Method Validation for the Simultaneous HPLC/MS/MS Quantitation of Midazolam and 1-Hydroxymidazolam in Human Plasma
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Overview
- Purpose: Validate an HPLC/MS/MS method to measure ratios of midazolam and 1-hydroxymidazolam in human plasma
- Methods: Liquid-Liquid extraction and HPLC/ESI/MS/MS (API3000)
- Results: Range from 0.3 to 80 ng/mL with accuracies and precision better than ±15%

Introduction
Midazolam and 1-hydroxymidazolam are important compounds in drug metabolism for phenotyping and/or drug interaction studies. Like midazolam, more than 50% of all drugs are metabolized by the P-450 CYP3A4 enzyme. Thus measurement of the ratios of midazolam and 1-hydroxymidazolam in the plasma of patients dosed with midazolam can determine their phenotype and if co-administered with another pharmaceutical can determine CYP3A4 drug-drug interactions. A rapid and sensitive HPLC/MS/MS method is needed to measure the ratios of midazolam and 1-hydroxymidazolam in human plasma.

Methods
- Extraction
  - Liquid-Liquid extraction (500 µL) from 100 µL of human plasma in 96 well plates
  - Extract, transfer organic, evaporate and reconstitute in 100 µL of mobile phase
- HPLC
  - Gradient HPLC from 25 to 90% organic in 2 minutes
  - 2 mM ammonium formate 0.1% formic acid and MeOH mobile phase at 0.6 mL/minute
  - Advantage Armor (Analytical Scale and Service) C5 2.1x30 mm column
  - Thirty µL Injections
- Mass Spectrometry
  - Scieix API3000 operating in MRM mode
  - Turbolonspray (400 °C)
  - Positive ion mode
  - MRM transitions - 326 → 291 (midazolam)
  - 342.2 → 202.7 (1-hydroxymidazolam)
  - 316.2 → 270.1 (conazepam internal standard)

Short-Term Stability
- Bench top stability - midazolam and hydroxymidazolam deviate less than 15% from T0 control concentrations when left on bench top for eight hours
- Freeze thaw stability - midazolam and hydroxymidazolam deviate less than 15% from T0 control concentrations when taken through three freeze-thaw cycles
- Autosampler stability - midazolam and hydroxymidazolam deviate less than 15% from original QC concentrations when re-injected up to 24 hours later

Conclusions
- Validated HPLC/MS/MS method to quantitate the ratios of midazolam and 1-hydroxymidazolam from human plasma
- Method is precise and accurate with quantitative recoveries
- Can process over 500 samples per day