Electronic Supplementary Material (ESI) for RSC Advances.


## Method

### 1. Validation procedures

Validation of the method was performed in accordance to the FDA guidelines for the assay in rat plasma [40].

#### *1.1. Specificity and selectivity*

The specificity of the method was assessed by analyzing plasma samples collected from six different rats to examine the interferences at the retention times of the peaks for analyte and IS using the proposed extraction procedure and chromatographic-MS conditions.

#### *1.2. Matrix effect*

The effect of plasma constituents in the ionization of S002-333 and IS was evaluated by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat standard samples (10 µL of required working stock sample spiked into 190 µL of acetonitrile instead of blank plasma) at the same concentrations[39-41]. The matrix effect for S002-333 was evaluated at QC low, QC medium and QC high concentrations whereas the matrix effect over the IS was determined at a single concentration of 500 ng/mL.

#### *1.3. Calibration curve*

The final concentrations of calibration standards obtained for plotting the calibration curve were 1.25, 1.56, 3.12, 12.5, 25, 100, 400 ng/mL. A plot of ratio of peak area (response) of S002-333 to that of IS against the concentration of calibration standards was used to prepare the calibration curve. The slope, intercept and the correlation coefficient of calibration curve were determined using linear regression analysis. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [40].

#### *1.4. Precision and accuracy*

The intra- and inter-day precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 1.25, 6.25, 50 and 200 ng/mL. The intra-day precision of the assay was estimated by calculating the relative standard deviation (RSD) for the analysis of QC samples in six replicates and inter-day precision was determined by the analysis of six replicates QC samples on three consecutive days. The accuracy was calculated on the basis of the given formula (mean concentration found/ concentration taken)×100. The acceptance criteria of the data in terms of accuracy and precision of the bioanalytical method is clearly defined by FDA guidelines for bioanalytical method validation [40].

## Results

### 2. Validation procedures

#### *2.1. Matrix effect, specificity and selectivity*

Due to interference of matrix seen in quantitative analysis of the biological sample, it has become essential to evaluate the matrix effect in bioanalytical validation [29, 30]. The chromatographic interferences were assessed by comparing chromatograms of blank plasma with that of sample spiked with S002-333 and IS in rat plasma and no interference was seen. The matrix effect for compound S002-333 at 6.25, 50 and 200 ng/mL concentration levels in rat plasma was <±15% (109.21 – 97.31%). The assessment of specificity and selectivity was carried out using independent plasma samples from six different rats. The supplementary figure 2

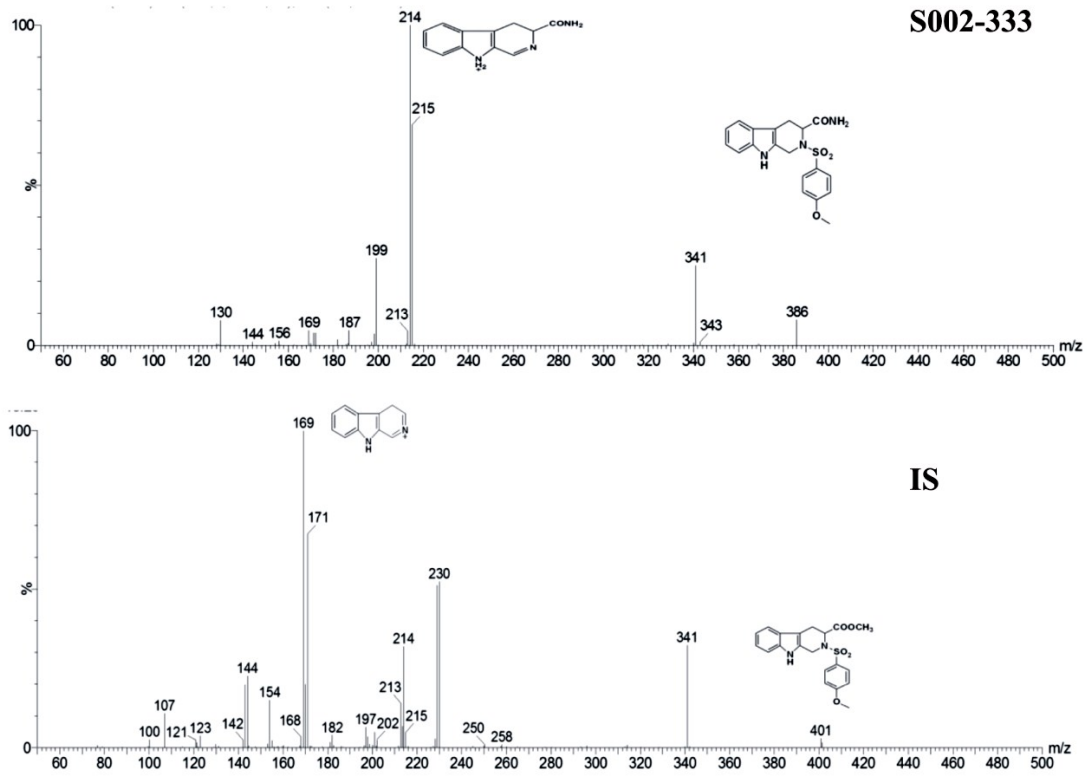
demonstrates a comparison of chromatogram representing the blank rat plasma, rat plasma spiked with S002-333 at LLOQ and IS, *in vivo* sample obtained at 2 h after oral administration of S002-333 in aqueous suspension and 1 h after oral administration of S002-333 in its liposomal formulation CH-LIP-F9. The chromatographic run time was 5 min with retention time of 1.43 and 3.77 min for S002-333 and IS, respectively.

***2.2. Calibration curve***

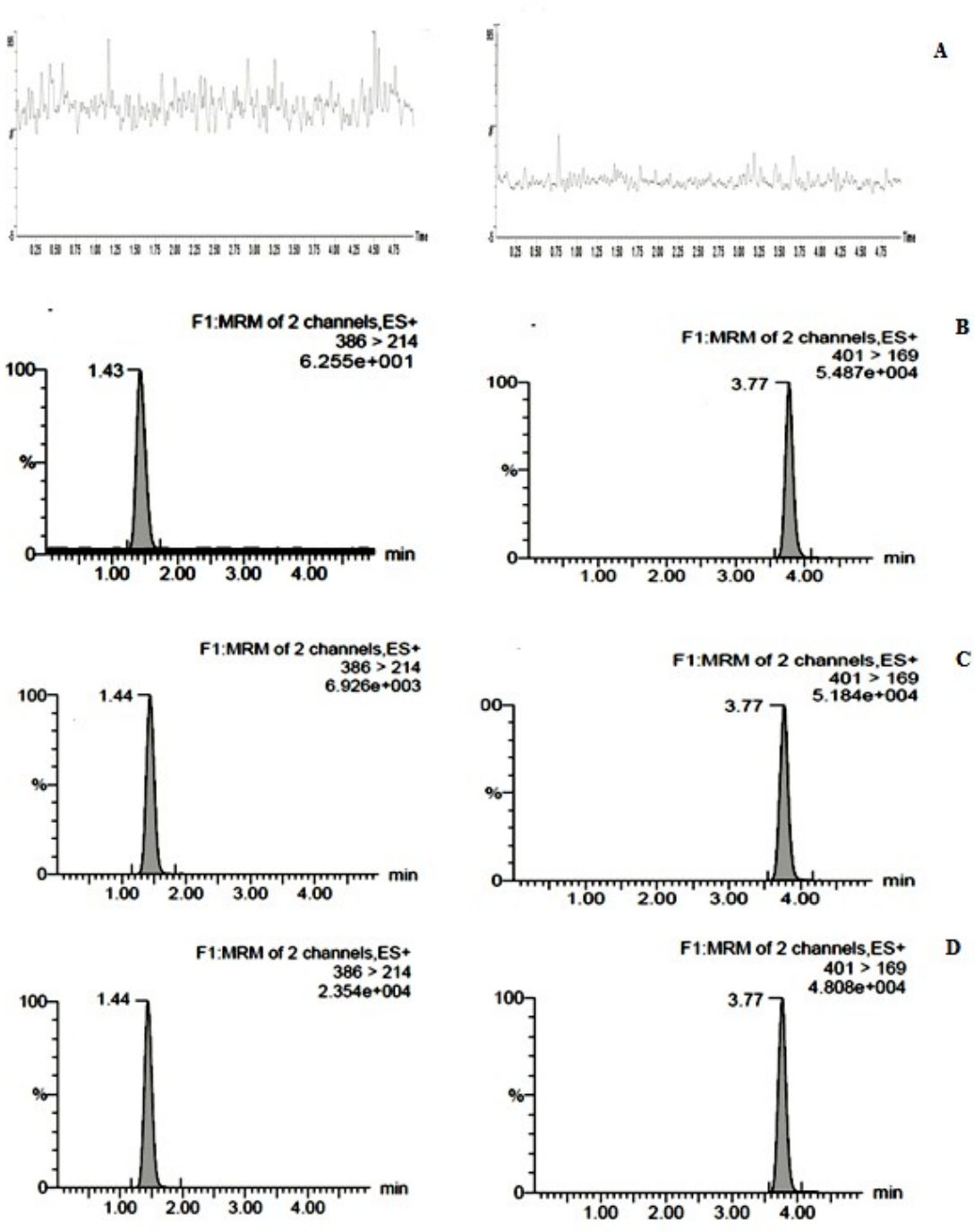
The calibration curve in plasma (n=3) showed a linear relationship between peak area ratios (peak area analyte/peak area IS) and concentration over the range of 1.2 – 400 ng/mL for compound S002 333. The standard curve was prepared using seven calibration standards and the curve was found to be reproducible over the entire calibration range. The correlation coefficient ($r^2$) of the calibration curve was found to be ≥0.997. The lowest concentration with R.S.D. < 20% was taken as LLOQ and was found to be 1.2 ng/mL. The LLOQ was measured according to the FDA guidelines (RSD < 20%) and was found to be 1.2 ng/mL.

***2.3. Accuracy and precision***

Accuracy and precision data for intra- and inter-day assay for plasma samples are given in Table 2. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.




**Supplementary figure 1:** MS/MS spectra of S002-333 and IS showing prominent precursor to product ion transitions.




**Supplementary figure 2:** Typical MRM chromatograms of S002-333 (left panel) and IS (right panel) in (A) rat blank plasma, (B) rat plasma spiked with S002-333 at LLOQ (1.2 ng/mL) and IS (500 ng/mL) (C) a 2 h *in vivo* plasma sample showing S002-333 peak obtained following oral dose of S002-333 in aqueous suspension and (D) a 1 h *in vivo* plasma sample showing S002-333 peak obtained after oral dose of the liposomal formulation CH-LIP-F9.

**Table 1:** Pharmacokinetic parameters of S002-333 from CH-LIP-F9 and aqueous suspension of group I, group II and group III of CH-LIP-F9 and Aqueous suspension.

| **Pharmacokinetic Parameter** | **CH-LIP-F9** | | | **Aqueous suspension** | | |
|---|---|---|---|---|---|---|
| | **Group I** | **Group II** | **Group III** | **Group I** | **Group II** | **Group III** |
| $t_{1/2}$ (h) | | 25.75±0.58 | | | 17.83±0.46 | |
| $AUC_{0-t}$ (h ng/ml) | | 7016.02 ±128.96 | | | 2382.02 ±77.17 | |
| $AUC_{0-\infty}$ (h ng/ml) | | 8576.10 ±158.33 | | | 2644.61 ±82.20 | |
| $C_{max}$ (ng/ml) | | 531.55± 36.2 | | | 241.39± 29.72 | |
| $T_{max}$ (h) | | 1±0.23 | | | 2±0.74 | |