8.75 h; P<0.05). 99-411 clearance (Cl/F) was decreased by 56.06 % and Vd/F decreased by 72.95 % with 99-411 co-administration.

➢ Interaction study of Piperaquine with 97-78 and vice versa.

In rats, 97-78 co-administration at a dose of 70 mg/kg significantly (P<0.05) decreased the AUC$_{0-\infty}$ (56.03 %) and C$_{max}$ (50.56 %) of orally administered PPQ. Consequently, the relative bioavailability (RB%) of PPQ in the presence of 97-78 is remarkably decreased (56.03 %) compared to the control. PPQ clearance (Cl/F) was significantly (P<0.05) increased by 91.50 % and Vd/F increased by 223.49 % with 97-78 co-administration; this resulted in a significant increase in plasma $t_{1/2}$ (36.56 h versus 21.44 h; P<0.05) since increase in Vd/F was 5 fold higher than increase in CL/F. PPQ co-administration at a dose of 50 mg/kg had no significant effect on pharmacokinetics of 97-78.