# Quantification of midazolam in KIKO mouse plasma by a novel and validated LC-MS/MS method

Anella Tabafunda
Mentored by Dr. Robert diTargiani

SMA Science and Mathematics Academy

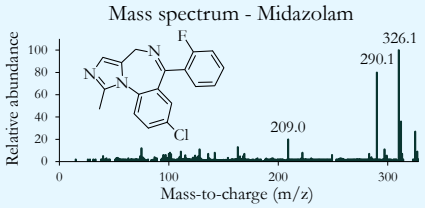
US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD

## Introduction

A pharmacokinetic (PK) study involves drug interaction and movement through its duration of exposure with the observation of drug absorption, distribution, metabolism, and excretion. To conduct an effective PK study, assays are required to be developed and validated, where all chromatographic assay performances must satisfy the Bioanalytical Method Validation Resource guidelines (U.S. Food and Drug Administration, 2018).

Midazolam (MDZ) is a short-acting benzodiazepine used as a countermeasure to combat epileptic seizures induced by a class of organophosphates called chemical warfare nerve agents (CWNAs). Humanized knock-in/knockout (KIKO) mice, modified by researchers at the U.S. Army Medical Research Institute of Chemical Defense (MRICD), are rodent models used to examine CWNAs and develop pharmacological treatments. In this study, liquid chromatography tandem mass spectrometry (LC-MS/MS) was utilized to quantify the concentration of MDZ in mouse plasma with a highly rapid and sensitive mass analyzer. The purpose of this experiment was to develop and validate a precise and accurate LC-MS/MS assay to quantify MDZ in KIKO mouse plasma.

Graph 1 (right): The positive-ion electrospray mass spectrum of MDZ is displayed with the chemical structure. Detection of product ions (m/z 290.1 and 209.0) from the fragmentation of the precursor ion (m/z 326.1) produce extracted ion chromatograms.




## Materials and Methods

The LC method was previously determined (Wessels et al., 2021) with a gradient elution of 20 mM of ammonium formate pH 3.5 (mobile phase A) and 100% methanol (mobile phase B). The MS conditions were optimized to detect MDZ and MDZ-d4 (refer to Tables 1 and 2).

| Analyte | Q1 | Q3 | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| MDZ | 326.1 | 209.0 | 40 | 6 | 48 | 5 |
| | | 290.5 | 40 | 6 | 33 | 5 |
| MDZ-d4 | 330.2 | 295.1 | 40 | 6 | 41 | 5 |
| | | 213.1 | 40 | 6 | 51 | 5 |

Table 1 (above): The MS method is displayed. Positive ion mode was used with multiple reaction monitoring (MRM) transition for quantifying ions 326.1 → 209.0 for MDZ and 330.2 → 295.1 for MDZ-d4.

| MS Source Conditions | |
|---|---|
| Curtain Gas | 30 |
| Ion Spray Voltage (V) | 5500 |
| Temperature (°C) | 60 |
| Ion Source (Gas 1) | 30 |
| Ion Source (Gas 2) | 30 |

Table 2 (above): Electrospray ionization was optimized for the detection and dissociation for both analytes.

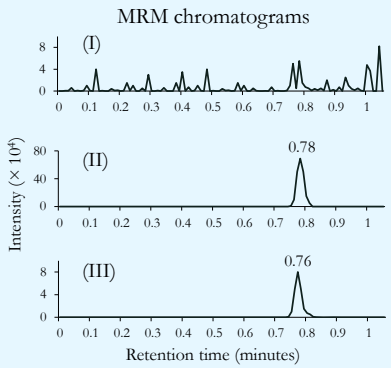
A stock solution of 1 mg/mL of MDZ in methanol and two working solutions (100 ng/mL and 1000 ng/mL) were prepared and stored in −20 °C before use. Calibration standard samples were prepared by spiking MDZ solutions into plasma. The calibration curve consisted of seven calibrators with a range from 1 to 100 ng/mL. Quality control (QC) samples were prepared at three concentration levels (low, medium, and high) with final concentrations of 30, 60, and 80 ng/mL. In each sample, 10 µL of 200 ng/mL of MDZ-d4 in methanol, internal standard (IS), was added. Samples had total volumes of 750 µL.

## Materials and Methods (continued)

Solid phase extraction (SPE) was chosen for sample preparation and purification. SPE wells were pretreated with 1 mL of methanol, followed by 1 mL of Milli-Q water. Wells were then loaded with 500 µL of each sample and washed with 500 µL of Milli-Q water. Analytes were eluted with 500 µL of acetonitrile. The eluate was evaporated under $N_2$ gas and heat. Residues were scraped, vortexed, and reconstituted with 100 µL of mobile phase A and transferred into 1.5 mL autosampler vials. At 60 °C, 1 µL aliquots of each sample were injected into the LC-MS/MS in triplicate for data analysis.

This assay was developed using an Agilent 1290 Infinity High Performance LC, coupled with a SCIEX QTRAP 6500 MS. Analytes were separated using a Halo C18 column (50 mm × 2.1 mm, 2.7 µm). An extraction manifold was used with Waters Oasis HLB 30 mg (1 cc) cartridges for interday SPE. A Waters Oasis HLB 96-well plate (30 µm) was used on an ASPEC Positive Pressure Manifold for intraday SPE. The QCs were collected daily for either an interday (two calibration curves, five sets of QCs) or intraday (one calibration curve, one set of QCs for five days) precision and accuracy study, measured in percent coefficient of variance (% *CV*) and percent error (% error).

## Results




Graph 2 (left): MRM chromatograms were integrated daily for either study through the SCIEX Analyst® Software. The chromatographic profile of blank KIKO plasma demonstrated no detection of MDZ or IS (Graph I). Graphs II and III are extracted ion chromatograms of plasma spiked with MDZ (100 ng/mL) and the IS, respectively. Calibration curves were fit to a linear regression with $R^2$ values of 0.994 ± 0.006. Interpolated QC concentrations were collected for analysis.

## Results (continued)

| Interday QCs | Interpolated Concentrations (ng/mL) Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | *M* | *SD* | % *CV* | % error |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 28.23 | 35.31 | 32.14 | 27.40 | 32.71 | 31.16 | 3.29 | 10.56 | −3.86 |
| 60 | 58.26 | 55.27 | 62.97 | 59.18 | 57.63 | 58.66 | 2.81 | 4.79 | 2.23 |
| 80 | 77.36 | 76.34 | 79.59 | 84.71 | 74.35 | 78.47 | 3.97 | 5.05 | 1.91 |

Table 3 (above): Interday trials were performed successfully throughout five days. For medium and high QCs, % *CV* must be less than 10% and % error must be ± 10%. For the low QCs, % *CV* must be less than 15% and % error must be ± 15%. −3.86

| Intraday QCs | Interpolated Concentrations (ng/mL) Rep 1 | Rep 2 | Rep 3 | Rep 4 | Rep 5 | *M* | *SD* | % *CV* | % error |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 33.01 | 34.51 | 33.99 | 33.65 | 32.71 | 33.57 | 0.73 | 2.17 | −11.91 |
| 60 | 58.89 | 61.34 | 58.02 | 56.75 | 57.63 | 58.52 | 1.75 | 3.00 | 2.46 |
| 80 | 72.34 | 73.47 | 73.78 | 76.04 | 74.35 | 74.00 | 1.36 | 1.83 | 7.50 |

Table 4 (above): Intraday trials were performed successfully with five replicates (rep) in one day. For medium and high QCs, % *CV* must be less than 10% and % error must be ± 10%. For the low QCs, % *CV* must be less than 15% and % error must be ± 15%.

## Discussion

This novel LC-MS/MS assay was developed and validated for the quantification of MDZ in KIKO mouse plasma. All interday and intraday QCs successfully met the FDA guidelines for precision and accuracy. Additionally, a stability test was conducted on a weekly basis for six consecutive weeks under the following conditions: freeze-thaw cycles with storage in −20 °C for aliquots and stock solutions. Samples satisfied the provided guidelines in these conditions.

For future research, an additional assay for the quantification of MDZ and its metabolites in KIKO plasma can be conducted, followed by a PK study on MDZ. The results of the study would provide further examination for *in vivo* metabolism, beneficial for pharmaceutical development in a sufficient animal model.

## Acknowledgements and References

I would like to thank MRICD for welcoming me, Noah Roberts for his continuous guidance throughout my project, and Katie Walker for her thoughtful advisement.

U.S. Food and Drug Administration. Center for Drug Evaluation and Research. (2018). Bioanalytical method validation: Guidance for industry. *Center for Drug Evaluation and Research*. https://www.fda.gov/media/70858/download

Wessels, A. M. A., Bolhuis, M. S., Bult, W., Nijsten, M. W. N., Kneyber, M. C. J., & Touw, D. J. (2021). A fast and simple method for the simultaneous analysis of midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam and 1-hydroxymidazolam glucuronide in human serum, plasma and urine. *Journal of Chromatography B*, *1162*, 122476. https://doi.org/10.1016/j.jchromb.2020.122476