

Automated Solid Phase Extraction of Benzodiazepines from Urine Using the Gilson ASPEC[®] 274 System

APPLICATION NOTE CL0211

Automated Solid Phase Extraction (SPE) prior to LC/MS-MS analysis of benzodiazepines in urine provides a confirmatory test for the positive or negative presence of these CNS depressants and is more informative than simple screening tests with a history of false positive results. This application involves three simple SPE steps, which saves time by not requiring the general SPE cartridge condition steps or multiple wash steps. Fast LC/MS-MS analysis in under five minutes creates further efficiency for benzodiazepines analysis. Acceptable resulting recoveries show with high confidence that sample analysis of 15 benzodiazepine analytes is possible from one urine sample.

INTRODUCTION

Central Nervous System (CNS) depressants can be used to introduce calm and induce sleep. Benzodiazepines are considered CNS depressants and are used in relieving anxiety, insomnia, and panic attacks. However, there has been reported misuse by mixing benzodiazepines with alcohol or opiates. Currently, 35 benzodiazepines are internationally regulated.¹

Positive identification of benzodiazepines using a confirmatory method of analysis is critical to correctly identifying patients using these pharmaceuticals. Simple screening tests have historically produced false positive results.²

This application note discusses a simple SPE urine extraction using the Gilson ASPEC[®] 274 System (refer to Figure 1) prior to LC/MS-MS to confirm the presence of 15 spiked benzodiazepine analytes.



Figure 1
Gilson ASPEC[®] 274 System

MATERIALS & METHODS

SPE Materials

SPE Cartridges: Phenomenex Strata™ X-Drug N 100 µm Polymeric Reversed Phase 100 mg/6 mL

SPE Solutions:

Wash: Acetonitrile: Water (20:80, v/v)

Elute: Ethyl Acetate: Isopropyl Alcohol 85:15, v/v)

Pre-Sample Treatment

1. Samples were spiked at 300 ng/mL with 15 benzodiazepine analytes.
2. For each 2 mL urine sample, add 1 mL betaglucuronidase (contains 5,000F units/mL *Patella vulgata* in 100 mM acetate buffer (pH 5.0)).
3. Mix/vortex.
4. Hydrolyze for three hours at 60°C.
5. Let cool.
6. Add 1 mL 100 mM Phosphate buffer (pH 6.0).
7. Centrifuge for five minutes at 5000 rpm and discard pellet.
8. Load directly onto SPE cartridge - NO CONDITIONING REQUIRED.

SPE Method

1. Load 4 mL of pre-treated sample onto SPE cartridge at 3 mL/min.
2. Wash with 2 mL solution at 6 mL/min.
3. Dry for ten minutes at 7-15 psi regulated gas (nitrogen, argon, or purified air).
4. Elute SPE cartridge with 2 mL solution at 3 mL/min.

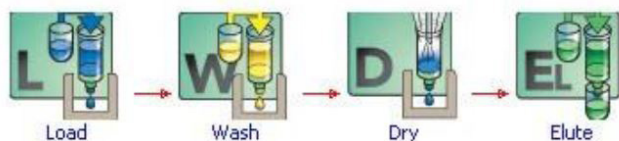


Figure 2

TRILUTION® LH benzodiazepines solid phase extraction method using the Gilson ASPEC® 274 System

Final Sample Treatment Prior to Analysis

1. Evaporate SPE eluent under a stream of nitrogen gas at 50°C.
2. Reconstitute with 1 mL of 35% Methanol.
3. Inject 5 µL on LC/MS-MS at ambient temperature, amu.

Analytical LC/MS-MS Materials

HPLC System: Binary Gradient Mobile Phase Pumps
MS-MS Detection: API 3000

Mobile Phase:

A: 0.1% Formic Acid

B: 0.1% Formic Acid in Methanol

Column: Phenomenex Kinetex™ 2.6 µm C18 100Å, 50x2.1 mm ID

Analytical HPLC Method

Table 1

Mobile Phase Gradient

STEP NO	TIME (MIN)	% A	% B
1	0	65	35
2	4	5	95
3	4.01	65	35
4	7	65	35

Flow rate: 300 µL/min

Column Temperature: ambient

Detection:

Source: Cur-11, CAD-12, IS-4500, Tem-4 50, NEB-12

Dwell: 25 ms

Polarity: Positive

RESULTS AND DISCUSSION

An automated SPE method was developed using TRILUTION LH software to control the Gilson ASPEC 274 system. The steps in the method are illustrated in Figure 2. Human urine samples were spiked with benzodiazepine analytes before the SPE procedure. After extraction, samples were subjected to LC/MS-MS analysis. Figure 3 shows a typical chromatogram of benzodiazepines extracted from urine.³

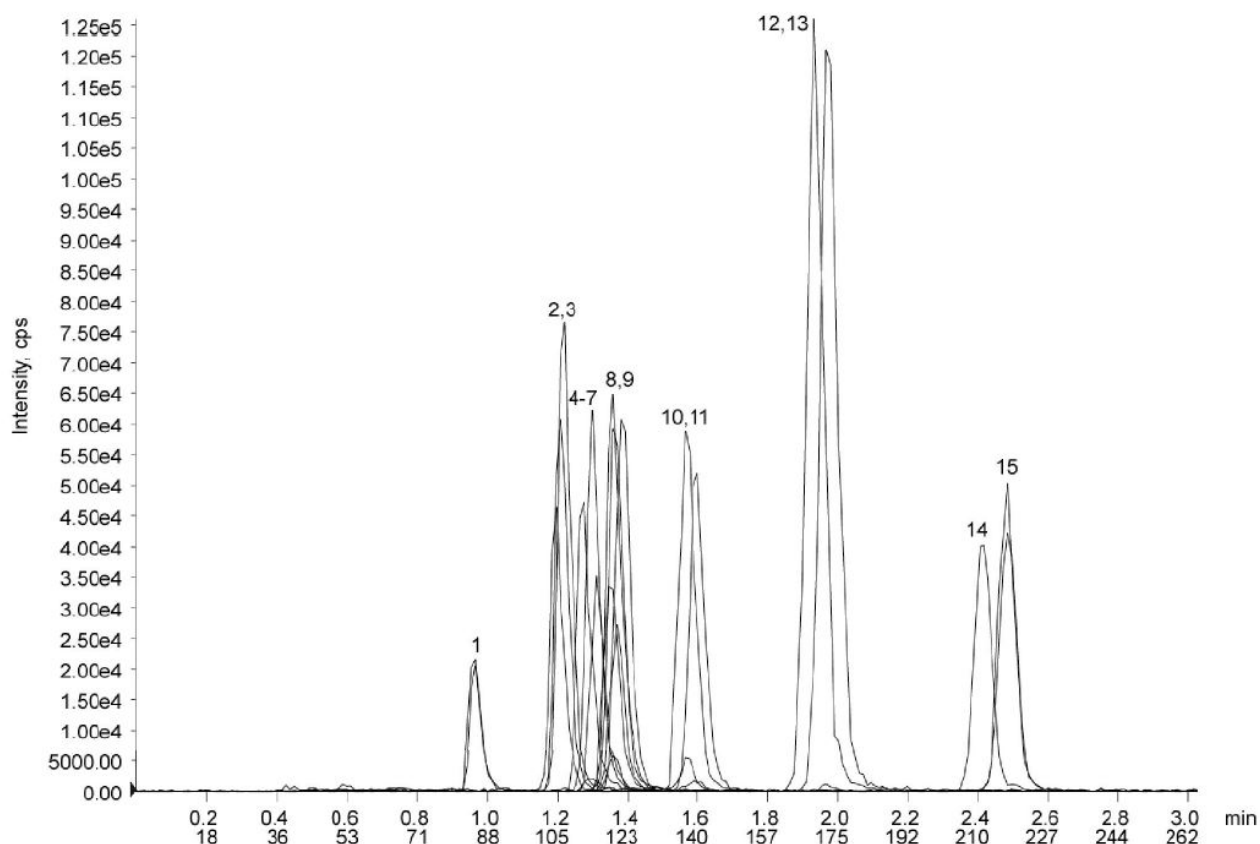


Figure 3

Example chromatogram of extracted benzodiazepines from urine. Peaks are numbered as follows: 1, a-Hydroxyalprazolam; 2, Oxazepam-D5; 3, Oxazepam; 4, Alprazolam-D5; 5, Alprazolam; 6, Nordiazepam-D5; 7, Nordiazepam; 8, Lorazepam-D4; 9, Lorazepam; 10, Clonazepam-D4; 11, Clonazepam; 12, Temazepam-D5; 13, Temazepam; 14, Diazepam-D5; 15, Diazepam.

Table 2

Recovery and % RSD for compounds evaluated in this study

PEAK #/ANALYTE	MASS RANGE 1	MASS RANGE 2	% RECOVERY	% RSD (n=3)
1 / a-Hydroxyalprazolam	325.4>279.2	325.4>204.9	92.4	10.6
2 / Oxazepam-D5	292.3>246.3	NA	N	NA
3 / Oxazepam	287.1>241.1	287.1>268.9	105.3	5.6
4 / Alprazolam-D5	314.2>210.2	NA	NA	NA
5 / Alprazolam	309.1>205.1	309.1>281.1	98.4	2.1
6 / Nordiazepam-D5	276.3>140.1	NA	NA	NA
7 / Nordiazepam	271.4>140.3	271.3>164.9	103.7	3.7
8 / Lorazepam-D4	325.1>279.2	NA	NA	NA
9 / Lorazepam	321.3>275.3	321.3>239.1	101.1	1
10 / Clonazepam-D4	320.1>274.1	NA	NA	NA
11 / Clonazepam	316.3>270.3	316.3>214	99.6	0.5
12 / Temazepam-D5	306.3>260.2	NA	NA	NA
13 / Temazepam	301.3>255.2	301.3>180.1	104.6	3.9
14 / Diazepam-D5	290.2>154.2	NA	NA	NA
15 / Diazepam	285.3>193.2	285.3>154.2	101.1	1

As shown in Table 2, recovery of these compounds was within the acceptable range. This method has advantages over simple screening tests with a history of false positive results. The assay provides a high confidence that sample analysis of 15 benzodiazepine analytes from one urine sample is possible with confirmatory results.

CONCLUSION

- Automated Solid Phase Extraction (SPE) prior to LC/MS-MS analysis of benzodiazepines in urine provides a confirmatory test for the positive or negative presence of these CNS depressants and is more conclusive than other screening tests with a history of false positive results.
- The automated method involves three simple SPE steps, and further time savings are achieved because the general cartridge conditioning step can be eliminated and multiple wash steps are not required.
- Fast LC/MS-MS analysis (under five minutes) creates further efficiency for benzodiazepine analysis.
- 15 benzodiazepine analytes were extracted and detected from one urine sample using this method.

REFERENCES

- <http://www.emcdda.europa.eu/publications/drug-profiles/benzodiazepine>
- Nasky, KM, Cowan, GL, Knittel, DR. False-positive urine screening for benzodiazepines: an association with sertraline? *Psy-chiatry (Edgemont)* 6, 36-39 (2009).
- Phenomenex Application Note 19714: Benzodiazepines from urine using Strata-X- Drug N on a Kinetex C18 2.6 μ m, 50x2.1mm.

ACKNOWLEDGEMENTS

This data was generated by Phenomenex (www.phenomenex.com) and is also reported in application note #19714.

Trademarks

All product and company names are trademarks™ or registered® trademarks of their respective holders. Use of the trademark(s) in this document does not imply any affiliation with or endorsements by the trademark holder(s).

Notice

This application note has been produced and edited using information that was available at the time of publication. This application note is subject to revision without prior notice.



VWR.COM

Prices, product, and/or services details are current when published and subject to change without notice. | Certain products or services may be limited by country, federal, state, provincial, or local regulations. | VWR, part of Avantor, makes no claims or warranties concerning sustainable/green products. Any claims concerning sustainable/green products are the sole claims of the manufacturer and not those of VWR International, LLC and/or Avantor, Inc. or affiliates. Offers valid in countries listed above, void where prohibited by law or company policy, while supplies last. | Trademarks are owned by Avantor, Inc. or its affiliates, unless otherwise noted. | Visit vwr.com to view our privacy policy, trademark owners, and additional disclaimers. © 2020 Avantor, Inc. All rights reserved.