

# 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  $\pm 15\%$

## 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

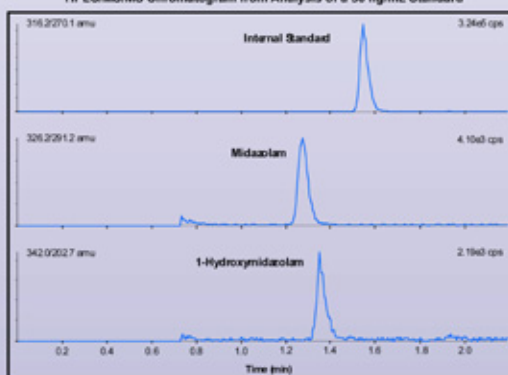
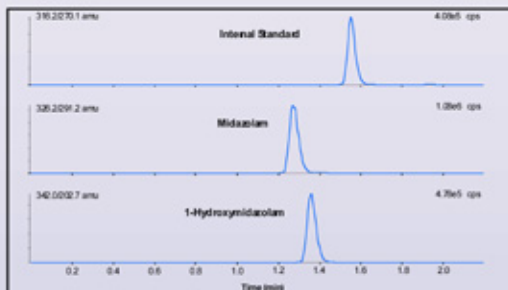
- Liquid-Liquid extraction (500  $\mu$ L) from 100  $\mu$ L of human plasma in 96 well plates
- Extract, transfer organic, evaporate and reconstitute in 100  $\mu$ L of mobile phase

### 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 Sales and Service) C8 2.1x30 mm column
- Thirty  $\mu$ L injections

### Mass Spectrometry

- Sciex API3000 operating in MRM mode
- Turboionspray (400 °C)
- Positive ion mode
- MRM transitions -
  - 326  $\rightarrow$  291 (midazolam)
  - 342.2  $\rightarrow$  202.7 (1-hydroxymidazolam)
  - 316.2  $\rightarrow$  270.1 (clonazepam-internal standard)



3 Different Days-5 Different Lots of Plasma	Midazolam QC Theoretical Concentrations (ng/mL)		
	0.600 (n=18)	5.00 (n=18)	65.0 (n=18)
Calculated Mean (ng/mL)	0.639	5.30	61.6
CV (%)	4.83	5.86	7.49
Accuracy (%)	106.6	106.0	94.8

3 Different Days-5 Different Lots of Plasma	1-Hydroxymidazolam QC Theoretical Concentrations (ng/mL)		
	0.600 (n=18)	5.00 (n=18)	65.0 (n=18)
Calculated Mean (ng/mL)	0.612	5.24	64.7
CV (%)	6.12	7.91	8.84
Accuracy (%)	102.0	104.8	99.5

## 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 reinjected 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