

## Overview

### Purpose:

- Develop and validate a method for simultaneous quantitation of midazolam and  $\alpha$ -hydroxymidazolam in human plasma.
- Validate selectivity and stability in the presence of coadministered drug.

### Methods:

Protein precipitation and HPLC-MS/MS (Sciex API 4000™).

### Results:

- Concentration range of 1.00 to 1000 ng/mL with accuracies and precision better than  $\pm$  15%.
- Demonstrated selectivity and stability in the presence of coadministered drug.

## Introduction

The short-acting, benzodiazepine drug midazolam is commonly used as a probe-substrate for drug-drug interaction studies involving cytochrome P450-3A4. The most important enzyme for drug metabolism in humans, CYP-3A4 participates in metabolizing an estimated 52% of drugs.<sup>1</sup> In order to provide bioanalysis for coadministered interaction studies, an HPLC-MS/MS method was developed and validated to quantify midazolam and its major metabolite  $\alpha$ -hydroxymidazolam in human plasma. The validated method was then applied to analyze clinical pharmacokinetic study samples.

## Methods

### Extraction:

- 50  $\mu$ L sample volume.
- Acetonitrile precipitation with 250  $\mu$ L of deuterated midazolam-D<sub>4</sub> and  $\alpha$ -hydroxymidazolam-D<sub>4</sub> internal standard working solution (5.00 ng/mL).
- 100  $\mu$ L supernatant transfer.
- 100  $\mu$ L addition of water.

### HPLC:

- Gradient HPLC using acetonitrile with 1% formic acid (v/v) and water with 1% formic acid (v/v) mobile phases.
- Flow Rate: 0.7 mL/min
- HPLC Column: HS C18 5cm x 2.1mm, 3 $\mu$ m (Supelco)
- Column Temperature: 50°C

### Mass Spectrometry:

- Sciex API 4000™ operating in MRM mode.
- ESI
- Positive Ion Mode
- MRM Transitions (m/z):  
Midazolam: 325.7  $\rightarrow$  291.3  
Midazolam-D<sub>4</sub>: 330.1  $\rightarrow$  295.2  
 $\alpha$ -Hydroxymidazolam: 341.8  $\rightarrow$  203.1  
 $\alpha$ -Hydroxymidazolam-D<sub>4</sub>: 346.0  $\rightarrow$  203.1

**Table 1:** Midazolam &  $\alpha$ -Hydroxymidazolam Validation Summary

Analyte	Midazolam	$\alpha$ -Hydroxymidazolam
Methodology	HPLC-MS/MS	
Matrix	Human Plasma	
Anticoagulant	K <sub>2</sub> EDTA	
Internal Standard (IS)	Midazolam-D <sub>4</sub>	$\alpha$ -Hydroxymidazolam-D <sub>4</sub>
Extraction Method	Acetonitrile Precipitation	
Validated Range	1.00 – 1000 ng/mL	
Calibration Model	Quadratic 1/x <sup>2</sup> Regression	
Standard Concentrations	1.00, 2.00, 10.0, 50.0, 100, 500, 900, 1000 ng/mL	
QC Concentrations	3.00, 45.0, 800 ng/mL	
Precision (% CV)		
Intrabatch:	1.8% to 9.4%	3.2% to 10.8%
Interbatch:	3.6% to 9.3%	4.0% to 11.3%
Accuracy (% Bias)		
Intrabatch:	-8.7% to 6.7%	-13.0% to 2.0%
Interbatch:	-6.6% to 6.0%	0.0% to 5.3%
Recovery:	Analyte: 81.5% to 89.1% IS: 93.3% to 102.0%	Analyte: 88.9% to 101.2% IS: 103.3% to 118.0%
Processed Sample Stability	67 hr at 10 °C	
Freeze-Thaw Cycles	Up to 4 cycles	
Benchtop Stability	Up to 6 hr	
Long Term Stability	288 days at -70 °C	

**Table 2:** Coadministered Drug Stability & Selectivity Experiments

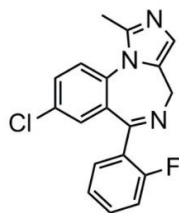
Analyte	Midazolam	$\alpha$ -Hydroxymidazolam
Precision (% CV)	2.9% to 5.4%	1.3% to 5.3%
Accuracy (% Bias)	6.0% to 9.0%	0.7% to 11.3%
Freeze-Thaw Cycles	Up to 4 cycles	
Benchtop Stability	Up to 6 hr	
Long Term Stability	294 days at -70 °C	

## Conclusions

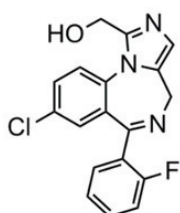
- Method can be utilized to quantify midazolam and  $\alpha$ -hydroxymidazolam simultaneously in human plasma by HPLC-MS/MS.
- Method is robust, simple, selective, and rapid with a short total run time of only 4.5 minutes.
- Validated stability and selectivity in the presence of coadministered drug.
- Successfully applied to analyze clinical pharmacokinetic study samples:
  - 600 plasma samples analyzed for midazolam,  $\alpha$ -hydroxymidazolam, and coadministered drug concentrations in one week.
  - 93% batch passing rate.
  - Demonstrated reproducibility upon incurred sample reanalysis.

## References

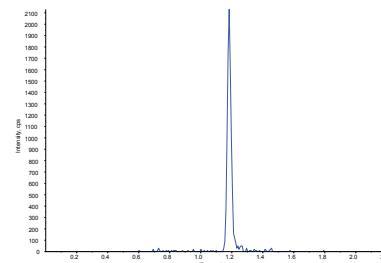
1. P. Anzenbacher, E. Anzenbacherova, Cell. Mol. Life Sci. 58 (2001) 737.



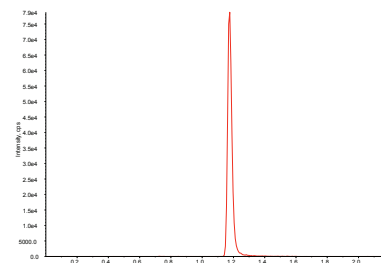
**Structure 1:** Midazolam



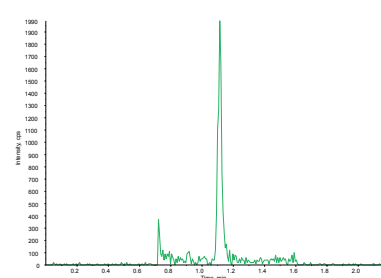
**Structure 2:**  $\alpha$ -Hydroxymidazolam



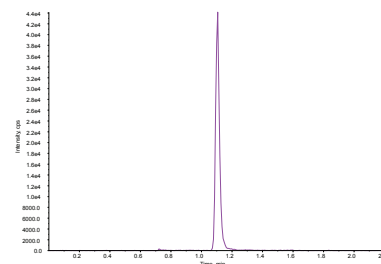
**Figure 1:** HPLC-MS/MS Chromatogram of Extracted Midazolam (1.00 ng/mL)



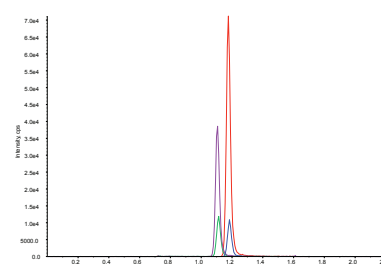
**Figure 2:** HPLC-MS/MS Chromatogram of Extracted Midazolam-D<sub>4</sub> Internal Standard



**Figure 3:** HPLC-MS/MS Chromatogram of Extracted  $\alpha$ -Hydroxymidazolam (1.00 ng/mL)



**Figure 4:** HPLC-MS/MS Chromatogram of Extracted  $\alpha$ -Hydroxymidazolam-D<sub>4</sub> Internal Standard



**Figure 5:** HPLC-MS/MS Chromatogram of Extracted 5.00 ng/mL Sample