

# Overcoming the Obstacles of Performing Dilutions and Internal Standard Addition to DBS Analysis Using HPLC/MS/MS

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# Overview

- ▶ Purpose To demonstrate an alternative to performing DBS dilutions with blank extract and determine the validity of adding internal standard to the card prior to spotting the sample.
- ► Methods DBS methanol extraction, followed by HPLC/MS/MS (API 4000).
- ➤ Results Range from 0.500-500 ng/mL with accuracies and precision better than ±8% when internal standard is added to the card prior to spotting the sample. Punch size dilutions resulted in accuracies within 15% with precision better than 9.0%.

# Introduction

Dried blood spot (DBS) analysis has emerged as a tool for bioanalysis. Two obstacles that have manifested during DBS analysis are performing dilutions and addition of the internal standard to the DBS card prior to sample spotting. Conventional methods for dilutions and internal standard addition are not optimal as these methods dilute the sample with blank sample extract or add the internal standard in blank solvent during extraction. Experiments were conducted to perform dilutions by reducing the size of the sample punched. A blank punch with a spot the size of the dilution punch removed was extracted with the dilution spot to account for any matrix effects that might have been reduced due to the smaller dilution sample punch. A simple method of adding internal standard to the card prior to spotting the sample was also investigated. Adding internal standard to the card prior to sample addition may compensate for any irregularities encountered during the sample storage, extraction, or analysis.1 HPLC/MS/MS was used for all analyses.

# Methods

### **Extraction**

- Alprazolam, α-Hydroxy-Alprazolam, and Midazolam extracted from human whole blood using a DBS extraction procedure
- Card type: Ahlstrom 226 (ID Biological Systems)
- Internal standard: Stable label internal standards added in extraction solvent and spotted onto card prior to sample addition
- Sample volume: 30 μL
- Punch diameter: 8 mm for standards, 2, 3, and 6 mm for dilutions
- Dilution samples prepared by adding dilution spot as well as an 8 mm blank spot minus the dilution spot
- Solvent: Methanol with and without internal standard

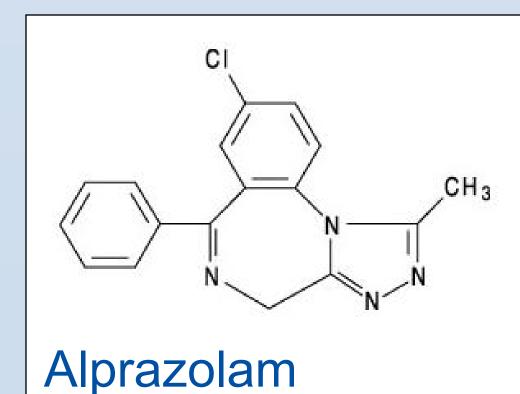
### HPLC

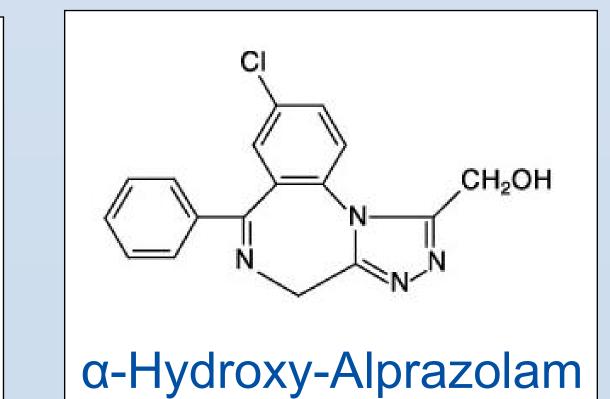
- Gradient HPLC using acetonitrile and water with 1% formic acid
- Flow rate: 0.7 mL/minute
- HPLC Column: HSC18 2.1x50 mm (Supelco)
- Column temperature: 50°C

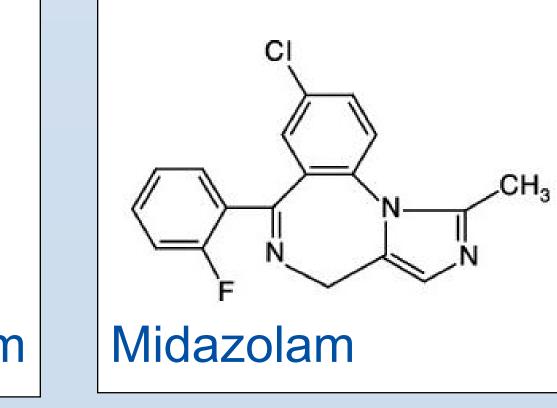
## **Mass Spectrometry**

- Sciex API 4000 operating in MRM mode
- **ESI**
- Positive ion mode
- MRM transitions:

Alprazolam:  $308.9 \rightarrow 280.0$   $\alpha$ -Hydroxy-Alprazolam:  $325.1 \rightarrow 297.7$ Midazolam:  $326.0 \rightarrow 290.3$ 







**Table 1:** Dilution data from utilizing 2, 3 and 6 mm punches from an 800 ng/mL sample. The standard curve was prepared using an 8 mm punch.

	Punch Diameter (mm)	Dilution Factor	Average Accuracy (%)	CV (%)
Alprazolam	2	16.0	88.4	5.1
	3	7.1	96.5	8.9
	6	1.8	85.9	1.6
	Punch Diameter (mm)	Dilution Factor	Average Accuracy (%)	CV (%)
α-Hydroxy- Alprazolam	2	16.0	85.6	6.0
	3	7.1	89.9	2.3
	6	1.8	89.7	3.1
Midazolam	Punch Diameter (mm)	Dilution Factor	Average Accuracy (%)	CV (%)
	2	16.0	87.2	8.1
	3	7.1	90.8	8.7
	6	1.8	87.9	5.0

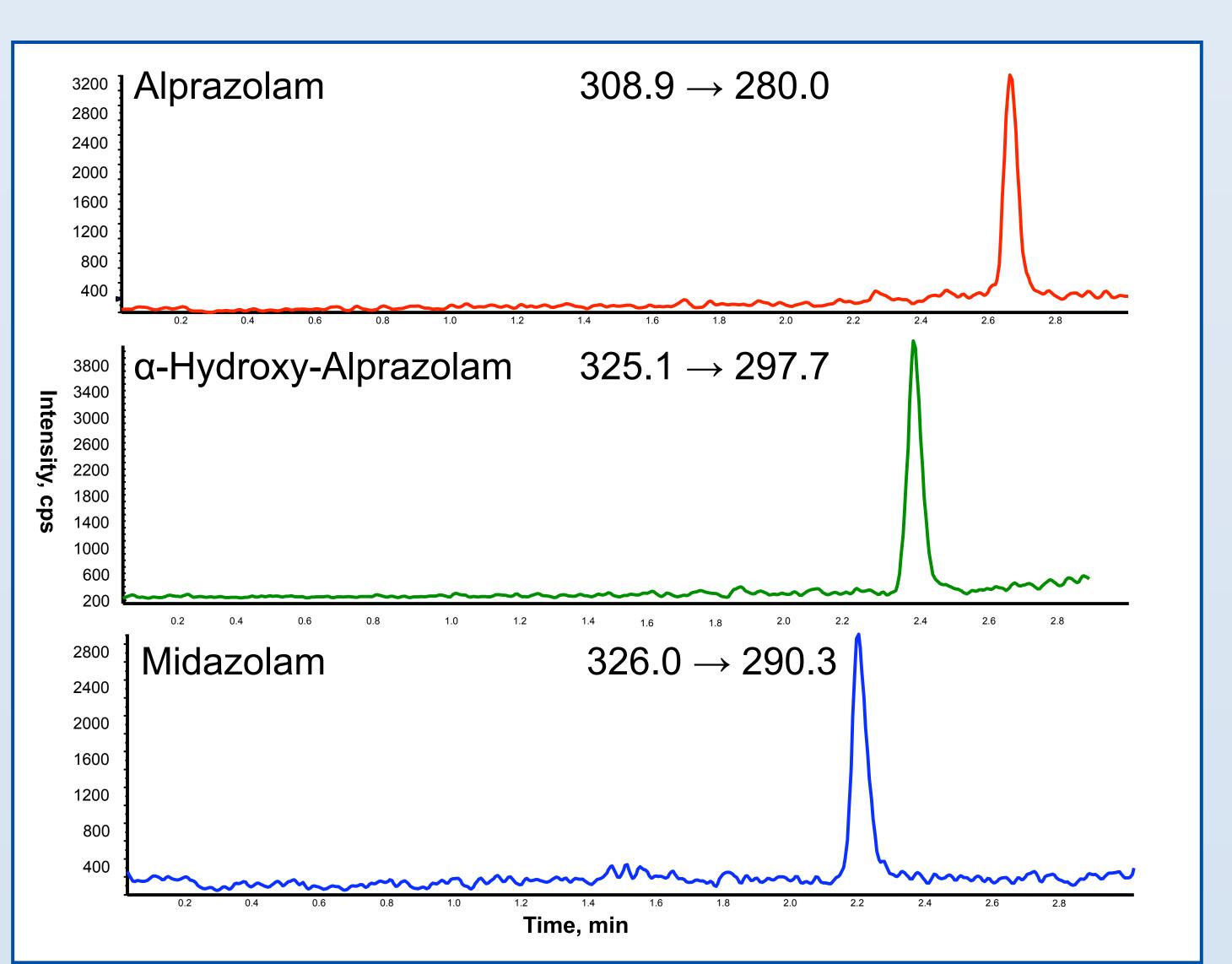


Figure 1. HPLC/MS/MS chromatograms from the analysis of a human DBS sample fortified with 0.500 ng/mL Alprazolam, α-Hydroxy-Alprazolam and Midazolam

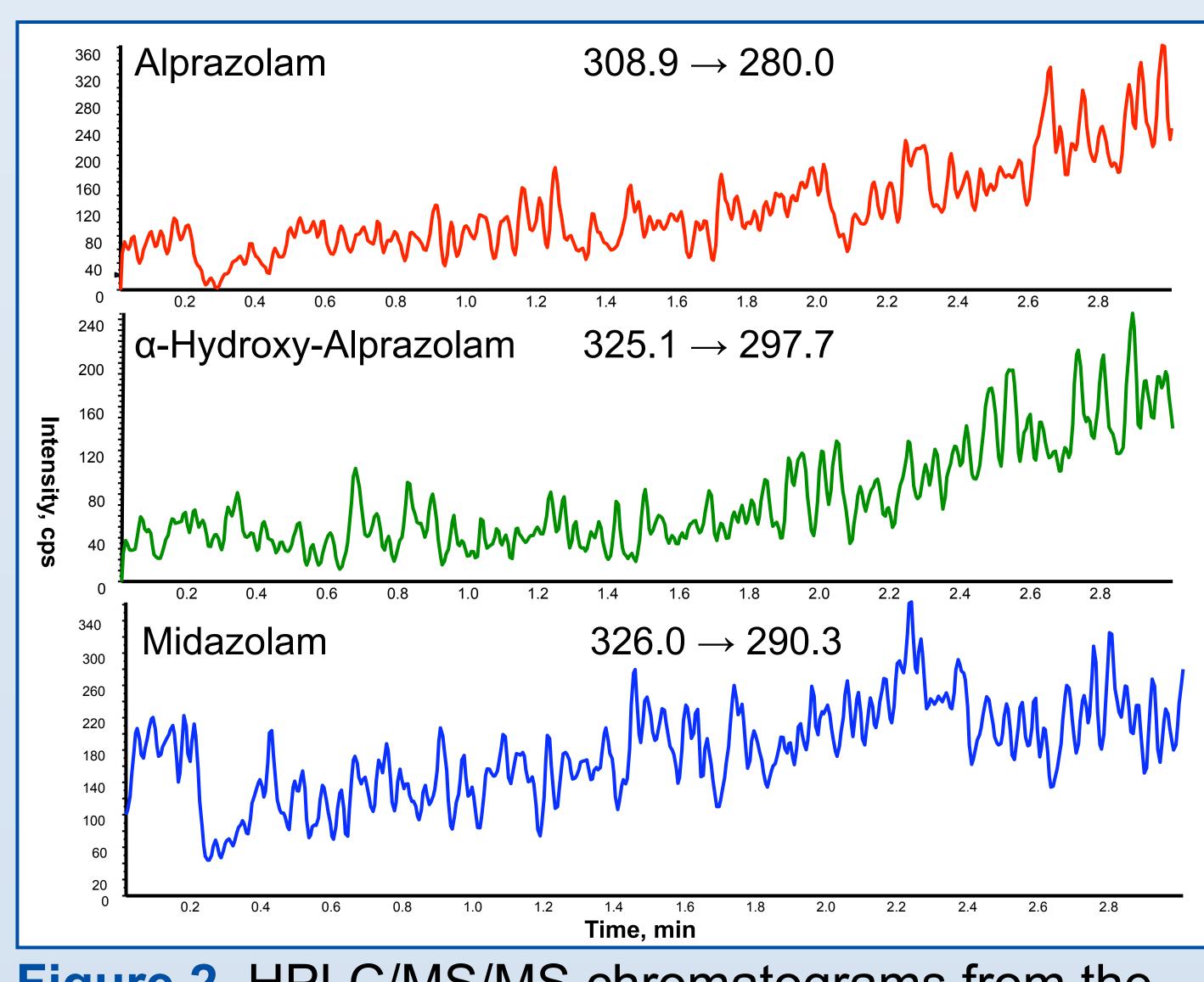


Figure 2. HPLC/MS/MS chromatograms from the analysis of a Blank Human DBS

Table 2. Quality Control results for DBS extractions performed with IS present on the card prior to sample addition and IS added in the extraction solvent.

		Avg Accuracy (%)	CV (%)		
IS ON CARD PRIOR TO SAMPLE ADDITION	Alprazolam				
	1.50 ng/mL	103	5.6		
	10.0 ng/mL	97.3	5.2		
	400 ng/mL	104	7.9		
	α-Hydroxy-Alprazolam				
	1.50 ng/mL	98.9	6.8		
	10.0 ng/mL	97.2	6.0		
	400 ng/mL	105	5.6		
	Midazolam				
	1.50 ng/mL	103	6.6		
	10.0 ng/mL	104	7.7		
	400 ng/mL	103	7.8		

<b>5</b>		Avg Accuracy (%)	CV (%)		
IS ADDED IN EXTRACTION SOLVENT	Alprazolam				
	1.50 ng/mL	105	4.5		
	10.0 ng/mL	100	10.2		
	400 ng/mL	97.4	6.0		
	α-Hydroxy-Alprazolam				
	1.50 ng/mL	108	0.7		
	10.0 ng/mL	98.5	6.6		
	400 ng/mL	95.5	6.3		
	Midazolam				
	1.50 ng/mL	107	1.0		
	10.0 ng/mL	103	6.0		
	400 ng/mL	99.0	4.4		

# Conclusions

Dilutions can be performed accurately and precisely by simply punching a smaller spot from the sample. A blank 8 mm punch minus the dilution punch was used to account for any possible matrix effects removed with the smaller dilution punch.

Internal standard can be added to the DBS cards prior to sample addition, which may allow the internal standard to compensate for any extraction irregularities.

# References

1. Christianson, et al. *Bioanalysis* 2010, 2(11):1829-1837.