Fast Analysis of 28 Benzodiazepines and Metabolites in Hydrolyzed & Non-Hydrolyzed Urine by LC-MSMS

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Background

Benzodiazepines, a drug group of tranquilizers with sedative, hypnotic properties, are commonly encountered in different types of forensic cases, such as overdoses and in victims of drug facilitated sexual assault (DFSA). Fast and easy-to-use multi-analyte procedures covering a wide analytical range and achieving the required sensitivity for challenging cases, such as DFSA, are necessary.

Aims

1) To develop fast and simple analytical methods for the simultaneous determination of 28 benzodiazepines and metabolites in hydrolyzed and non-hydrolyzed urine. The target analytes were: 3-hydroxyflubromazepam, 3-hydroxyphenazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, alpha-hydroxy-alprazolam, alpha-hydroxy-midazolam, alpha-hydroxy-triazolam, alprazolam, bromazepam, clobazam, clonazepam, delorazepam, desalkylflurazepam, diazepam, etizolam, flubromazepam, flunitrazepam, flurazepam, lorazepam, lorazepam-glucuronide, midazolam, nordiazepam, oxazepam, oxazepam-glucuronide, phenazepam, temazepam, temazepam-glucuronide, and triazolam.
2) To compare the hydrolysis efficacy and performance of 2 rapid enzymes, B-One® recombinant β-Glucuronidase and BG-Turbo® glycerol free high efficiency recombinant β-Glucuronidase (Kura Biotech, Puerto Varas, Chile).

Methods

Urine Prep No Hydrolysis

Thomson (Oceanside, CA)

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

Urine Prep Hydrolysis B-One® & BG-Turbo® Recombinant β-Glucuronidase (Kura Biotech)

Mix → Wait → Filtrate → LC-MSMS

1) 50 µL urine
2) 25 µL Istd mixture
3) 130 µL water
4) 20 µL B-One

5 min

1) 50 µL urine
2) 25 µL Istd mixture
3) 20 µL buffer
4) 10 µL BG-Turbo
5) 120 µL water

No wait

Results

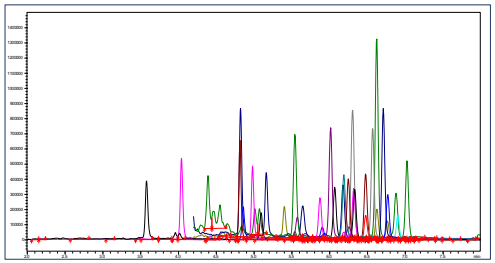

Fig. 1. MRM chromatogram of 28 benzodiazepines and metabolites in non-hydrolyzed urine at 10 ng/mL.

- All the methods, without hydrolysis and with hydrolysis with both enzymes, 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 nor endogenous interferences were observed (n=10).

- In fortified samples with lorazepam-glucuronide, oxazepam-glucuronide and temazepam-glucuronide at 10 and 100 ng/mL, both enzymes performed a complete hydrolysis.
- In the authentic cases, both enzymes completely hydrolyzed lorazepam-glucuronide, oxazepam-glucuronide and temazepam-glucuronide at concentrations above 1000 ng/mL.
- In the authentic cases, the hydrolysis with both enzymes also improved or allowed the detection of certain benzodiazepines (non-hydrolyzed sample concentration vs. hydrolyzed sample concentration) of midazolam (0 vs. 62.5-102ng/mL), hydroxy-midazolam (0-12.9 vs. >100ng/mL), hydroxy-alprazolam (0-4.7 vs. >100ng/mL) and nordiazepam (0-99.6 vs. >100ng/mL).

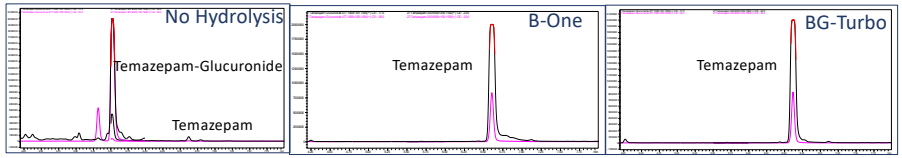

Fig. 2. MRM chromatogram of authentic sample 2 in non-hydrolyzed and hydrolyzed urine by B-One and BG-Turbo enzymes.

Table 1. Matrix effect (ME, %) in urine (n=10) of 25 benzodiazepines at 10 and 100 ng/mL for B-One and BG-Turbo hydrolyzed samples. Ion suppression (red), ion enhancement (lavender).

	B-One		BG-Turbo	
Analyte	**ME Urine 10**	**ME Urine 100**	**ME Urine 10**	**ME Urine 100**
Alprazolam	87.9	97.1	117.1	145.7
Alpha-OH-Alprazolam	79.0	73.3	83.6	86.2
Bromazepam	113.7	101.3	106.9	110.2
Clonazepam	86.0	85.9	90.1	91.6
7-Aminoclonazepam	95.6	97.5	86.1	93.7
Clobazam	158.4	123.5	133.6	114.1
Diazepam	69.7	64.6	74.6	71.3
Nordiazepam	115.5	108.2	114.2	114.3
Etizolam	127.6	112.5	130.3	114.2
Flubromazepam	79.2	76.8	91.3	92.1
3-OH-Flubromazepam	66.7	64.3	77.4	79.4
Flunitrazepam	75.6	69.9	82.2	83.9
7-Aminoflunitrazepam	63.2	76.0	71.8	83.5
Flurazepam	141.6	123.7	176.7	126.7
Desalkylflurazepam	64.7	62.2	73.4	72.2
Lorazepam	92.6	89.5	99.8	106.4
Delorazepam	123.2	113.0	123.3	116.9
Midazolam	56.8	49.4	50.0	59.4
Alpha-OH-Midazolam	79.9	78.9	78.9	83.7
Oxazepam	83.6	85.2	87.3	90.2
Phenazepam	111.1	105.7	114.4	112.3
3-OH-Phenazepam	82.6	78.2	96.5	97.2
Triazolam	103.9	93.0	112.4	104.5
Alpha-OH-Triazolam	79.6	76.7	95.3	92.2
Temazepam	99.7	95.4	104.4	106.8

Conclusions & Discussion

1) We described a fast and easy procedure for the analysis of 28 benzodiazepines in urine (25 and 3 glucuronides).
2) The methods were sensitive, achieving a LOD between 1 and 5 ng/mL in 50 µL urine.
3) The sample preparation consisted in sample dilution and filtration within the injection vial.
4) The enzymatic hydrolysis was performed in the vial and at room temperature in less than 5 min.
5) Both enzymes showed excellent efficacy and minimal matrix effects.

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