Quick and Easy Sample Preparation of Urine for the Analysis of Psychoactive Drugs using the Thomson eXtreme Filter Vials® by LC-MS/MS

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Abstract

This improved sample preparation method allows for the quantitative measurement of Benzodiazepines in urine. Benzodiazepines are psychoactive drugs that enhance the effect of the neurotransmitter GABA at the $GABA_A$ receptor. The urine samples were prepared using the eXtreme|FV®, followed by LC/MS/MS analysis. The most critical aspects of reliable urine analysis are the reduction of interferences from the sample matrix and analyte recovery. eXtreme|FV®, were compared to SPE for sample preparation to reduce the sample matrix causing interference prior to analysis. SPE is time consuming, adversely impacts recovery, uses large amounts of solvent and are expensive. The improved sample preparation method using the Thomson eXtreme|FV® allows for the analysis of 9 Benzodiazepines.

Experimental

Table 1. Drugs analyzed in this Benzodiazpine Panel

7-Aminoclonazepam (7AMINO)	**Hydroxy-Midazolam (OH-MID)**	**Oxazepam (OXAZ)**
α-hydroxy-Alprazolam (OH-AL)	**Lorazepam (LOR)**	**Temazepam (TEM)**
Diazepam (DIAZ)	**Nordiazepam (NDIAZ)**	**Zolpidem (ZOLP)**

Equipment:

Vortex Mixer
Dry Block Heater set at 55°C±2°C
Bulk Liquid Nitrogen
MLA pipette or equivalent
Finnpipette
Eppendorf MixMate
ABI 3200 Mass Spectrometer
Shimadzu Prominence HPLC
Autosampler: SIL-20AC HT
Pumps A, B and C: LC-20AD
Communication Bus Module: CBM-20A
Column Oven: CTO-20AC
Degasser: DGU-$20A_5$
Ultra Biphenyl Columns 5um 50 x 2.1 mm - Restek #9109552
Analyst / Multiquant Software
Thomson 48 Position Thomson Vial Press p/n 35015
Thomson eXtreme|FV 0.2µm PVDF , p/n 85531

Reagents:

Methanol (HPLC Grade)
Water (HPLC Grade)
Drug Free Urine
≥ 96% Formic Acid (ACS Grade)
B-Glucuronidase - IMCSzyme™, a purified beta glucuronidase #04-E1F-010 (IMCS - Integrated Micro- Chromatography Systems)
Rapid Hydrolysis Buffer
0.1% Formic Acid in HPLC Grade Water (Mobile Phase A)
2% Mobile Phase B
2% Methanol in HPLC Grade Water

Improved Sample Preparation

Hydrolysis:

1. Allow standards, specimens and controls to come to room temperature.
2. Label one 1.5 mL Safe-Lock Tube and one Thomson vial for each blank, standard, control and client specimen.
3. Place 350 µL 40% Methanol into the 12 x 75 glass tube for the LC Check
4. To each 1.5 mL Safe-Lock Tube add 50 µL of Rapid Hydrolysis Buffer.
5. Prepare 1.5 mL Safe-Lock Tubes for analysis according to the following table:

Tube	Working UBENZO I.S. (µL)	Working UBENZO STD #1 (µL)	Working UBENZO STD #2 (µL)	Urine (µL)
Blank	25	-	-	200
Level 1	25	5	-	200
Level 2	25	20	-	200
Level 3	25	-	5	200
Level 4	25	-	25	200
Level 5	25	-	50	200
Controls*	25	-	-	200
Specimens	25	-	-	200
Orasure Serum Sample	25	-	-	400 (Serum)

7. Cap and vortex for 30 seconds using the Eppendorf Mix Mate.
8. Uncap and add 40µL IMCS ß-glucuronidase to each tube.
9. Cap and vortex for 2 minutes to ensure sample is mixed.
10. Uncap and incubate at 55°C ± 2°C for 30 minutes.
11. Allow tubes to come to room temperature.
12. Microcentrifuge at 14000 rpm for 10 minutes.

Sample Prep

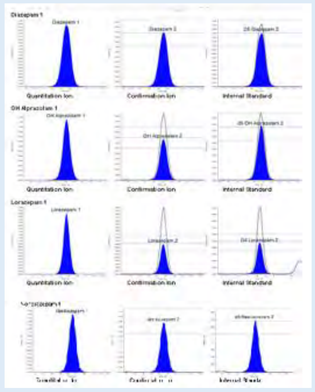
1. Add 300 µL of 40% Methanol to each Thomson Vial.
2. Give each Eppendorf tube a quick vortex and add 50 µL of the hydrolyzed urine sample to its respective Thomson Vial.
3. Place Thomson Filter Plunger on top of Thomson Vial.
4. Press filter plunger down approximately ¼ of the way into each of the Thomson Vials.
5. Vortex for 2 minutes at 1750 rpm using the Eppendorf Mix Mate.
6. Slowly press the filter plunger the rest of the way down using the Thomson 48 position press.
7. Samples are now ready for LC/MS/MS analysis

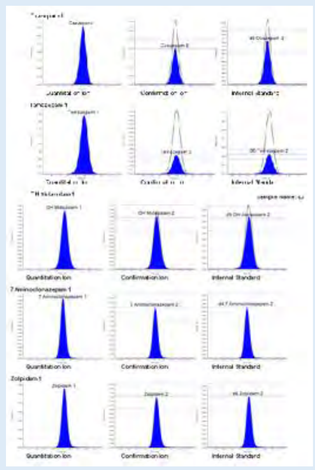
Results

- Alleviate the need to use and dispose of Hexane, Glacial Acetic Acid, Potassium Hydroxide.
- Solvent used is reduced from 532mL to 0mL, when extracting 96 samples.
- Solvent waste generated is reduced from 236mL to 0mL, extracting 96 samples .
- Extraction time for 96 samples is reduced from 4 hours to 20 minutes.

Data

Positive Results





Previous Sample Preparation

Hydrolysis:

1. Allow standards, specimens and controls to come to room temperature.
2. Turn Block Heater on to 37°C±2°C
3. Label one 13 x 100 mm screw top tube for each blank, standard, control and client specimen.
4. All samples will be analyzed in the order they are extracted on the Rapid Trace.
5. Prepare a LC Check Standard (equivalent to a Level 1 concentration)
6. To all tubes, add 1 mL of 1.1 M pH 5.2 Acetate Buffer.
7. Prepare the 13 x 100 mm screw top tubes for hydrolysis according to the following table:

Tube	Working UBENZO I.S. (µL)	Working UBENZO STD #1 (µL)	Working UBENZO STD #2 (µL)	Urine (µL)
Blank	25	-	-	200
Level 1	25	5	-	200
Level 2	25	20	-	200
Level 3	25	-	5	200
Level 4	25	-	25	200
Level 5	25	-	50	200
Controls*	25	-	-	200
Specimens	25	-	-	200
Orasure Serum Sample	25	-	-	400 (Serum)

9. Vortex for 10 seconds.
10. Add 20 µL ß-glucuronidase.
11. Vortex for 10 seconds to ensure sample is mixed.
12. Incubate at 37°C±2° for 3 hours.
13. Allow tubes to come to room temperature.

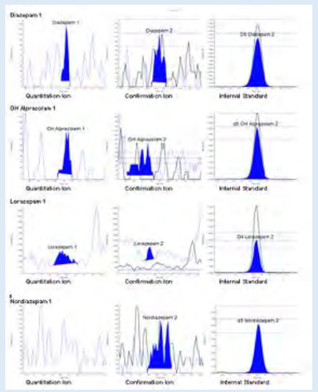
Sample Prep

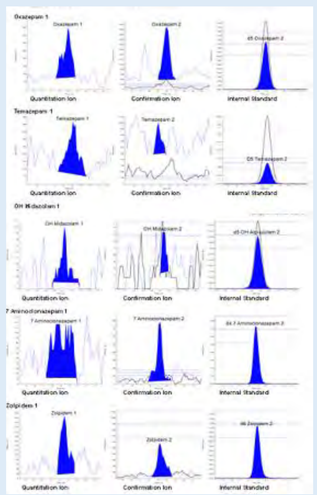
1. Uncap
2. Add 3 mL 0.1 M pH 6.0 Potassium Phosphate Buffer.
3. Place the Benzo.set reagents on the Zymark Rapid Trace and purge the reagent lines.
4. Vortex for 10 seconds.
5. Centrifuge tubes at 3000 rpm for 5 minutes.
6. Transfer samples into corresponding labeled 13 x 100 mm tubes.
7. The tubes are now ready for automated extraction.
8. After the elution is complete on the Rapid Trace®, remove the racks with the tubes intact.
9. Include QC Check at his point.
10. Dry down extracts/tubes under a gentle stream of nitrogen.
11. Add 2mL of 10% Methanol.
12. Vortex for 30 seconds.
13. Transfer supernatant using a glass borosilicate pipet to the appropriately labeled autosampler vials. Cap and place vials on autosampler tray.
14. Extracts are ready for LC/MS/MS analysis.

Final concentrations for the various analytes are as follows:

	Final Concentration All other analytes (ng/mL)	Final Zolpidem Concentration (ng/mL)
Level 1	75	75
Level 2	300	300
Level 3	1000	500
Level 4	5000	2500
Level 5	10000	5000

Negative Results





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