

# Separation of Synthetic Benzodiazepines by SPE and LC-MS/MS

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## Overview

Benzodiazepines are some of the highest prescribed drugs in the US, with significant abuse potential. “Synthetic” benzodiazepines (which are not FDA approved) are increasingly found in the population as well. It is important to be able to track these substances as they arise so they can be monitored and detected. They show cross reactivity with other benzodiazepines in immunoassays, so developing effective LC-MS/MS techniques are important for proper identification and quantitation.

In this application note, we quantify 14 compounds from a urine matrix with high sensitivity using fast LC analysis in less than 4 minutes. 13 of the analytes are “synthetic” benzodiazepines, and Alprazolam was added to the panel because it is an isomer of Deschloroetizolam, and therefore differentiation is necessary. Strata®-X-Drug B Plus was chosen as an ideal SPE plate to maximize recovery, eliminate matrix interferences, and optimize the workflow by combining the hydrolysis and SPE within the same platform. The high efficiency Kinetex® Biphenyl LC column was used for chromatographic separation.

The Kinetex 2.6 µm Biphenyl, 50 x 3.0 mm column provided ideal selectivity for the benzodiazepines. It was able to separate all analytes in the panel quickly, resolving all 14 analytes in under 4 minutes (**Figure 1**). In high throughput labs, this short run time is essential for optimizing instrument time. The critical pair of Alprazolam and Deschloroetizolam were separated with baseline resolution, as shown in **Figure 2**. The Strata-X-Drug B Plus plate achieved optimal recovery across all 14 analytes using the elution solvent system Ethyl acetate/Isopropanol/Ammonium hydroxide (7:2:1, v/v/v). The absolute recovery yield in the urine sample was 76-114 % (analyte spiked at 20 ng/ml) with a CV value of less than 10 % (**Figure 3**).

We established a method that is sensitive, accurate, and precise for synthetic benzodiazepines. The constant changing environment of novel psychoactive substances presents a challenge, but by utilizing the Kinetex Biphenyl column’s unique selectivity and Strata-X-Drug B Plus, we can adapt to the new challenges and compounds as they arise to quickly develop effective methods.

## Sample Preparation

**Sample Pre-treatment:** Combine 200 µL of urine sample spiked with 40 µL mix of internal standards (IS) (250 ng/mL), 60 µL hydrolysis buffer, and 20 µL of IMCSzyme® RT enzyme on the Strata-X-Drug B Plus, 30 mg plate (Part No.: [8E-S128-TGB-P](#)). Incubate at room temperature for 15 minutes.

**Load:** 200 µL 0.1 % formic acid in water. Shake/vortex for 1 min, then apply vacuum.

**Wash 1:** 1 mL 0.1 % formic acid in water.

**Wash 2:** 1 mL 30 % methanol.

**Dry:** 5 min at high vacuum (15-20 in Hg).

**Dry Down:** Sample under gentle stream of nitrogen at 40-45 °C.

**Reconstitute:** Dried samples in 200 µL initial mobile phase.

## LC Conditions

**Column:** Kinetex 2.6 µm Biphenyl

**Dimensions:** 50 x 3.0 mm

**Part No.:** [00B-4622-Y0](#)

**Mobile Phase:** A: 0.1 % formic acid in water  
B: 0.1 % formic acid in methanol

Gradient:	Time (min)	%B
	0	60
	0.5	95
	2.5	95
	2.51	60
	4	60

**Flow Rate:** 500 µL/min

**Injection Volume:** 5 µL

**Temperature:** 40 °C

**LC System:** Agilent® 1260 Infinity

**Detection:** MS/MS

**Detector:** SCIEX® Triple Quad™ 4500

## MS Conditions

**Ion Source:** Positive

**Source Temperature:** 550 °C

**GS1:** 40

**GS2:** 60

**CUR:** 20

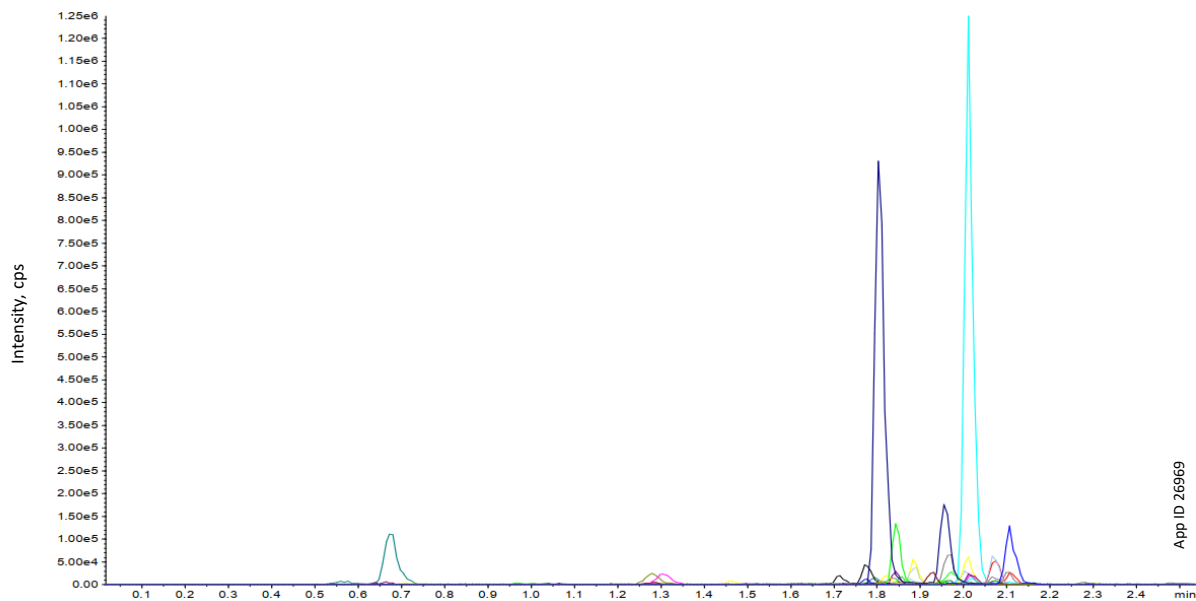
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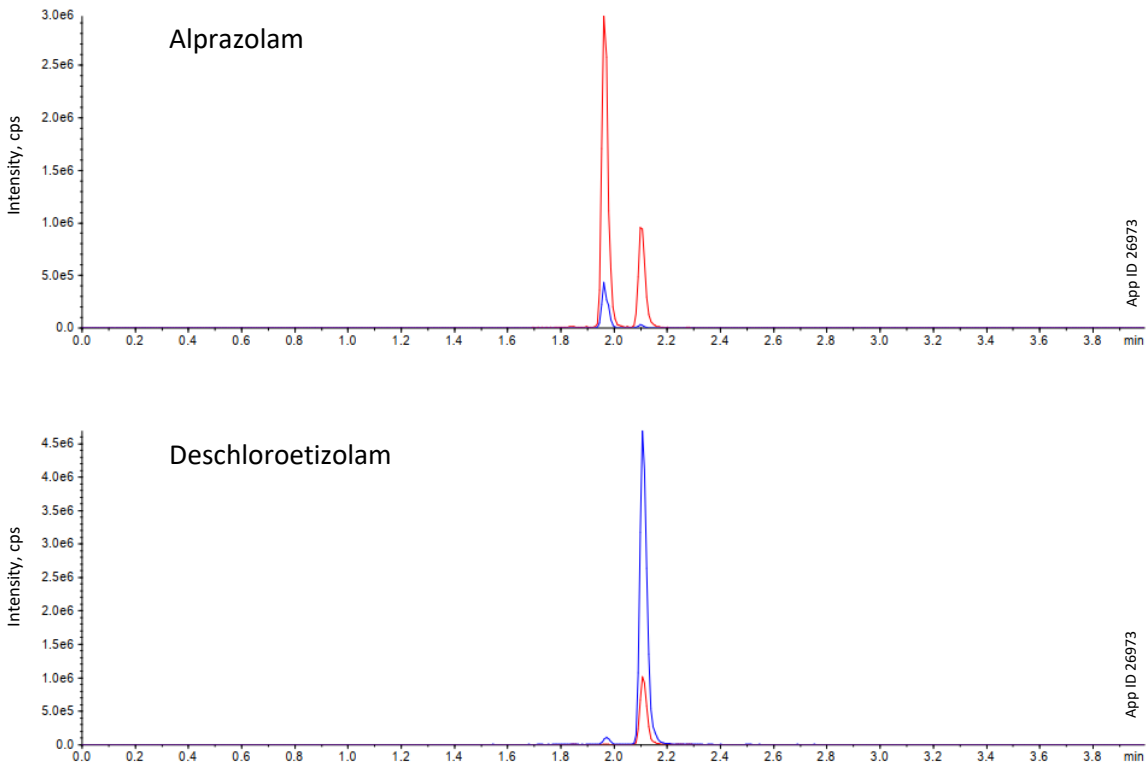
**Table 1.** MRM Transitions

Analyte	Q1 (m/z)	Q2 (m/z)
Adinazolam	352.1	58
Bromazolam	352.9	324.8
Clonazolam	354	308
Deschloroetizolam	309.1	280.1
Diclazepam	319.01	154
Etizolam	343.05	314.1
Flualprazolam	327.01	299
Flubromazepam	333.03	226
Flubromazolam	371	292.02
Flunitrazolam	338	292
Methylclonazepam	329.8	283.9
Nitrazolam	320	246.2
Phenazepam	351.09	185.8
Alprazolam	309.1	205.2
Diclazepam-D4 (IS)	323	231
Etizolam-D3 (IS)	346.01	317
Phenazepam-D4 (IS)	355	183
Clonazolam-D4 (IS)	358	312.02

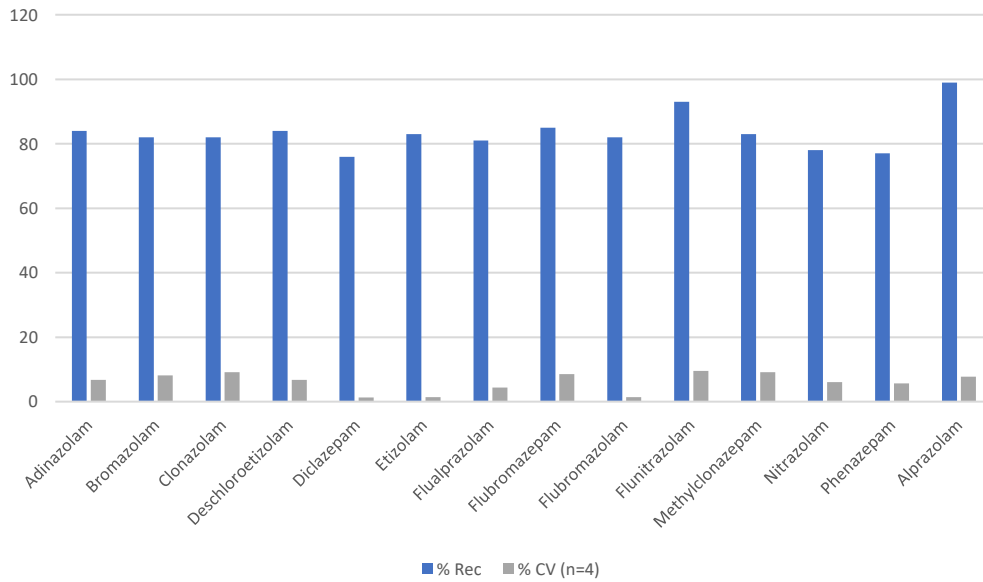
**Figure 1.** Separation of 14 Synthetic Benzodiazepines and Internal Standards using the Kinetex® 2.6 µm Biphenyl Column



**Figure 2.** Separation of Isomeric Alprazolam and Deschloroetizolam



**Figure 3.** Absolute Recovery and % CV for 14 Benzodiazepines



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