METHODOLOGY

The proposed plan of work is summarized below in phased manner. Research work will be undertaken in a period of 2 years in following phases:

Phase I (0-2 months): Review of Literature

Phase II (3-6 months): Procurement of chemicals and selection/optimization of suitable HPLC and/or LC-MS/MS method.

Phase III (7-12 months)

Development of analytical methods:
Development of analytical method requires a thorough understanding and correlation of available literature and its application to attain a best suitable strategy for chromatographic resolution and detection. Validation of analytical method will performed in accordance to following guidance documents.

- As per (APVMA), Australian pesticides and veterinary medicines authority guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products, October 2004.
- ICH Guidelines: validation of analytical procedures: text and methodology q2 (r1).

Phase IV (13-15 months)

Validation of bioanalytical methods:
Bioanalytical method development leading to method validation is proposed to involve certain essential stability considerations also; besides optimization of selectivity, sensitivity and accuracy. Stability studies involve investigations of drug stability in bio matrices e.g. blood, plasma, tissue homogenate etc. and access drug stability in storage solution/s.

These studies will be conducted by experiment design created with reference to validation guidance issued by US-FDA regulatory agency.


Following experiments will be part of the validation plan

1. **SYSTEM SUITABILITY**

   System suitability test(s) will be performed prior to each day of method validation at the medium QC level. Analytical batch of system suitability will consists of six injections of medium QC level in solution. % CV of Peak Area Ratio (Analyte area/Internal standard area) and retention time will be ascertained for variation.

2. **SELECTIVITY**

   A minimum of six independent lots of blank plasma will be processed and analysed. Similarly, same six lots of blank plasma, spiked at LLOQ (lower limit of quantification concentration), will also be analyzed. Any interference for analyte (with reference to spiked standard sample) will be a determinant of non-selectivity.

3. **AUTOSAMPLER CARRYOVER TEST**

   A sequence comprising of extracted plasma blank (PB), aqueous LLOQ (LLOQ) and extracted ULOQ (Upper limit of quantification) (EXT-ULOQ) samples will be injected to observe whether any carry over is present at the retention times of the analyte and internal standard.

4. **LINEARITY OF CALIBRATION STANDARDS**
A standard curve comprised of at least 8 non-zero standards including lowest and highest concentration in duplicate (excluding Standard Blank and Standard Zero) will be analysed to access linearity of proposed method.

5. **PRECISION AND ACCURACY (PA)**

The precision and accuracy samples will comprise six sets each of the LLOQQC, LQC, MQC and HQC representing the entire standard curve range with concentrations at:

- The lowest concentration, LLOQQC will be slightly ≥ LLOQ (i.e. ~5 to 10% higher)
- LQC will be within 3 times the LLOQ concentration
- MQC will be around the mid-range of the curve
- HQC will be approximately in the upper range of the standard curve (i.e. ≥ 80% of ULOQ)

Three or more PA batches will be analysed to access the precision and accuracy of method.

6. **RECOVERY**

Recovery of the analyte will be determined using 6 replicates at 3 concentrations of LQC, MQC and HQC. The recovery of internal standard will be determined at its working concentration in presence of analyte (combined at 3 levels, LQC, MQC and HQC).

7. **MATRIX EFFECT**

Six interference free plasma samples (from different lots) and one hemolyzed plasma (in duplicate) will be processed and process blanks will be spiked at low and high QC concentration (post-extraction spiking, n = 6 + 2). Aqueous un-extracted (neat solution)
will be prepared at concentration identical to LQC and HQC and matrix factor will be evaluated as per the following formula.

\[
\text{Matrix Factor} = \frac{\text{Peak response in presence of matrix (post-extracted)}}{\text{Mean peak response in neat solution}}
\]

8. **DILUTION INTEGRITY**

QC samples having concentration above ULOQ will be prepared and these QC samples will be diluted with interference-free blank plasma to evaluate the integrity of analyte during dilution. Samples (6 replicates) will be processed along with freshly prepared CC standards and batch QC samples or with any PA batch. Dilution integrity will be evaluated for 5 and 10 folds.

9. **HEMOLYSIS EFFECT**

Working/spiking solutions of low QC and high QC concentrations will be spiked into the interference free hemolyzed plasma to get 6 replicates at each level. These samples will be processed along with freshly prepared CC standards and batch QC samples (in non-hemolysed plasma) or with any PA batch.

10. **STABILITY OF ANALYTE IN MATRIX**

Stability of analyte will be evaluated in biological matrix at LQC and HQC concentrations. The stability samples will be analyzed using a freshly spiked calibration curve and quality control samples (minimum of 2 replicates of LQC, MQC and HQC) or with any PA batch. Stability samples will be prepared and stored in bulk and can be aliquoted as per requirement during the analysis. Six replicates of stability samples will be analyzed per concentration, and mean back-calculated concentrations of stability samples will be compared against mean of first day (initially observed) concentrations for stability evaluation.
**10.1. Bench-top Stability**

Bench-top stability of the analyte will be evaluated at room (19 - 25°C) temperature. Stability will be determined for approximately 4 h or more. Six replicates of stability samples will be analyzed per concentration, and back-calculated concentrations of stability samples will be compared against nominal concentrations for stability evaluation.

\[
\% \text{ Change} = \left( \frac{\text{Mean Conc. of analyte in stability samples}}{\text{Initial Conc. of analyte}} \right) - 1 \times 100
\]

**10.2. Freeze-thaw Stability at -70 ± 10°C in Plasma**

The effect of five freeze-thaw cycles on analyte stability in the matrix will be determined. All stability samples will be frozen at -70 ±10°C in multiple sets for determining stability after the fifth, fourth and third cycles. Stability samples will be stored at the intended storage temperature initially for about 24 h and then thawed unassisted at room temperature (19 - 25°C). When completely thawed (first freeze-thaw cycle), samples will be refrozen for at least 12 - 24 h for continuation of freeze-thaw cycling. Analysis for samples of lower number of cycle will be performed only if analyte is found to be unstable after five freeze-thaw cycles.

**10.3. Long-term Stability**

Long-term stability of the analyte in matrix will be determined at -70 ±10°C for the durations of 30 ± 5 and 60 ± 15 days.
10.4. **Re-injection Reproducibility**

Stability of the analyte in processed sample at auto-sampler temperature will be evaluated by performing re-injection reproducibility.

After analysis of any accepted PA batch, calibration curve standards and quality control samples (LLOQQC, LQC, MQC and HQC) will be stored in the auto-sampler for a minimum of 12 h at the auto-sampler temperature (~15°C or as specified in method of analysis), and the entire batch will be re-injected.

After completion of validation, method will be used for *in vitro* and *in vivo* studies.

**Phase- V (16-24 months)**

**Application of bioanalytical method to *in vitro* and *in vivo* studies:**

Following *in-vitro* and *in-vivo* studies will be performed to understand ADME characteristics:

- **AQUEOUS SOLUBILITY:**
  Solubility determination is measure of solvent capacity to dissolve the solute. For conduct of any study, we need to spike a required concentration of compound in desired medium and a measure of solubility ascertains that level of compound concentration can be added/ maintained in assay conditions. Kinetic solubility is typically a measure of compound solubility in aqueous buffer when added from an existing stock solution in DMSO. [Kerns EH and Di L. (2005)]

- **MICROSOMAL/ METABOLIC STABILITY:**
  The metabolic stability determination using liver microsomes represents a well-established in-vitro model to estimate hepatic clearance. This model has been widely utilized for prediction of hepatic clearance for compound known to possess desired biological activity, accelerating the identification of lead compounds. [Dermot F. et.al. (2004), Obach RS (1999), Riley RJ et.al. (2005)].
• **CYP INHIBITION:**
  The in-vitro pooled human liver microsomes CYP450 inhibition assay is used to evaluate the potential of compounds to cause drug-drug interactions, through inhibition of clinically important CYP450 isoforms. Co-incubation of the probe substrate with the test inhibitor allows the calculation of percent inhibition/IC50 measurement. A decrease in the formation of the metabolite(s) compared to the vehicle control is used to calculate the inhibition/IC50 value.[ Miia Turpeinen et.al. (2004), Dierks EA et.al. (2001), Chu I et.al. (2000), Mankowski DC (1999) and Rendic S et.al. (1997)].

• **PERMEABILITY:**
  P-glycoprotein (P-gp) is the most widely studied drug transporter due to its potential role in drug disposition and efficacy, and drug-drug interactions (DDI). It is abundantly expressed in both the intestinal wall and blood-brain barrier where it serves as a drug permeability barrier while simultaneously facilitating drug elimination in the liver and kidney. It is also abundantly expressed in tumors where it can facilitate the elimination of chemotherapeutics, a phenomenon known as multidrug resistance (MDR). Permeability of drug across Madin Darby canine kidney (MDCK) monolayer (MDR1 transfected) is a measure of its oral absorption and indicative of its mechanism of transport. Apparent permeability of test compound from the basal to apical and apical to basal side of the cellular monolayer will be determined for efflux possibilities. [C L Lam K et.al. (2012)].

• **PLASMA PROTEIN BINDING:**
  The extent of drug binding to plasma proteins influences the way drug distributes in body tissues. Fraction unbound for plasma binding is an indicator of higher proportion of free drug available for pharmacological action. Plasma protein binding will be determined by equilibrium dialysis method. [Goodman and Hilman’s (1996) and Kariv I et.al. (2001)].
• PHARMACOKINETIC STUDY:

Sprague dawley (SD) rats will be dosed to evaluate pharmacokinetic profile. The plasma concentration vs time data will be transcribed into a Microsoft® Excel spreadsheet. The pharmacokinetic parameters will be calculated using WinNonlin Enterprise 5.3 / Phoenix 1.3 (Pharsight Co., Mountain View, CA, USA). Plasma concentrations less than limit of quantification will not be considered for calculation. Individual/mean plasma concentration vs. time and pharmacokinetic data will be tabulated and presented in graphical form, where appropriate. The $C_{\text{max}}$ and $t_{\text{max}}$ will be direct observations from the plasma concentration vs. time data. $C_0$ (for IV) will be calculated by extrapolating the initial distribution phase log transformed in plasma concentrations versus time graph extending the best-fit line back to the y-axis (preferably initial 3 time points) using Microsoft excel. The bioavailability ($F$) will be calculated from dose adjusted $\text{AUC}_{0-\text{inf}}$ value. In cases, where the differences between $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\text{inf}}$ is more than 20% (for p.o. or i.v. or for both arm), or there is no $\text{AUC}_{\text{inf}}$ value (due to a lack of a defined terminal phase), $F$ will be calculated using $\text{AUC}_{0-t}$. 