**INTRODUCTION**

**Background**
- Method validation results, which was obtained with samples prepared in human plasma.
- Displayed the method validation results, which was obtained with samples prepared in human plasma.
- Criteria for the method development:
  - Injection Volume: 10 µL
  - Sample Temperature: Stored at –20 °C
  - Columns tested for this study include: Waters® Atlantis® dC18 column, 3 µm.

**Project Goal**
- To develop a LC/MS/MS method for the simultaneous determination of isoniazid and ethambutol in human plasma.

**EXPERIMENTAL CONDITIONS**

**HPLC Conditions**
- Mobile Phase: Water/Methanol 95/5, 0.3% Formic Acid
- Column: Waters Atlantis® dC18, 3 µm, 30 x 2 mm
- Guard Columns: Phenomenex SecurityGuard C18, 5 µm, 4 x 2 mm
- Sample Temperature: Stored at –20 °C
- Analyzed at room temperature
- Flow Rate: 0.2 mL/min
- Injection Volume: 10 µL

**MS Conditions**
- Target M/z: 204, 205
- SIM m/z: 204.1 [M+H]+ and 205.1 [M+H+CH3CN]+
- Collision Energy: 10 eV
- Full Scan m/z: 50-700
- Time Per Scan: 0.25 s
- Drug Structures: Metformin (IS), Isoniazid (INH), Ethambutol (EB)
- Structures shown acceptable peak shape for isoniazid, broad or tall peak for ethambutol.

**Plasma Sample Preparation Procedure**
- Place ready to inject samples on the auto-sampler rack at 22 °C for 24 hours.
- Store standard spiked plasma samples at -20 °C for 30 days.
- Spot the standard plasma samples at 4°C for 30 days prior to analysis.
- Freeze-dry three times (60°C to 70°C) at 0°C prior to analysis.

**METHOD DEVELOPMENT**

**MS Condition Optimization**
- Isoniazid and ethambutol are both basic polar compounds.
- Both target compounds were monitored using both positive and negative ionization modes.
- Significant ion suppression was observed using ESI+.
- Positive ionization mode was chosen to be the ionization mode for the analysis.
- MS/MS Water/water is the chosen mobile phase.
- Injection Volume: 10 µL
- Mobile Phase: Water/Methanol 95/5, 0.3% Formic Acid
- Sample Temperature: Stored at –20 °C
- Columns tested for this study include: Waters® Atlantis® dC18 column, 3 µm.
- Time Per Scan: 0.25 s
- Drug Structures: Metformin (IS), Isoniazid (INH), Ethambutol (EB)
- Structures shown acceptable peak shape for isoniazid, broad or tall peak for ethambutol.

**METHOD VALIDATION IN HUMAN PLASMA**

**Linearity and LLOQ**
- Calibration curves for both analytes in human plasma were constructed with external calibration.
- Method was the internal standard.
- Linearity was achieved from 100 ng/mL to 500000 ng/mL in human plasma.
- LLOQ of isoniazid and ethambutol (LLOQ) was established at 10 ng/mL for both analytes.
- Accuracy equals to [mean measured conc. - spiked conc. – spiked conc.]/spiked conc. × 100%.

**Stability Tests**
- Stability of both analytes in human plasma were evaluated.
- Blank spiked plasma samples, at 50 ng/mL and 4500 ng/mL, were used for stability studies.
- Three replicates per sample per analytical run.
- Spiked samples were stored at -20 °C for 24 hours.
- Short-term stability evaluation:
  - Store ready to inject samples at 4°C for 30 days prior to analysis.
  - Freeze-dry three times (60°C to 70°C) at 0°C prior to analysis.

**Precision and Accuracy**
- Precision was assessed by running QC samples:
  - at three concentration levels (10, 400, and 4500 ng/mL).
- Accuracy was evaluated using linear regression methods at three concentration levels.

**PK STUDY IN CLINICAL TRIAL**
- Twenty two healthy male volunteers were included in the study.
- An oral dosage tablet was given to each of the volunteers.

**CONCLUSIONS**
- An APCI LC/MS/MS method was developed and validated for the simultaneous determination of isoniazid and ethambutol in human plasma.
- The major advantage of this method is the sample preparation procedure, the rapidity of the method, and the reproducibility of the drug analysis.
- This method was successfully applied to several pharmacokinetic studies for evaluation of efficacy and safety of isoniazid and ethambutol, and was found to be adequate and reliable.