Validation of an HPLC/MS/MS Bioanalytical Method for the Quantitative Analysis of Alprazolam, α-OH-Alprazolam and 4-OH-Alprazolam in Human Plasma

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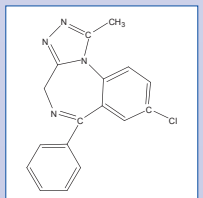
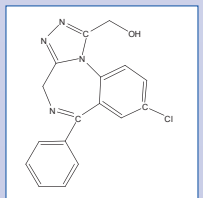
Overview

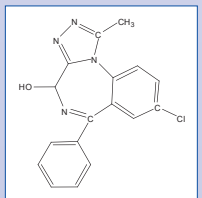
- **Purpose** - Develop and validate an HPLC/MS/MS method to determine concentrations of Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam in human plasma
- **Methods** – 96 well-plate SPE extraction and HPLC/MS/MS (API4000)
- **Results** – Range from 0.025 - 20 ng/mL with accuracies and precision better than +/-15% using HPLC/MS/MS

Introduction

Alprazolam is a benzodiazepene, which is used for treatment of anxiety and other disorders. Two metabolites of alprazolam, 4-OH-alprazolam and α-OH-alprazolam, have been shown to be active. To accurately study the pharmacokinetics, all three analytes should be quantified from plasma. Under new dose regimens with alprazolam, concentrations of 4-OH-alprazolam and α-OH-alprazolam could b e <0.05 ng/mL in plasma 24 hours post-dose. Previous LC/MS/MS assays quantified alprazolam and only α-OH-alprazolam to the 0.05 ng/mL level. Other assays quantified the drug and both metabolites to the 0.05 ng/mL level; however, the assay suffered from poor precision (>15% RSD). Here we report on an accurate and precise validated LC/MS/MS assay for the determination of alprazolam, α-OH-alprazolam and 4-OH-alprazolam from human plasma.


Alprazolam
Molecular Weight: 308.76


α-OH-Alprazolam
Molecular Weight: 324.76


4-OH-Alprazolam
Molecular Weight: 324.76

Methods

Extraction

- Alkalinize plasma sample
- SPE using Strata-X 96 well plate
- Elute with acetonitrile, then dry down
- Reconstitute in 8:2 H_2O:Organic

HPLC

- Binary Gradient 20% to 95% over 4.3 minutes.
- Flow rate = 0.5 mL/minute
- HS C18 50X2.1 mm, 3 µm (Supelco)
- Forty µL injections

Mass Spectrometry

- Sciex API4000 operating in MRM mode
- ESI Positive ion mode
- MRM transitions for Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam, respectively:
  - 309 ➔ 204
  - 325 ➔ 216
  - 325 ➔ 239

Table 1. Typical Standard Curve and QC Results for the HPLC/MS/MS Analysis of Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam from Human Plasma.

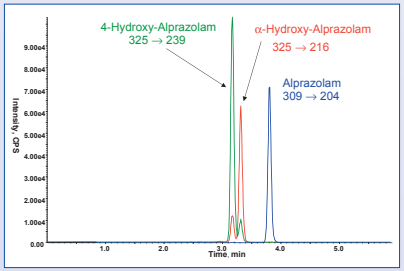
Correlation Coefficient Alprazolam: 0.9977
Correlation Coefficient α-Hydroxy-Alprazolam: 0.9988
Correlation Coefficient 4-Hydroxy-Alprazolam: 0.9983

Curve Conc. (ng/mL)	Alprazolam Calc. Con. (ng/mL)	Alprazolam **Accuracy (%)**	α-Hydroxy-Alprazolam Calc. Con. (ng/mL)	α-Hydroxy-Alprazolam **Accuracy (%)**	4-Hydroxy-Alprazolam Calc. Con. (ng/mL)	4-Hydroxy-Alprazolam **Accuracy (%)**
0.025	0.0266	**106**	0.0240	**96.2**	0.0248	**99.2**
0.025	0.0229	**91.5**	0.0248	**99.3**	0.0238	**95.2**
0.075	0.0790	**105**	0.0804	**107**	0.0855	**114**
0.075	0.0747	**99.5**	0.0791	**105**	0.0767	**102**
0.5	0.542	**108**	0.522	**104**	0.518	**104**
0.5	0.520	**104**	0.506	**101**	0.506	**101**
3	3.15	**105**	3.09	**103**	3.00	**100**
3	3.10	**103**	3.01	**100**	2.94	**98.1**
16	15.3	**95.4**	15.4	**96.4**	15.5	**96.8**
16	15.0	**93.8**	15.3	**95.5**	15.2	**95.1**
20	18.7	**93.5**	18.6	**93.1**	19.2	**96.0**
20	18.8	**94.1**	19.6	**98.1**	19.7	**98.3**
0.075 QC	0.0807	**108**	0.0819	**109**	0.0769	**103**
0.075 QC	0.0745	**99.3**	0.0734	**97.8**	0.0812	**108**
3 QC	3.12	**104**	3.02	**101**	3.02	**101**
3 QC	3.16	**105**	3.11	**104**	3.13	**104**
16 QC	15.0	**94.0**	15.2	**95.3**	15.1	**94.2**
16 QC	15.4	**96.4**	15.4	**96.5**	15.7	**98.0**

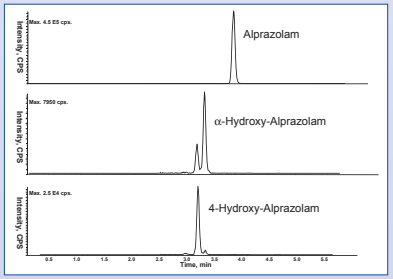
Table 2. Short-Term Stability Results for the HPLC/MS/MS Analysis of Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam from Human Plasma.

QC Level (ng/mL)	**Freeze Thaw Stability** (% Difference from Control)	**Benchtop Stability** (Hours)	**Extract Stability** (Hours)
	Alprazolam		
0.075-16.0	+/-15	> 6	> 21
	α-Hydroxy-Alprazolam		
0.075-16.0	+/-15	> 6	> 21
	4-Hydroxy-Alprazolam		
0.075-16.0	+/-15	> 6	> 21

HPLC/MS/MS Chromatogram from the Analysis of a 3 ng/mL Standard of Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam Extracted From Human Plasma



HPLC/MS/MS Chromatogram from the Analysis of a Human Plasma Sample



Conclusions

- Developed and validated an HPLC/MS/MS method to quantify Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam in human plasma
- Method supports human PK studies for Alprazolam, α-Hydroxy-Alprazolam, and 4-Hydroxy-Alprazolam