



# Automated Solid Phase Extraction of Benzodiazepines from Urine using the Gilson GX-274 ASPEC™ 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, saving even more 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 benzodiazepine 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.<sup>1</sup>

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.<sup>2</sup>



Figure 1. Image of the Gilson GX-274 ASPEC System

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



## Materials & Methods

### SPE Materials

#### SPE Cartridges:

Phenomenex Strata™ X-Drug N 100u  
Polymeric Reversed Phase 100 mg/6 mL

#### SPE Solutions:

Wash: Acetonitrile:Water (20:80)  
Elute: Ethyl Acetate:Isopropyl Alcohol  
(85:15)

### Pre-Sample Treatment

1. Samples were spiked @ 300 ng/mL with 15 benzodiazepine analytes
2. For each 2mL urine sample, add 1mL beta-glucuronidase (contains 5,000 F units/mL *Patella vulgata* in 100mM acetate buffer(pH 5.0))
3. Mix/vortex
4. Hydrolyze for 3 hours at 60°C
5. Let cool
6. Add 1mL 100mM Phosphate buffer (pH 6.0)
7. Centrifuge for 5 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 10 minutes at 7-15 psi regulated gas (nitrogen, argon, or purified air)
4. Elute SPE cartridge with 2 mL solution at 3 mL/min

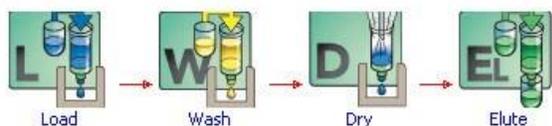


Figure 2. TRILUTION® LH Benzodiazepine Solid Phase Extraction Method Using the Gilson GX-274 ASPEC™ System

### Final Sample Treatment Prior to Analysis

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

### 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™ 2.6u C18 100A,  
50 x 2.1 mm ID

### Analytical HPLC Method

#### Mobile Phase Gradient:

Step No.	Time (min)	Pct A	Pct 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: 25ms  
Polarity: Positive



## Results and Discussion

An automated SPE method was developed using TRILUTION LH software to control the Gilson GX-271 ASPEC 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.<sup>3</sup>

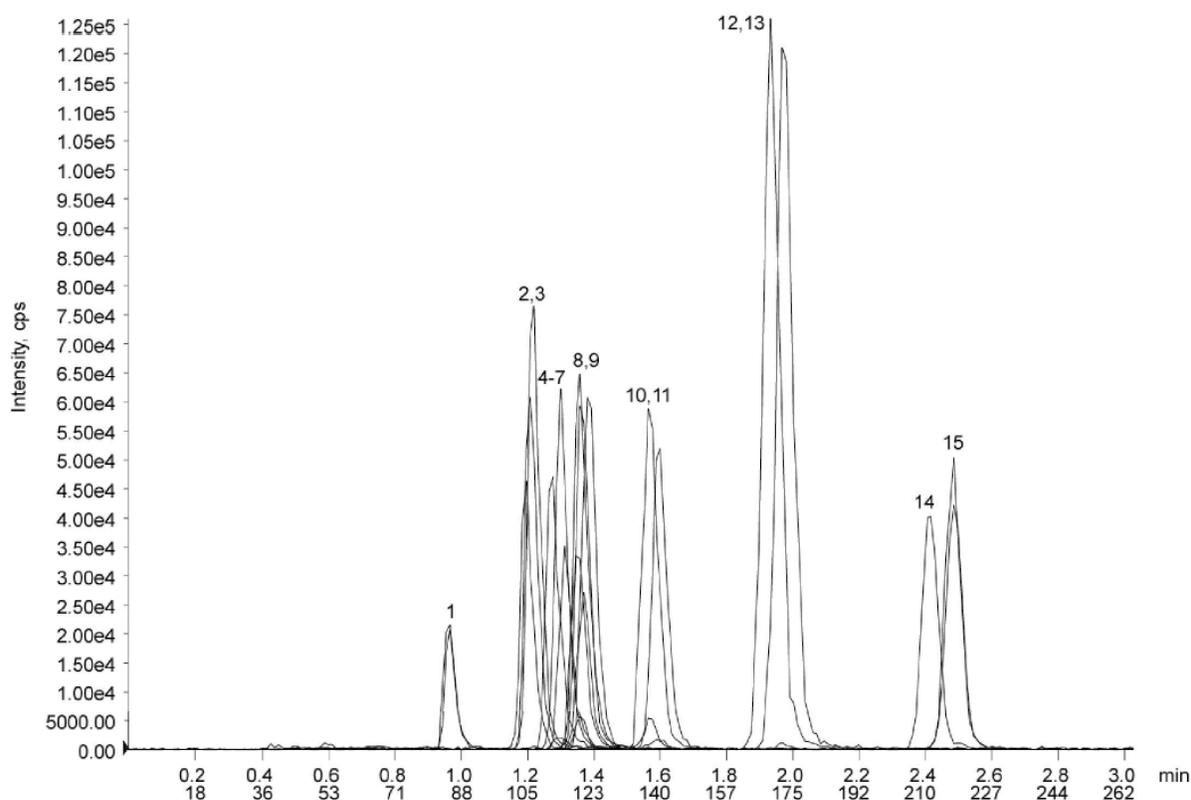


Figure 3. Example Chromatogram of Extracted Benzodiazepines from Urine. Peaks are numbered as follows: 1,  $\alpha$ -Hydroxyalprazolam; 2, Oxazepam-D5; 3, Oxazepam; 4, Alprazolam-D5; 5, Alprazolam; 6, Nordiazepam-D5; 7, Nordiazepam; 8, Lorazepam-D4; 9, Lorazepam; 10, Clonazepam-D4; 11, Clonazepam; 12, Temazepam-D5; 13, Temazepam; 14, Diazepam-D5; 15, Diazepam.



Peak #/Analyte	Mass Range 1	Mass Range 2	% Recovery	% RSD (n=3)
1 / <i>a</i> -Hydroxylprazolam	325.4>279.2	325.4>204.9	92.4	10.6
2 / Oxazepam-D5	292.3>246.3	NA	NA	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

Table 1. Recovery and % RSD for compounds evaluated in this study.

As shown in Table 1, 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.

## References

1. <http://www.emcdda.europa.eu/publications/drug-profiles/benzodiazepine>

2. Nasky, KM, Cowan, GL, Knittel, DR. False-positive urine screening for benzodiazepines: an association with sertraline? *Psychiatry (Edgemont)* **6**, 36-39 (2009).
3. Phenomenex Application Note 19714: Benzodiazepines from urine using Strata-X-Drug N on a Kinetex C18 2.6µm, 50x2.1mm

## Acknowledgements

These data were generated by Phenomenex ([www.phenomenex.com](http://www.phenomenex.com)) and are also reported in application note #19714.

## Summary or Conclusions

- 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.