Quantitation of Benzodiazepines

Hydrolysis with Finden BGTurbo® Enzyme

Finden, from Kura Biotech

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 properties. These group of tranquilizers are 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. These properties make benzodiazepines useful treating anxiety insomnia, agitation, seizure, muscle spasms, alcohol withdrawal and as a premedication for medical or dental procedures

Due to their widespread availability, they are frequently abused, taken with either alcohol or other medications, 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 benzodiazepines-glucuronides, there are potential 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 and sample preparation times and use highly purified enzymes.

Genetically enhanced Kura BGTurbo® enzyme provides an efficient hydrolytic activity for the broad spectrum of conjugated analytes. Based on its specific affinity with “hard-to-cleave” glucuronides and its purity, BGTurbo® delivers optimum conditions for a complete and fast recovery of analytes being compatible with D&S methods due to its purity, without needing additional clean-up steps, or hydrolysis can be performed directly on Tip on Tip technology as well as filter plates being integrated into the sample prep, or using a Support Liquid Extraction.

Nov 7, 2024, v3.0

1201 West Peachtree Street NW,
Suite 2625, Atlanta, Georgia 30309
+1 800 332 1448

Compatible Methods

Dilute & Shoot: This method is simple to implement and cost-effective. However, an additional clean-up step, like a protein crash followed by centrifuging, is often applied to eliminate the protein load added by traditional β-glucuronidase preparations. In practice, achieving desired sensitivity and reproducibility can be challenging. BGTurbo® presents very high glucuronidase activity at very low concentrations of enzyme, becoming compatible with D&S method, avoiding column clogging and interfering peak formation.

Filter & Shoot: Filter cartridges contain a solid phase sorbent bed and small pore frits. Positive pressure is used to prepare urine samples for LC-MS/MS analysis. This methodology eliminates centrifugation steps and removes particulates greater than 1 µm. Samples can be diluted at a ratio as low as 1:1, which is useful for detecting analytes at very low concentrations, providing higher sensitivity than dilute and shoot method. With BGTurbo® hydrolysis can be performed directly on the filter plates.

Solid Phase Extraction and Supported Liquid Extraction: These techniques are designed for rapid and selective sample preparation and purification prior to chromatographic analysis. During the last years, these techniques have been simplified on Automated Liquid Dispensers (ALD´s). However, hydrolysis, which is upstream of extraction, has been treated separately as an off-line manual process. BGTurbo® solves this bottleneck, not only does it provide flash hydrolysis but enables it to integrate and run hydrolysis directly on SPE/SLE plates in line with the extraction and LC-MS process, becoming a fully integrated sample preparation.

Objectives

- Achieve recovery target of benzodiazepines >95% at ULOQ 10,000 ng/mL.
- Optimize sample preparation by eliminating incubation time..
- Simplify and potentially automate workflow.
- Preserve integrity of labile analytes (Oxazepam and Temazepam).
- Keep low added background-noise using chromatographically purified β-glucuronidase.
- Maintain a low protein-content enabling D&S/F&S/SPE/SLE without column clogging..
- This highly purified enzyme doesn’t need post hydrolysis clean-up.

Nov 7, 2024, v3.0

1201 West Peachtree Street NW,
Suite 2625, Atlanta, Georgia 30309
+1 800 332 1448

BGTurbo® Hydrolysis Protocol

1. Optional: Centrifuge urine sample for 5 minutes at 4°C at 20,000 x g.
2. With a pipette, add 50 µL of urine sample to plate or column.
3. Add Instant Buffer I + BGTurbo® + ISDs + distilled water to the urine sample according to Table 1.
4. Mix by slowly inverting a capped test tube. If an automated pipetting station is used mixing can be done by repeating aspirate/dispense actions.
5. No heat and no incubation time is needed.
6. Proceed with the preferred extraction method.

Table 1. Hydrolysis Mix Composition

Compound	Volume (µL)
Urine	50
Instant Buffer I	20
BGTurbo Enzyme	**10**
Distilled Water	55
Internal Standards (50% - 100% MeOH)	15
Total	150
Incubation Room Temperature (20°C) for 0 min.	

Notes

- The protocol above is based on an initial volume of 50 µL of urine. The mix could be adapted to any required urine volume by keeping the given proportions.
- It's important to keep a minimum enzyme: urine ratio of 1:5 in order to achieve expected recoveries instantly, in spiked urine and mainly in authentic specimens.
- BGTurbo® is active from 0-20% MeOH but is optimal from 5 to 15% in the total hydrolysis mix.
- Mastermix containing Instant Buffer I, enzyme, DI-water and ISDs can be prepared to simplify workflow. Store at 2–8°C. Use within 14 days.

Testing & Validation

Table 2. Hydrolysis Control

Drug-Class	Recommended Hydrolysis Control (10,000 ng/mL of parent drug)
Benzodiazepines	Nordiazepam-O-Glucuronide

- Kura Biotech recommends performing validation in two steps:
  1. Run assay with spiked urine, using the hydrolysis controls mentioned above.
  2. Benchmark with authentic specimens.

Nov 7, 2024, v3.0

1201 West Peachtree Street NW,
Suite 2625, Atlanta, Georgia 30309
+1 800 332 1448

Learn More

- BGTurbo® Datasheet
- Quick Start Guide BGTurbo®

References

1. Da Silva, K; Kwan, R; Rabbia, V et al. Poster Streamlining Sample Preparation with Second Generation Enzymes. Presented at Society of Forensic Toxicology Conference, Inc.2016.
2. J. F. Emory, N. Bianchini, et al. Complete Integration of a Fully Automated Flash Hydrolysis Protocol of Glucuronides in Urine with LC-MS/MS Quantification. Presented by Shimadzu at the American Society for Mass Spectrometry 2018.
3. C. Pierre, C. Gineste, et al. Poster Incomplete Hydrolysis of Midazolam-Glucuronide can cause false negatives on Urine Drug Confirmation by Liquid Chromatography Tandem Mass Spectrometry. Presented at Mass Spectrometry Application to the Clinical Lab 2019.
4. R. Peralta, R. Caroca, et al. Poster Hydrolysis Efficiency Comparison of Two Beta-Glucuronidases BG100® and BGTurbo®. Presented at Society of Forensic Toxicology Conference 2017.
5. Kura Biotec; Poster Catalyze Complete Hydrolysis of All Glucuronides in only 10 Minutes with BGTurbo®.
6. ElSohly Laboratories, Inc. 2016. Evaluation realized for Kura Biotec®. BGTurbo® glucuronidase evaluation for the hydrolysis of morphine-3-Glucuronide and codeine-6-Glucuronide at different concentrations at 10 minutes and comparison with EBG and IMCSzyme at different incubation times.
7. A. Shellinger, P. Carr. 2004. Solubility of Buffers in Aqueous–Organic Effluents for Reversed-Phase Liquid Chromatography.

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.

PATENTS & TRADEMARKS

Kura Biotech®, Finden®, Finden Kura®, B-One®, BGTurbo® and BG100® are registered trademarks of Kura Biotech SpA in Chile and in the USA.

EBG™, BAMf™, BG2.5™, ASPC™, BGS™, Instant Buffer I™ and Instant Buffer II™ are trademarks of Kura Biotech SpA in Chile and in the USA.

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

©2024 Kura Biotech Inc.
1201 West Peachtree Street NW,
Suite 2625, Atlanta, Georgia 30309
+1 800 332 1448