finden KURA

APPLICATION NOTE

KURA biotech

Benzodiazepines and Metabolites in Urine

Hydrolysis with Finden B-One® Enzyme vs No Hydrolysis

Overview

Benzodiazepines are a class of agents that work on the central nervous system, acting selectively on gamma-aminobutyric acid-A (GABA-A) receptors in the brain. GABA is a neurotransmitter that inhibits or reduces the activity of neurons within the brain resulting in sedative, hypnotic, anxiolytic, anticonvulsant, and muscle relaxant effects. These properties make benzodiazepines useful for treating anxiety, insomnia, agitation, seizures, muscle spasms, alcohol withdrawal and as a premedication for medical or dental procedures. This group of tranquilizers is commonly encountered in different types of forensic cases, such as overdoses and in victims of drug facilitated sexual assault (DFSA). High doses of shorter-acting benzodiazepines may also cause anterograde amnesia and dissociation. Due to their widespread availability and the sedative effect they produce, they are frequently abused, taken with either alcohol or other medications and this combination can be dangerous or even lethal. Benzodiazepines have been used as a "date rape" drug because they can markedly impair and even abolish functions that normally allow a person to resist or even want to resist sexual aggression or assault. In recent years, the detection and conviction of people involved in this event has increased dramatically.

During hydrolysis of benzodiazepine-glucuronides, there is the potential of reductive conversions of Oxazepam and Temazepam to Nordiazepam and Diazepam respectively, with the potential risk of false positives, particularly in the case of specimens presenting high Oxazepam and Temazepam concentrations. To avoid this conversion, it is necessary to maintain a short incubation, reduce sample preparation times and use highly purified enzymes.

Finden® by Kura produces B-One®, a recombinant and highly purified β-glucuronidase enzyme that is clean, stable at room temperature, and doesn't require heating of samples for hydrolysis. B-One® can reach over 99% recoveries within 5 minutes at room temperature. A unique benefit of B-One® is that it does not require a supplemental buffer which allows the user to simply add B-One® and iSTD to the urine sample; no additional mixing reagents are required. B-One® delivers optimum conditions for a complete and fast recovery of analytes compatible with dilute and shoot (D&S) and other extraction methods due to its purity.

For research use only. Not for use in diagnostic procedures. It is particularly useful for demanding and high-throughput clinical or forensic urine drug-investigation laboratories.

Objectives

- This procedure aims to develop a fast and simple analytical method for the simultaneous determination of 28 benzodiazepines and metabolites in hydrolyzed and non-hydrolyzed urine.

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Methodology:

The targeted analytes were:

3-hydroxyflubromazepam	Alprazolam	Etizolam	Nordiazepam
3-hydroxyphenazepam	Bromazepam	Flubromazepam	Oxazepam
7-aminoclonazepam	Clobazam	Flunitrazepam	Oxazepam-glucuronide
7-aminoflunitrazepam	Clonazepam	Flurazepam	Phenazepam
Alpha-hydroxy-alprazolam	Delorazepam	Lorazepam	Temazepam
Alpha-hydroxy-midazolam	Desalkylflurazepam	Lorazepam-glucuronide	Temazepam-glucuronide
Alpha-hydroxy-triazolam	Diazepam	Midazolam	Triazolam

Urine Prep without hydrolysis protocol:

1) Transfer 50 µL of urine into shell vial
2) Fortify with 25 µL of Istd mixture (9 deuterated analogs) in water at 100 ng/mL
3) Add 150 µL of water
4) Vortex
5) Filter in the vial (nanoFilter Vial™ PES 0.2 µm)
6) Inject into the LC-MSMS

Table 1.

Compound	Volume (µL)
Urine	50
Internal Standards	25
Water	150

Urine Prep with hydrolysis protocol with **B-One®**:

1) Transfer 50 µL of urine intoa shell vial
2) Fortify with 25 µL of Istd mixture (9 deuterated analogs) in water at 100 ng/mL
3) Add 130 µL of water
4) **Add 20 µL of B-One®**
5) Wait 5 minutes
6) Filtrate in vial (nanoFilter Vial™ PES 0.2 µm)
7) Inject into the LC-MSMS

Table 2.

Compound	Volume (µL)
Urine	50
Internal Standards	25
Water	130
B-One®	20

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Clean Up Protocol

Filtration was performed using a nanoFilter Vial™ PES 0.2 µm. The plunger with filter was slightly inserted into the shell vial, vortexed and then inserted all the way. The filtered samples (20 µL) were directly injected into the LC-MS/MS. The chromatographic separation was performed in a Kinetex C18 2.6 µm, 2.1x100 mm column (Phenomenex), using as mobile phases 0.1% formic acid in water and in acetonitrile. The instrument employed was a triple quadrupole LCMS-8050 from Shimadzu. The mass spectrometer worked in positive mode, monitoring 2 transitions per analyte.

Notes

- B-One® is active from 0-20% MeOH but is optimal from 5 to 15% in the total hydrolysis mix.
- Mastermix containing B-One®, DI-water and ISDs can be prepared to simplify workflow. Store at Room Temperature (20°C). Use within 14 days.
- B-One® can be stored at room temperature (19°C-22°C) for up to 3 months or up to 15 months at 2-8°C.

Results

All methods, without hydrolysis and with hydrolysis with B-One®, were linear between 5 and 100 ng/mL. Limits of detection (LOD) were between 1 and 5 ng/mL, depending on the analyte. No loss was observed within +/- 20% for any of the 28 analytes due to filtration with PES filters. No exogenous or endogenous interferences were observed (n=10). Matrix effect was not observed for 15 analytes, 3 showed ion enhancement (127.6-158%), and 7 ion suppression (49.4-73.3%) at 10 and 100 ng/mL.

In fortified samples with Lorazepam-glucuronide, Oxazepam-glucuronide and Temazepam-glucuronide at 10 and 100 ng/mL, B-One® performed complete hydrolysis (100% recovery of free drug). In authentic cases, B-One® completely hydrolyzes Lorazepam-glucuronide, Oxazepam-glucuronide and Temazepam-glucuronide at concentrations above 1000 ng/mL. Even in cases with concentrations above 1000 ng/mL for these glucuronides, after the hydrolysis with B-One®, no glucuronides were left.

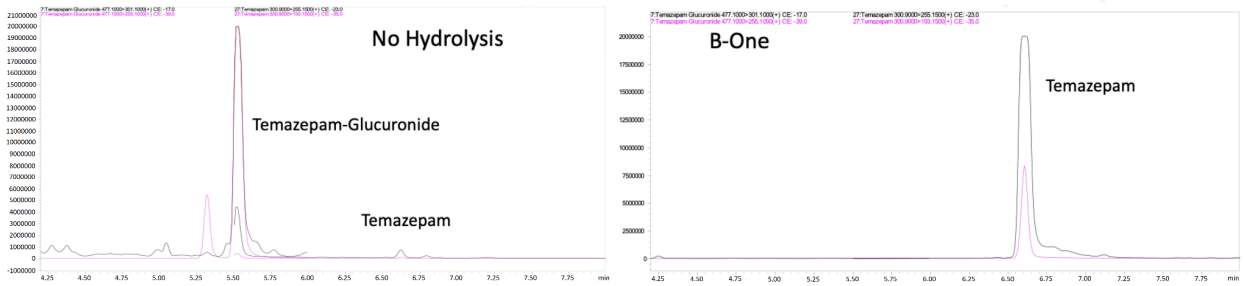

Fig. 1: MRM chromatogram (intensity vs. time in min) of authentic sample 2 in non-hydrolyzed and hydrolyzed urine by B-One®.

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In authentic cases, B-One® improved or allowed the detection of certain benzodiazepines such as midazolam, hydroxy-midazolam, hydroxy-alprazolam, and nordiazepam, as shown in table 3.

Table 3

Benzodiazepine	Detection without hydrolysis (ng/mL)	Detection after adding B-One® (ng/mL)
Midazolam	0	62.5 - 102
Hydroxy-midazolam	0 - 12.9	>100
Hydroxy-alprazolam	0 - 4.7	>100
Nordiazepam	0 - 99.6	>100

Conclusions

- This protocol describes a fast and easy procedure for the analysis of 28 benzodiazepines in urine (25 and 3 glucuronides)
- The methods were sensitive, achieving a LOD between 1 and 5 ng/mL in 50 µL of urine.
- The sample preparation consisted of sample dilution and filtration within the injection vial.
- The enzymatic hydrolysis was performed in the vial and at room temperature in less than 5 minutes.
- B-One® showed excellent efficacy and minimal matrix effects.
- B-One® is free of routine steps/bottlenecks (Multiple manual interventions, offline heating devices, offlines centrifuges and time of incubation).
- Every automated step minimizes errors and sample processing.

Learn More

- B-One® Technical Datasheet
- Quick Start Guide B-One®
- B-One® Stability Report

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References

1. Daniel Aguilar, Isaiah Jewell, Gary Milman and Marta Concheirol. Fast Analysis of 28 Benzodiazepines and Metabolites in Hydrolyzed & Non-Hydrolyzed Urine by LC-MSMS. Poster presentation at Society of Forensic Toxicologists 2021 meeting, Nashville, TN.

PRECAUTIONS AND DISCLAIMER

This document is for R&D use only, not for use in diagnostic procedures. Please consult the Safety Data Sheet (SDS) for information regarding hazards and safe handling practices.

CONTACT AND SUPPORT

To ask questions, solve problems, suggest protocol or product enhancements or report new applications, please contact us at www.kurabiotec.com or email us at help@kurabiotech.com.

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U.S. Patent Nos. 20180067116 and 202117324067 are still pending. United Kingdom Patent Nos.GB2553142 patent are granted.

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