Incomplete hydrolysis of midazolam-glucuronide can cause false negatives on urine drug confirmation by liquid chromatography tandem mass spectrometry

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Introduction

Many controlled drugs, including benzodiazepines, undergo conjugation with glucuronic acid during their metabolism to increases their solubility for renal excretion[1]. Current urine drug testing algorithms utilize immunoassay screens with reflex confirmation by liquid chromatography tandem mass spectrometry (LC-MS/MS) for positive or indeterminate results, due to the high false positive rates of drug screening immunoassays[2]. LC-MS/MS is the gold standard for urine drug testing due to the high sensitivity and specificity of this method[3]. Prior to LC-MS/MS analysis, urine samples are hydrolyzed by β-glucuronidase enzymes to remove glucuronide groups[4]. Removal of the glucuronide group increases the sensitivity of the assay, since both parent drug and metabolites can be detected in urine for some drugs and glucuronide metabolites are often unstable in LC-MS/MS assays[4].

Midazolam is a benzodiazepine that is widely used as a sedative, sleep aid and in clinical anesthesia. Both midazolam and its major metabolite α-hydroxymidazolam undergo conjugation with glucuronic acid prior to renal excretion. During the validation of an in-house lab-developed LC-MS/MS assay for benzodiazepines confirmation, we observed poor agreement between midazolam measurements in our method comparison studies. ***We hypothesized that inefficient glucuronidase-mediated hydrolysis may account for the differences in midazolam measurements observed between laboratories.***

Methods

Benzodiazepine confirmation LC-MS/MS assay method comparisons were performed on 20 midazolam positive urine samples collected at University of Virginia Hospital. Sample aliquots were measured in-house and sent to a commercial reference laboratory (Lab 1) and a university hospital clinical laboratory (Lab 2) for analysis. Both midazolam and α-hydroxymidazolam were measured in-house and at the commercial reference laboratory, while the university hospital laboratory only measured midazolam. For quantitative analyses, only samples with concentrations above the lower limit of quantitation were included (11 samples total).

In-house LC/MS/MS measurements were performed on a Waters Acquity I Class Liquid Chromatography module and a Waters Micro TQS API (Atmospheric Pressure Ionization) mass spectrometer. LC was performed on a UPLC C18 column using an acidified acetonitrile gradient at a flow rate of 0.3 mL/min. D4-Midazolam was utilized as an internal standard for all measurements. Multiple reaction monitoring (MRM) scans in positive ion mode of the molecular ion and at least two of the most predominant fragments for each analyte were utilized. Selected MRM transitions for each analyte are listed in Table 1. A standard curve was prepared using midazolam and α-hydroxymidazolam standards to enable quantification (25-2500 ng/mL). Midazolam-glucuronide and α-hydroxymidazolam-glucuronide were measured in unhydrolyzed samples based on their relative response.

Table 1- LC-MS/MS MRM transitions

Analyte	Parent Ion (m/z)	Qualifier Transitions (m/z)
midazolam	326.0	291.1, 209.1,222.1
α-hydroxymidazolam	342.1	203.1, 168.1, 176.0
midazolam glucuronide	502	291, 326
α-hydroxymidazolam glucuronide	518	168, 203.1, 342.1

The efficiencies of 3 different commercially-available β-glucuronidase enzymes for midazolam-glucuronide and α-hydroxymidazolam-glucuronide hydrolysis was assessed (Table 2.). Five midazolam positive urines were pooled and incubated for either 15, 30 or 60 minutes with enzymes prepared according to manufacturer recommendations prior to LC-MS/MS measurement.

Table 2- β-glucuronidase enzymes used for midazolam-glucuronide and α-hydroxymidazolam-glucuronide hydrolysis

Supplier	Enzyme
Sigma Aldrich	β-glucuronidase from *E. coli*
Kura Biotech	BG100 *H. rufescens* β-Glucuronidase
Kura Biotech	BGTurbo high-efficiency recombinant β-glucuronidase

Results

Section 1: The Problem

Large variations in quantitative midazolam measurements between labs

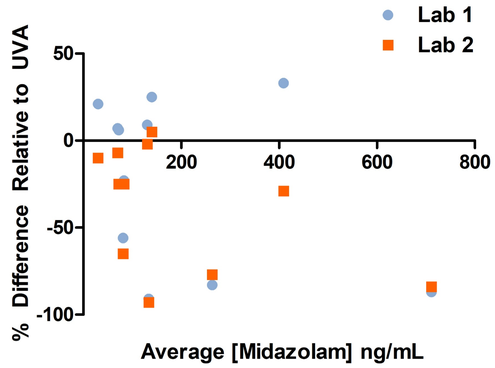

Figure 1- Difference plot showing significant negative biases in midazolam measurements by LC-MS/MS by 2 different labs relative to UVA

Differences in qualitative interpretation of midazolam positivity between labs

	Midazolam Test Result		α-hydroxymidazolam Test Result		Interpretation Agreement
Lab	+	-	+	-	
UVA	19	1	20	0	N/A
Lab 1	14	6	20	0	100%
Lab 2	11	9	N/A	N/A	60%

Table 3- Lab 2 incorrectly identified 9 samples as negative for midazolam, while UVA and Lab 1 correctly identified all samples as positive for midazolam either based on the presence of α-hydroxymidazolam only or both α-hydroxymidazolam and midazolam. The cutoff for a positive result at UVA, Lab 1 and Lab 2 were 25, 20 and 25 ng/mL respectively.

Section 2: The Investigation

Inefficient hydrolysis may contribute to inter-lab differences in midazolam measurements

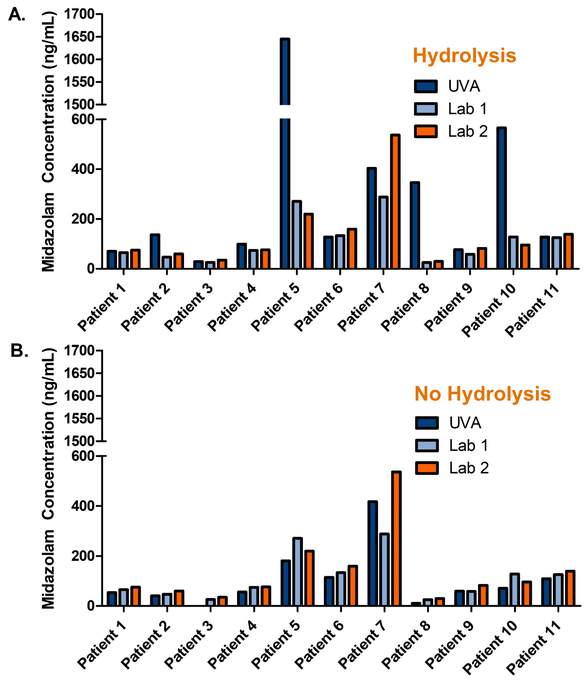

Figure 2- A. Significant differences in midazolam measurements were observed between laboratories. B. UVA midazolam measurements more closely matched measurements from Lab 1 and Lab 2 when no hydrolysis was performed

Higher concentrations of midazolam glucuronide correlate with larger percent differences in midazolam measurements between labs

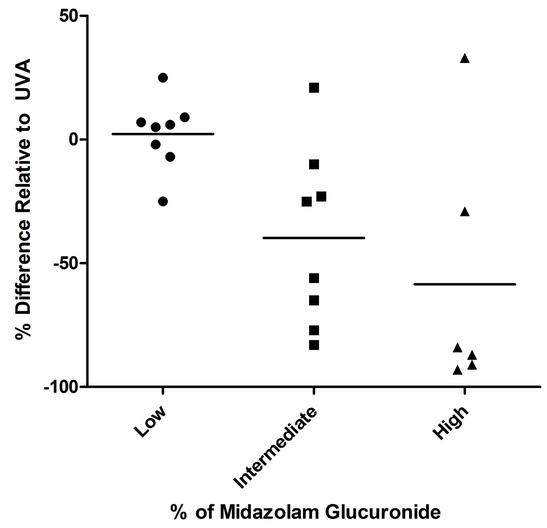

Figure 3- Percentage of midazolam glucuronide in each sample was estimated by dividing the relative response for midazolam glucuronide by the relative response for midazolam. Measurements for samples with lower proportions of midazolam glucuronide agreed more closely and samples with higher proportions differed more significantly between labs. (% of Midazolam Glucuronide- Low: 5-19%, n=8; Intermediate= 23-42%, n= 8, High= 74-100%, n=6)

B-glucuronidase enzymes vary in their efficiency for hydrolysis of midazolam glucuronide

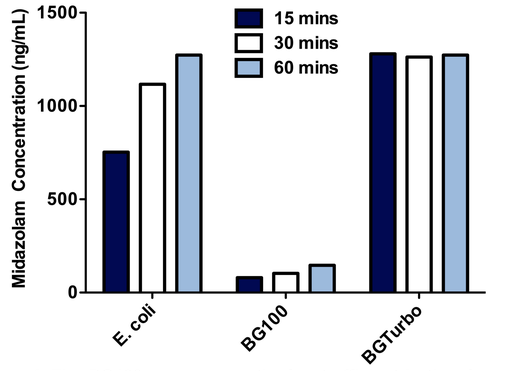

Figure 4. The BG100 enzyme was the least efficient in the cleavage of midazolam glucuronide. The *E.coli* enzyme required a 75% longer incubation than the BGTurbo enzyme to recover equivalent concentrations of midazolam.

Section 3: Relevance

3 out of 10 labs measure midazolam as the only analyte for midazolam confirmation

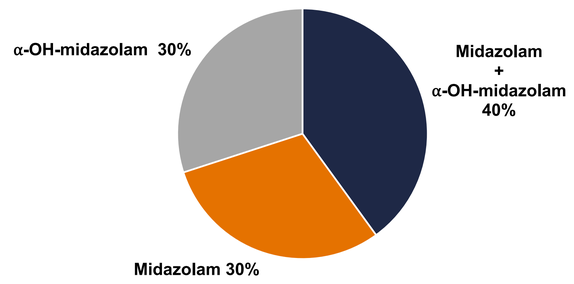

Figure 5- Review of benzodiazepine LC-MS/MS confirmation assays revealed that seven out of ten labs measure α-hydroxymidazolam as a marker for midazolam in urine, with four of those measuring both midazolam and α-hydroxymidazolam. Three labs only measured midazolam

Conclusions

1. Midazolam-glucuronide is susceptible to inefficient hydrolysis, which may lead to underestimation of its concentration in urine by LC-MS/MS
2. α-hydroxymidazolam is a more sensitive marker for midazolam and should be adopted as the primary marker for midazolam detection
3. Several routinely abused narcotics undergo glucuronidation. Failure to detect these narcotics due to inefficient hydrolysis may have life-threatening consequences in instances of drug overdose.

References

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