



## Quantitative Analysis of Benzodiazepines in Whole Blood by QuEChERS and LC-MS/MS

UCT Part Numbers:

**Enviro-Clean<sup>®</sup> ECQUUS15CT** (15 mL centrifuge tube with 400 mg MgSO<sub>4</sub> and 100 mg NaOAc)

**Enviro-Clean<sup>®</sup> CUMPSC18CT** (2 mL dSPE tube with 150 mg MgSO<sub>4</sub>, 50 mg PSA and 50 mg C18)

**SLDA100ID21-3UM** (Selectra<sup>®</sup> DA LC column, 100 x 2.1 mm, 3 μm)

**SLDAGDC21-3UM** (Selectra<sup>®</sup> DA guard column, 10 x 2.0 mm, 3 μm)

**SLDGRHLDR** (Guard cartridge holder)

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### Summary:

Benzodiazepines (Benzos) are psychoactive drugs widely prescribed for treating anxiety, insomnia, agitation, seizures, muscle spasms, and alcohol withdrawal. Benzos are deemed safe and effective for short term use. However, frequent use of these drugs may lead to dependence and abuse. Because of this attribute clinical, forensic and toxicological laboratories are interested in monitoring these compounds in biological samples. Common sample preparation methods for biological samples include a protein precipitation step followed by liquid-liquid extraction (LLE) or solid phase extraction (SPE). This application describes an easy, fast, and effective method using QuEChERS for the quantitative analysis of benzodiazepines in whole blood.

1 mL of negative whole blood sample is extracted using 2 mL of acetonitrile (MeCN) with 0.4 % formic acid (FA). 400 mg magnesium sulfate (MgSO<sub>4</sub>) and 100 mg sodium acetate (NaOAc) (pre-packed in 15-mL centrifuge tube) are employed to enhance the phase separation and the partition of benzodiazepines into the organic phase. After shaking and centrifugation, 1 mL of the supernatant is purified by a 2-mL dSPE tube containing 150 mg MgSO<sub>4</sub>, 50 mg PSA, and 50 mg C18. MgSO<sub>4</sub> absorbs residual water in the extract, while PSA and C18 remove organic acids and fatty matrix co-extractives, resulting in a clean extract for LC-MS/MS analysis.

Matrix matched calibration curves were constructed for the benzodiazepines quantification. The responses for 10 representative compounds were linear with  $R^2$  ranging from 0.9963 to 1.0000 over the concentration range of 10 - 500 ng/mL. Matrix effects were evaluated by comparing the slopes of the matrix matched calibration curves to those of the calibration curves of solvent standards. The matrix effects were found to be minor, ranging from -22 to 18%. This indicated that the QuEChERS method with dSPE cleanup sufficiently removed matrix interferences that may cause significant ion suppression or enhancement. Excellent recoveries (85.5 - 105%) and relative standard deviations ( $RSD\% \leq 10.7\%$ ) were obtained. This method was also applied to 8 real whole blood samples, no benzodiazepines were detected above the limit of quantitation of 10 ng/mL.

## **Procedure:**

### **QuEChERS extraction**

- a) Add 2 mL of MeCN with 0.4% FA to 15-mL centrifuge tube containing 400 mg  $MgSO_4$  and 100 mg NaOAc (**ECQUUS15CT**).
- b) Add internal standards (IS), and appropriate amounts of benzos spiking solution to fortified samples.
- c) Add 1 mL of the negative whole blood into the 15-mL tubes
- d) Cap and shake for 1 min at 1000 strokes/min using a Spex 2010 Geno-Grinder.
- e) Centrifuge at 3000 g for 5 min.

### **dSPE cleanup**

- a) Transfer 1 mL of the supernatant to a 2-mL dSPE tube (**CUMPSC18CT**).
- b) Shake 1 min at 1000 strokes/min using the Spex 2010 Geno-Grinder.
- c) Centrifuge at 3000 g for 5 min.
- d) Transfer 0.4 mL of the cleaned extract into a 2-mL auto-sampler vial, add 0.4 mL of reagent water, and vortex for 30 sec.
- e) The samples are ready for LC-MS/MS analysis.

## LC-MS/MS method:

| <b>System:</b> AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump SL     |  |   |
|---|--|---|
| <b>Column:</b> UCT Selectra <sup>®</sup> DA LC column, 100 x 2.1 mm, 3 µm         |  |   |
| <b>Guard Column:</b> UCT Selectra <sup>®</sup> DA guard column, 10 x 2.0 mm, 3 µm |  |   |
| <b>Column Temperature:</b> 50 °C  |  |   |
| <b>Column Flow Rate:</b> 0.3 mL/min   |  |   |
| <b>Injection Volume:</b> 10 µL  |  |   |
| <b>Gradient Program:</b>  |  |   |
| Time (min)  | % Mobile Phase A<br>(0.1% FA in water) | % Mobile Phase B<br>(0.1% FA in methanol) |
| 0   | 70                                     | 30  |
| 0.5   | 70                                     | 30  |
| 2   | 25                                     | 75  |
| 6.5   | 25                                     | 75  |
| 7   | 0                                      | 100                                       |
| 9   | 0                                      | 100                                       |
| 10.1  | 70                                     | 30  |
| 14  | 70                                     | 30  |

| MRM transitions (ESI positive, dwell time: 50 ms) |          |        |          |          |
|---|----------|--------|----------|----------|
| Compound  | Rt (min) | Q1 ion | Q3 ion 1 | Q3 ion 2 |
| 7-aminoclonazepam                                 | 7.58     | 286.1  | 222.3    | 250.2    |
| Alpha-Hydroxy-Alprazolam                          | 9.26     | 325.2  | 297.1    | 216.3    |
| Alprazolam  | 9.72     | 309.2  | 205.3    | 281.2    |
| Clonazepam  | 9.03     | 316.1  | 270.2    | 241.2    |
| Diazepam  | 9.87     | 285.1  | 193.2    | 154.1    |
| Lorazepam   | 8.94     | 321.1  | 303.3    | 275.0    |
| Midazolam   | 8.53     | 326.0  | 291.0    | 222.0    |
| Nordiazepam                                       | 9.30     | 271.1  | 140.1    | 165.2    |
| Oxazepam  | 9.00     | 287.1  | 241.3    | 104.2    |
| Temazepam   | 9.45     | 301.1  | 255.2    | 177.2    |
| Alprazolam D5                                     | 9.69     | 314.2  | 286.3    |          |
| Oxazepam D5                                       | 8.98     | 292.1  | 246.2    |          |

## Results:

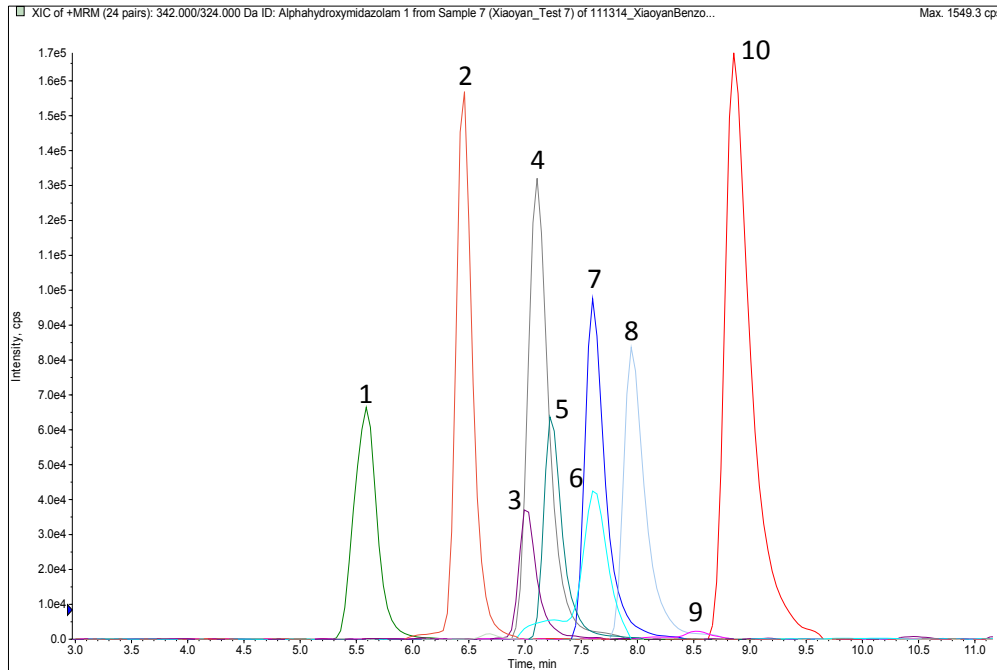
### Linearity and Matrix Effect

| Compound                 | Solvent standard |                | Matrix-matched standard |                | Matrix effect |
|--------------------------|------------------|----------------|-------------------------|----------------|---------------|
|                          | Slope            | Linearity (R2) | Slope                   | Linearity (R2) |               |
| 7-aminoclonazepam        | 0.00823          | 0.9993         | 0.00646                 | 0.9998         | -22           |
| Alpha-Hydroxy-Alprazolam | 0.00646          | 0.9990         | 0.00764                 | 0.9996         | 18            |
| Alprazolam               | 0.000413         | 0.9990         | 0.000486                | 0.9989         | 18            |
| Clonazepam               | 0.00443          | 0.9995         | 0.00497                 | 0.9999         | 12            |
| Diazepam                 | 0.0133           | 0.9997         | 0.0146                  | 0.9996         | 10            |
| Lorazepam                | 0.00306          | 0.9999         | 0.0034                  | 0.9997         | 11            |
| Midazolam                | 0.00656          | 0.9989         | 0.00675                 | 0.9963         | 3             |
| Nordiazepam              | 0.00703          | 0.9999         | 0.00754                 | 0.9998         | 7             |
| Oxazepam                 | 0.00987          | 1.0000         | 0.0107                  | 1.0000         | 8             |
| Temazepam                | 0.00641          | 0.9998         | 0.00709                 | 0.9999         | 11            |

### Recovery and RSD% from Whole Blood Spiked at 3 Levels

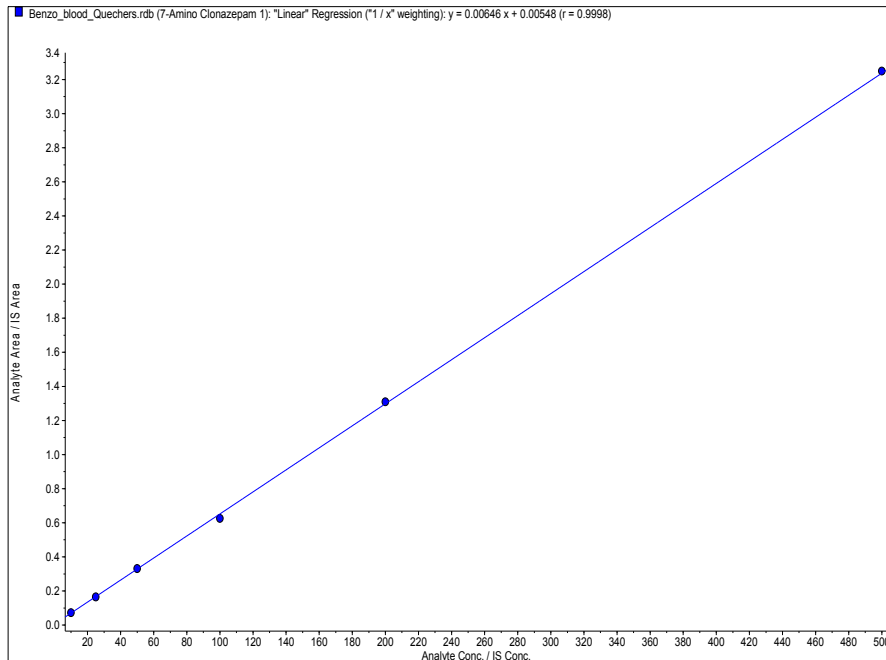
| Compound                 | Spiked at 10 ng/mL |            | Spiked at 50 ng/mL |            | Spiked at 200 ng/mL |            |
|--------------------------|--------------------|------------|--------------------|------------|---------------------|------------|
|                          | Recovery%          | RSD% (n=6) | Recovery%          | RSD% (n=6) | Recovery%           | RSD% (n=6) |
| 7-aminoclonazepam        | 88.6               | 7.5        | 96.9               | 2.1        | 99.7                | 3.8        |
| Alpha-Hydroxy-Alprazolam | 101.2              | 3.4        | 91.0               | 2.0        | 90.3                | 2.7        |
| Alprazolam               | 92.3               | 10.7       | 90.2               | 4.0        | 86.5                | 3.5        |
| Clonazepam               | 96.4               | 3.6        | 105.0              | 3.2        | 103.0               | 2.0        |
| Diazepam                 | 85.5               | 3.3        | 103.0              | 2.7        | 100.4               | 1.9        |
| Lorazepam                | 96.9               | 5.1        | 93.7               | 4.1        | 91.6                | 2.7        |
| Midazolam                | 96.7               | 2.7        | 101.6              | 2.7        | 100.6               | 1.9        |
| Nordiazepam              | 88.4               | 3.9        | 99.7               | 2.5        | 97.8                | 2.3        |
| Oxazepam                 | 86.5               | 1.9        | 93.8               | 2.4        | 92.6                | 1.7        |
| Temazepam                | 96.7               | 2.7        | 101.6              | 2.7        | 100.6               | 1.9        |

## Chromatogram of a whole blood sample spiked with 200 ng/mL benzos



**Peak list:** 1. 7-aminoclonazepam; 2. Midazolam; 3. Lorazepam; 4. Oxazepam; 5. Clonazepam; 6. Alpha-Hydroxy-Alprazolam; 7. Nordiazepam; 8. Temazepam; 9. Alprazolam; 10. Diazepam

## Matrix Matched Calibration Curve of 7-aminoclonazepam ( $R^2=0.9998$ )



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