

## INTRODUCTION

## Overview

- Described an LC/MS/MS quantification method developed for the simultaneous detection of isoniazid and ethambutol in human plasma.
- Displayed the method validation results, which was obtained with samples prepared in human plasma.
- Displayed the results of the LC/MS/MS method applied to a clinical trial.
- This is the very first assay protocol ever reported for the simultaneous detection of the isoniazid and ethambutol in human plasma.

## Project Goal

- To develop a LC/MS/MS quantification method for the simultaneous determination of isoniazid and ethambutol in human plasma.
- Criteria for the method development:
  - Plasma sample preparation needs to be simple and fast so that it can be easily adapted to large batches of samples.
  - The LC/MS/MS method needs to be sensitive and specific enough so that it can be easily applied to clinical trials.

## EXPERIMENTAL CONDITIONS

## HPLC Conditions

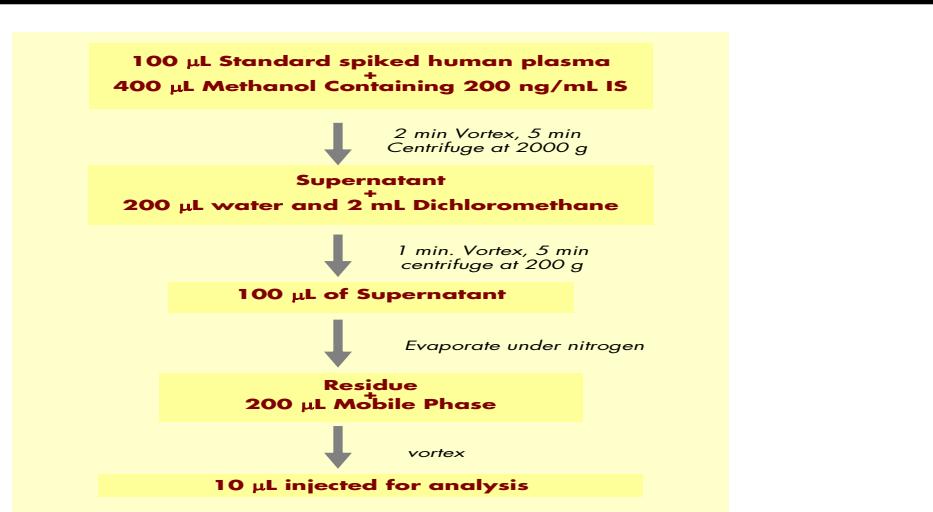
- Mobile Phase: Water/Methanol 90/10, 0.3% Formic Acid
- Column: Waters® Atlantis™ dC<sub>18</sub> column, 3 μm
- Guard Column: Phenomenex SecurityGuard C<sub>18</sub>, 5 μm, 4 x 2.0 mm
- Sample Temperature: Stored at -20°C, Analyzed at room temperature
- Flow Rate: 0.2 ml/minute
- Injection Volume: 10 μL

## MS Conditions

- Tune Page Parameters:
  - Ionization Mode: APCI<sup>+</sup>
  - Corona Current (μA): 4.0
  - Heated Capillary Temperature (°C): 320
  - Vaporizer Temperature (°C): 420
  - Sheath Gas (Arb): 35
  - Auxiliary Gas (Arb): 8
- SRM transitions: 0.25s dwell each transition
 

Isoniazid	m/z 138 > 79	CE 30v
Ethambutol	m/z 205 > 116	CE 20v
Metformin (IS)	m/z 130 > 60	CE 20v

## Plasma Sample Preparation Procedure



## Matrix Effect After The Sample Prep

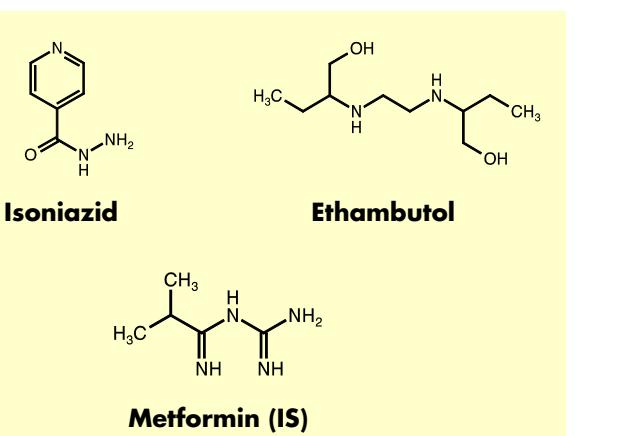
- No significant interference from endogenous substances was observed at the retention time of the analytes.
- Two other drugs were co-administered in clinical trial. They were rifampicin and pyrazinamide. No interferences from these two compounds were observed
  - selectivity obtained by running SRM MS detections.
  - Possible interference from these two drugs was also further reduced by the dichloromethane wash after the protein precipitation

## METHOD DEVELOPMENT

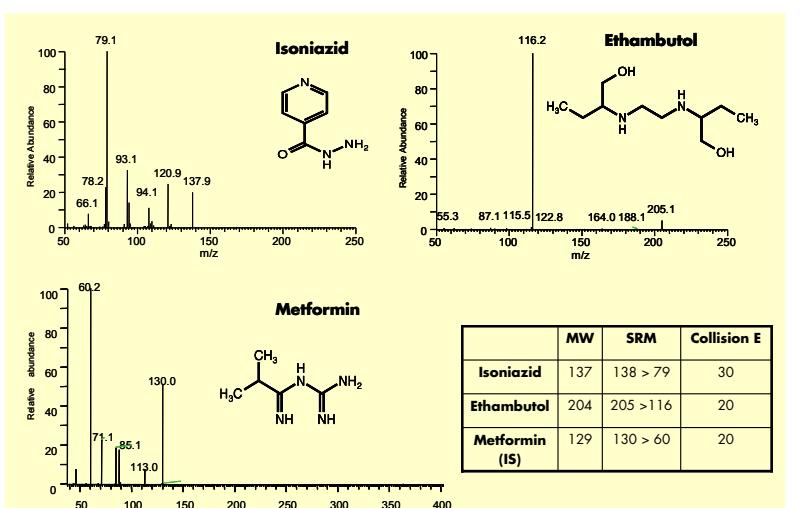
## MS Condition Optimization

- Isoniazid and ethambutol are both basic polar compounds.
  - Both prefer positive ionization mode.
  - Both showed higher signal in ESI vs. APCI.
- Significant ion suppression was observed using ESI<sup>+</sup>. Hence APCI<sup>+</sup> was chosen to be the ionization mode for the analysis.
- Methanol/water was the chosen mobile phase:
  - Acetonitrile/water vs Methanol/water compared
  - Isoniazid showed weak [M+H]<sup>+</sup> ion in acetonitrile/water, most abundant ion was the solvent cluster ion [M+H+CH<sub>3</sub>CN]<sup>+</sup>.
  - Strong [M+H]<sup>+</sup> ions were shown for both analytes in Methanol/water mobile phase.

## Chemical Structures of Standards

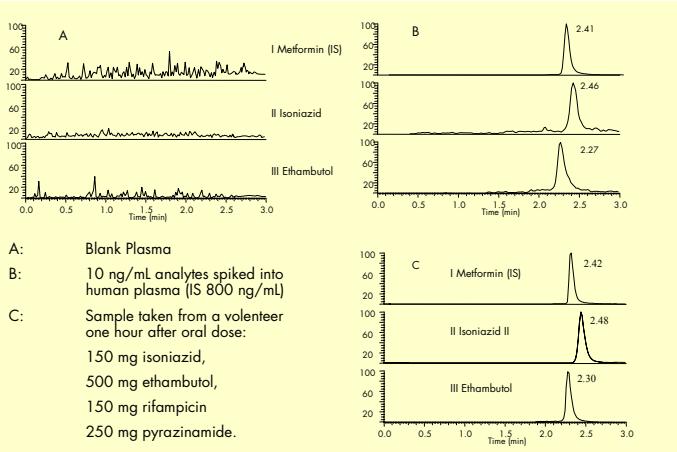


## Daughter Ion Spectra of Standards



## HPLC Condition Optimization

- Isoniazid and ethambutol are both basic polar compounds
  - May have interactions with acid silanol groups on a silica based HPLC column.
  - Can result in tailing and ghost peaks as well as irreproducible retention times.
- Columns tested for this study include:
  - Diamnosil C<sub>18</sub>
  - Phanomenex Luna C<sub>18</sub>
  - Zorbax AB C<sub>18</sub>
  - Capcell Pack C<sub>18</sub>
- These columns shows acceptable peak shape for isoniazid, but broad or tailing peak for ethambutol.
- The final column of choice was the Waters Atlantis dC<sub>18</sub> column, which gave symmetric peak shape for both analytes.



## METHOD VALIDATION IN HUMAN PLASMA

## Linearity and LLOQ

- Calibration curves for both analytes in human plasma were constructed with internal calibration
    - Meformin was the internal standard
    - Linear range for both analytes was 10.0 – 5000 ng/mL in human plasma.
  - Lower Limit of Quantification (LLOQ) was established at 10.0 ng/mL for both analytes.
  - Equations of the calibration curves were
    - Isoniazid:  $y^a = 2.484 \times 10^2 + 2.292 \times 10^{-3}x^b$   $r^2 = 0.9970$
    - Ethambutol:  $y = 0.881 \times 10^2 + 2.872 \times 10^{-3}x$   $r^2 = 0.9980$
- a. Y = Relative peak area (analyte vs internal standard)  
b. X = Measured plasma concentration of the analytes

## Stability Tests

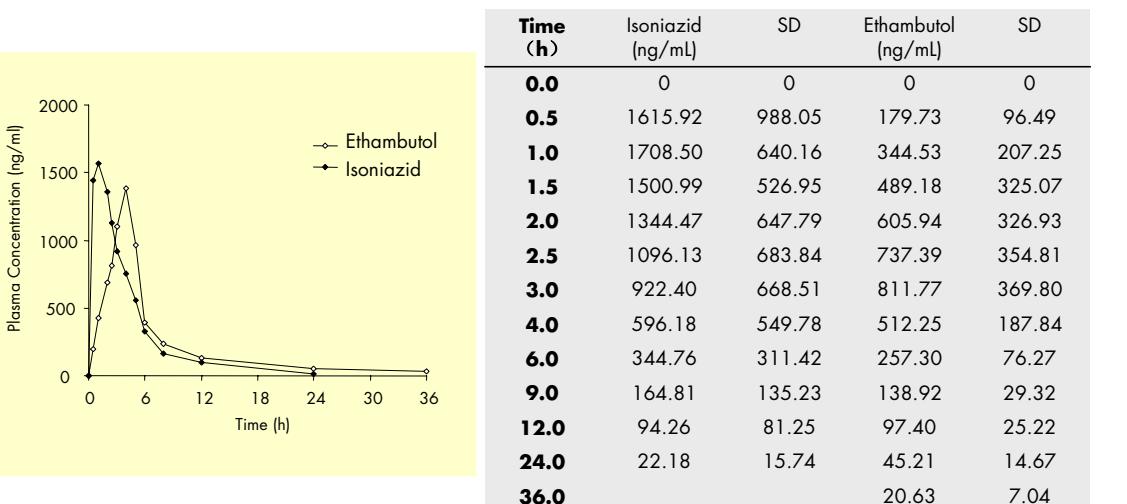
- Stabilities for both analytes in human plasma were evaluated
  - Standard spiked plasma samples at 50 ng/mL and 4500 ng/mL were used for the evaluation
  - Three replicates per sample per analyte
  - Results compared with freshly prepared plasma samples
- Short-term stability evaluation
  - Expose the standard spiked plasma samples at 22°C for **two hours**
  - Place ready to inject samples on the auto-sampler rack at 22°C for 24 hours
- Long-term stability evaluation
  - Store standard spiked plasma samples at -20°C for **30 days** prior to analysis
- Freeze-thaw stability evaluation
  - Freeze-thaw three cycles (-20°C to 22°C) on three consecutive days prior to analysis

## Precision and Accuracy

- Precision and accuracy was assessed by running QC samples
  - at three concentration levels (10, 400, and 4500 ng/mL).
  - 6 replicates per concentration level repeated on 3 separate days.
  - Independent calibration curves run on each of the validation days.
- Accuracy equals to [(mean measured conc.-spiked conc. – spiked conc.)/spiked conc.] x 100
- Precision for each QC level was the relative standard deviation (RSD)
- Requirements for precision and accuracy are
  - For intra- and inter-day assays: precision below than 15%, accuracy within  $\pm 15\%$  except at LLOQ.
  - At LLOQ, precision should be below 20% and accuracy within  $\pm 20\%$ .

	Concentration (ng/ml)		RSD/%	Relative error (%)
	Added	Found (mean)		
<b>Isoniazid</b>	10.0	10.2	4.8	5.7
	400.0	406.4	5.1	2.4
	4500	4553	4.8	2.1
<b>Ethambutol</b>	10.0	10.4	6.4	4.5
	400.0	400.2	4.1	0.1
	4500	4558	4.4	1.9

## PK STUDY IN CLINICAL TRIAL



## CONCLUSIONS

- An APCI<sup>+</sup> LC/MS/MS method was developed and validated for the simultaneous determination of isoniazid and ethambutol in human plasma.
- The major advantages of this method are the simple sample preparation, the speed of separation, the efficiency obtained by analyzing two drugs simultaneously, which is an important characteristic when dealing with large batches of samples.
- This method was successfully applied to several pharmacokinetic studies for multi-component formulations containing isoniazid, ethambutol and other components, and was found to be adequate and reliable.