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

UCT Part Numbers

ECQUUS15CT
Enviro-Clean® QuEChERS
15 mL centrifuge tube with
400 mg MgSO4 and 100 mg NaOAc

CUMPSC18CT
Enviro-Clean® dSPE
2 mL centrifuge tube with
150 mg $MgSO_4$, 50 mg PSA and
50 mg C18

SLDA100ID21-3UM
Selectra® DA HPLC column
100 x 2.1 mm, 3 µm

SLDAGDC21-3UM
Selectra® DA guard column
10 x 2.0 mm, 3µm

SLGRDHLDR
Guard Column Holder

CLINICAL FORENSICS

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 ($MgSO_4$) 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 $MgSO_4$, 50 mg PSA, and 50 mg C18. $MgSO_4$ 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% ≤ 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.

QuEChERS Procedure:

1. Add 2 mL of MeCN with 0.4% FA to 15-mL centrifuge tube containing 400 mg $MgSO_4$ and 100 mg NaOAc
2. Add internal standards
3. Add 1 mL whole blood
4. Cap and shake for 1 minute at 1000 strokes/minute using a Spex 2010 Geno-Grinder
5. Centrifuge for 5 minutes at 3000 g

dSPE Cleanup:

1. Transfer 1 mL of supernatant to 2 mL dSPE tube
2. Cap and shake for 1 minute at 1000 strokes/minute using a Spex 2010 Geno-Grinder
3. Centrifuge for 5 minutes at 3000 g
4. 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.
5. Samples are now ready for LC-MS/MS analysis

LC-MS/MS Parameters:

Instrumentation	
HPLC system	Agilent 1200 Binary Pump SL
MS system	API 4000 QTRAP (MS/MS)
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 3 µm
Guard column	UCT Selectra® DA, 10 × 2.1 mm, 3 µm
Column temperature	50°C
Flow rate	300 µL/min
Injection volume	10 µL

Mobile Phase Gradient		
Time (min)	**% Mobile Phase A** (0.1% Formic Acid in Water)	**% Mobile Phase B** (0.1% Formic Acid in MEOH)
0.0	70	30
0.5	70	30
2.0	25	75
6.5	25	75
7.0	0	100
9.0	0	100
10.1	70	30
14.0	70	30

MRM transitions				
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	NA
Oxazepam D5	8.98	292.1	246.2	NA

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Results:

Linearity and Matrix Effect

Compound	Solvent standard		Matrix-matched standard		Matrix effect (%)
	Slope	Linearity (R^2)	Slope	Linearity (R^2)	
7-aminoclonazepam	0.00823	0.9993	0.00646	0.9998	-22
α-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 (n=6)

Compound	10 ng/mL		50 ng/mL		200 ng/mL	
	Recovery%	RSD%	Recovery%	RSD%	Recovery%	RSD%
7-aminoclonazepam	88.6	7.5	96.9	2.1	99.7	3.8
α-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

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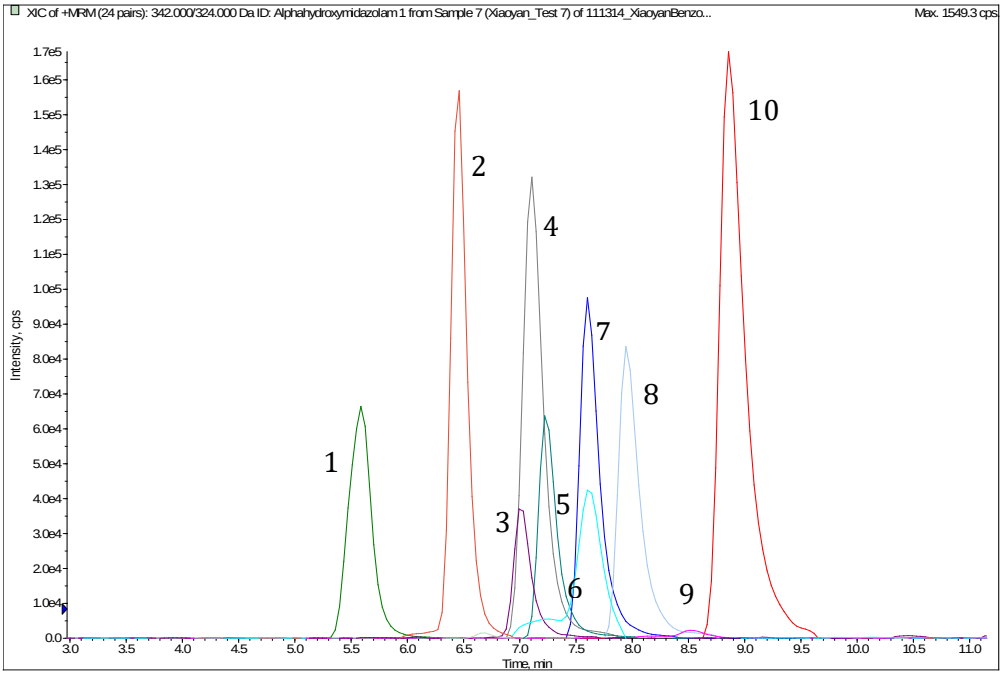
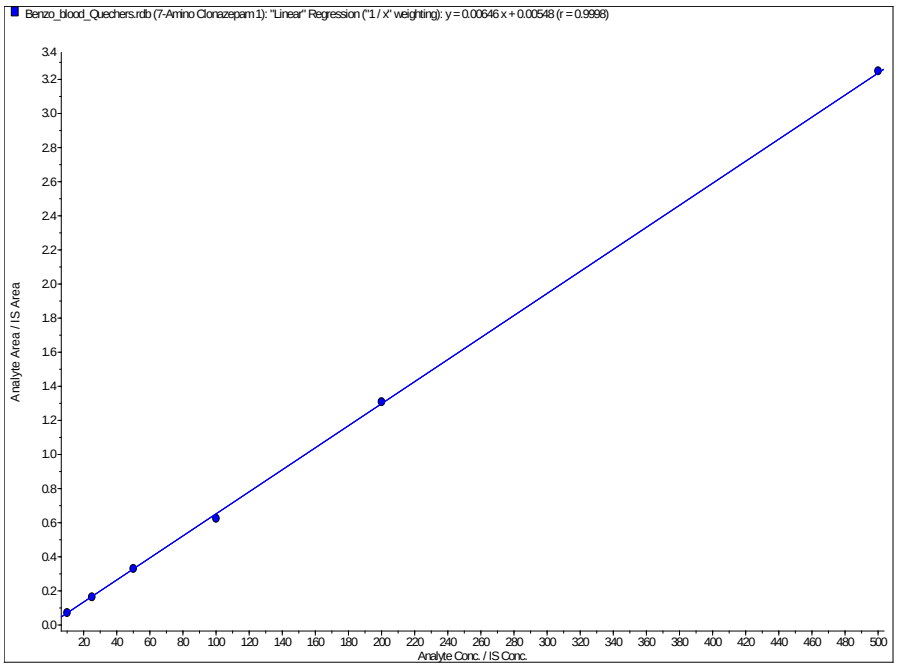
Chromatogram of a whole blood sample spiked with 200 ng/mL of drug



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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