Supplementary material

The downfall of TBA-354 – a possible explanation for its neurotoxicity via mass spectrometric imaging

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Figure S1 Chemical structures of TBA-354 (A: target analyte) and pretomanid (B: internal standard).

**Preparation of standards and quality control samples**

The 100 µg/ml stock solutions of TBA-354 and the internal standard (IS) were prepared separately in MeOH and stored at -20 °C where they were found to be stable. A series of TBA-354 working standard solutions were prepared by diluting the stock solution using the extraction solvent (ACN or MeOH) along with the IS working standard solution at 5 µg/ml. For calibration standards, 100 µL of blank samples from untreated Sprague-Dawley rats were spiked with working standard solutions of TBA-354 (50 µL) and the IS (50 µL). The TBA-354 concentrations were starting from 20 to 1500 ng/mL and 10 to 1400 ng/g for plasma and brain homogenates, respectively with the IS kept at 250 ng/mL in each sample. Following the same procedure, four quality control (QC) samples were investigated, including the lower limit of quantification (LLOQ), low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC) samples.

**Method validation procedures**

The method used in this study was validated according to the European Medicines Agency (EMA) guidelines on bioanalytical method validation (E.M.A., 2009). Validation parameters covered in this study include specificity, selectivity, linearity, LLOQ, accuracy, precision, matrix effect, recovery, and the stability.
Specificity and selectivity
The experiments conducted to evaluate the specificity and selectivity of the method involved comparison of the chromatograms from six different sets of blank biological samples obtained from six different sources. Each blank biological sample was tested under the LC-MS/MS conditions mentioned above to evaluate if there were any interfering peaks at the retention times of TBA-354 and the IS. Possible interferences may come from endogenous compounds in the biological sample.

Linearity and LLOQ
The calibration curves for quantification of TBA-354 in rat biological samples were constructed by plotting the peak area ratio of analyte to IS against the theoretical concentrations of the analyte, using $1/x^2$ weighted linear regression. The LLOQ was defined as the lowest quantifiable concentration in the calibration range with a minimum signal-to-noise (S/N) ratio of 5:1.

Accuracy and precision
The evaluation of intra-day and inter-day precision and accuracy involved analysis of four QC samples in six replicates. Accuracy and precision were expressed in terms of percentage of concentration found to the nominal concentration and percentage relative standard deviation (% RSD), respectively. According to EMA guidelines, the mean concentration should be within ± 15% of the nominal concentration value for the QC sample, except for the LLOQ which should be within ± 20% of the nominal concentration value.(E.M.A., 2009)

Matrix effect and extraction recovery
Matrix effect was tested for six different lots of drug-free rat plasma and brain homogenates. The matrix effect for each lot was evaluated at LQC, MQC, and HQC ($n = 6$). According to EMA
guidelines, the variability of matrix factor should be within ± 15% (E.M.A., 2009). The extraction recovery of TBA-354 was determined by comparing the peak area from the extracted samples with the peak area from un-extracted samples.

**Stability**

The short-term stability was studied by keeping the QC samples in a bench-top at room temperature for 6 h. Freeze-thaw stability was evaluated after three freeze-thaw cycles. The post-preparation stability was evaluated by analyzing the extracted samples stored in the auto-sampler for 24 h before analysis.

**Results**

**Sample extraction method development and optimization**

SPE is used as a clean-up to remove endogenous substances from biological matrices, which may affect the analysis. Recoveries were determined by using different cartridges and solvents (MeOH or ACN), as protein precipitants. The best recoveries were achieved using the Discovery® DSC-PS/DVB SPE C\textsubscript{18} (100 mg, 1 mL) and MeOH for plasma and Supel™-Select HLB (30 mg, 1 mL) and ACN for brain homogenate.

Table S1. Extraction recoveries of TBA-354 using different SPE cartridges.

| Matrix | Cartridge                        | % Recovery | % RSD |
|--------|----------------------------------|------------|-------|
| Plasma | HLB SPE (30 mg, 1 mL)            | 90         | 4     |
|        | HybridSPE-Phospholipid (30 mg, 1 mL) | 107       | 4     |
|        | C\textsubscript{18} (50 mg, 1 mL) | 104        | 2     |
|        | C\textsubscript{18} (100 mg, 1 mL) | 98         | 4     |
| Brain  | HLB SPE (30 mg, 1 mL)            | 96         | 3     |
|        | HybridSPE-Phospholipid (30 mg, 1 mL) | 92       | 6     |
|        | C\textsubscript{18} (50 mg, 1 mL) | 108        | 9     |
Method validation results

Specificity and selectivity

The detection of TBA-354 (retention time = 6.5 min) and IS (retention time = 5.9 min) was highly selective and there were no interfering endogenous substances at the retention time. The results confirmed that the method is selective for the analysis of TBA-354 in rat plasma (see Figure S2).

Figure S2. MRM chromatogram of (1) 250 ng/mL IS and (2) 500 ng/mL TBA-354 in rat plasma.

Accuracy and precision

The intra-day and inter-day accuracy and precision for TBA-354 in the two biological samples were within variability limits set by EMA (see Table S2). (E.M.A., 2009)
Table S2. Accuracy and precision of the method for TBA-354 in biological samples.

| Sample | Parameter                        | LLOQ | LQC  | MQC  | HQC  |
|--------|----------------------------------|------|------|------|------|
| Plasma | Nominal concentration (ng/mL)    | 20   | 50   | 750  | 1400 |
|        | Average concentration found      | 19.3 | 48.5 | 725.9| 1366.3|
|        | Accuracy (%)                     | 96.5 | 96.9 | 96.8 | 97.59|
|        | % RSD                            | 1.9  | 1.9  | 2.7  | 3.1  |
|        | Intra-day (n = 6)                |      |      |      |      |
|        | Inter-day (n = 6)                |      |      |      |      |
| Brain  | Nominal concentration (ng/g)     | 10   | 30   | 600  | 1200 |
|        | Average concentration found      | 10.2 | 27.4 | 537.2| 1073.2|
|        | Accuracy (%)                     | 101.7| 91.3 | 89.5 | 89.4 |
|        | % RSD                            | 4.9  | 2.4  | 2.8  | 2.1  |

Stability

From the stability study conducted, we found that TBA-354 is stable in any one of the conditions that were investigated since the mean concentrations at each QC level were within ±15% of the nominal concentrations (see Table S3).

Table S3. Stability of TBA-354 in different rat biological samples (n = 6).
| Sample | Nominal concentration (ng/mL or ng/g) | Storage condition | Accuracy (%) | % RSD | Accuracy (%) | % RSD | Accuracy (%) | % RSD |
|--------|--------------------------------------|-------------------|--------------|-------|--------------|-------|--------------|-------|
|        | Bench top for 6 h (RT)               | Auto-sampler, 24 h| Three freeze/thaw cycles |
|        |                                      |                   |              |       |              |       |              |       |
| Plasma | 50                                   | 93.5              | 94.9         | 1.3   | 95.8         | 7.4   |
|        | 750                                  | 92.5              | 93.3         | 1.0   | 90.4         | 4.2   |
|        | 1400                                 | 94.8              | 90.8         | 1.2   | 85.7         | 5.9   |
| Brain  | 30                                   | 92.5              | 102.8        | 1.2   | 99.4         | 5.2   |
|        | 750                                  | 90.4              | 86.9         | 1.5   | 88.7         | 1.7   |
|        | 1200                                 | 93.1              | 94.1         | 0.4   | 92.5         | 0.8   |

RT = room temperature

**MSI**

Figure S4. MSI image at 6.0 h postdose displaying high intensity of m/z 437.1 in the CTX of the rat brain.
References

E.M.A., 2009. European Medicines Agency, C.H.M.P. Committee for Medicinal Products for Human Use [online]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. [Accessed 15 March 2016].