Benzodope and Beyond: Detection of Benzodiazepine and Opioid Mixtures in Blood and Alternative Matrices using LC-MS/MS

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INTRODUCTION

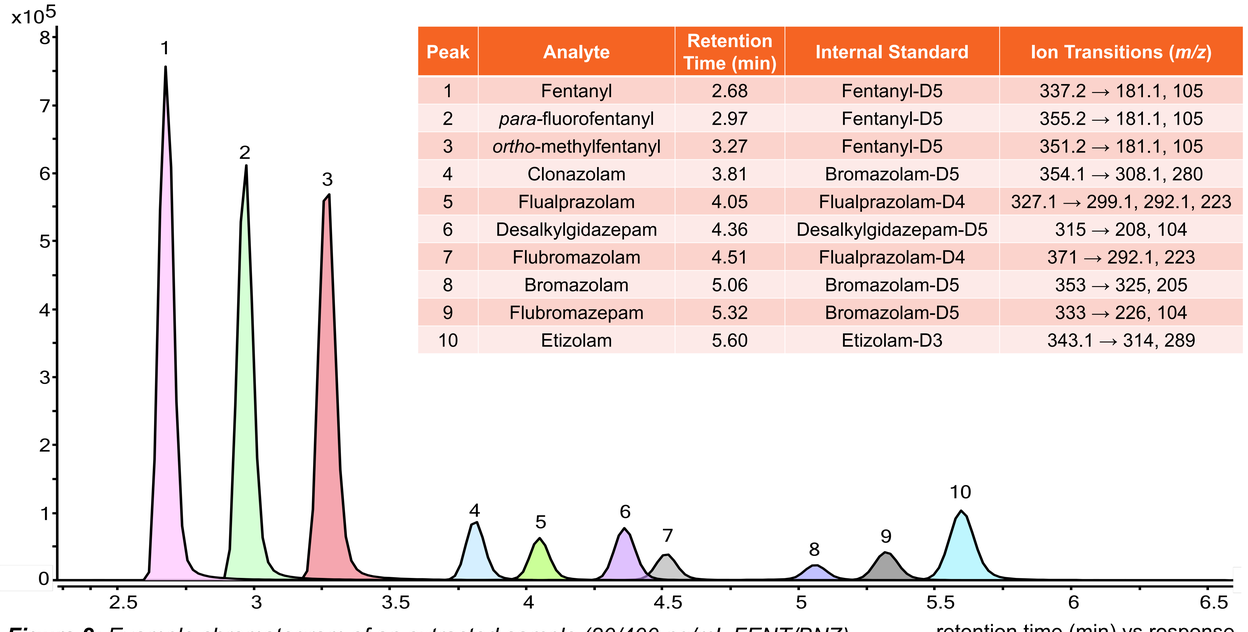
In recent years, more novel psychoactive substances (NPS) from benzodiazepine and opioid classes have been identified in forensic casework[1]. Both benzodiazepines and opioids are CNS depressants, producing synergistic effects when used in combination. This increases the likelihood of adverse effects with potentially fatal consequences, particularly among illicit drug users who combine fentanyls and benzodiazepines (benzodope), necessitating detection of these compounds in forensic matrices. While blood is the preferred toxicological matrix, there are instances where blood may not be readily available, such as in cases of decomposition. For those scenarios, other matrices such as tissues must be considered to support analytical results[2]. This study supports the application of solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) to detect possible benzodope combinations in blood and tissues.

MATERIALS & METHODS

Prep	• Homogenize tissue (liver or heart) 1:4 with DI water • Fortify 250 µL matrix with calibrator or QC, ISTD
Buffer	• Add 2.5 mL phosphate buffer (100 mM, pH 6.0) • Centrifuge at 4200 rpm for 10 minutes
Load	• Apply sample to SPE columns (Cerex® Clin II) • Gently elute matrix at ≤3 psi
Wash	• 1 mL DI water • 1 mL acetic acid (0.1 M), dry 5 minutes • 1 mL hexanes, dry 30 seconds • 1 mL ethyl acetate • 1 mL methanol
Elute	• Place glass conical vials for elution • 2 mL 95:5 ethyl acetate:ammonium hydroxide
Dry & Recon	• Dry down under nitrogen gas (40°C) • Reconstitute in 50 µL of mobile phase (85:15)

Figure 1: *SPE protocol*

RESULTS & DISCUSSION



Peak	Analyte	Retention Time (min)	Internal Standard	Ion Transitions (*m/z*)
1	Fentanyl	2.68	Fentanyl-D5	337.2 → 181.1, 105
2	*para*-fluorofentanyl	2.97	Fentanyl-D5	355.2 → 181.1, 105
3	*ortho*-methylfentanyl	3.27	Fentanyl-D5	351.2 → 181.1, 105
4	Clonazolam	3.81	Bromazolam-D5	354.1 → 308.1, 280
5	Flualprazolam	4.05	Flualprazolam-D4	327.1 → 299.1, 292.1, 223
6	Desalkylgidazepam	4.36	Desalkylgidazepam-D5	315 → 208, 104
7	Flubromazolam	4.51	Flualprazolam-D4	371 → 292.1, 223
8	Bromazolam	5.06	Bromazolam-D5	353 → 325, 205
9	Flubromazepam	5.32	Bromazolam-D5	333 → 226, 104
10	Etizolam	5.60	Etizolam-D3	343.1 → 314, 289

Figure 2: *Example chromatogram of an extracted sample (80/400 ng/mL FENT/BNZ)*

Table 2: *Validation Summary Results*

Parameter	Fentanyl Analogs	Benzodiazepines
Calibration Model	Blood (BL) curve with tissue (TS) QCs	
	Quadratic 1/x weighted all analytes	
	0.1 – 100 ng/mL	0.5 – 500 ng/mL
Precision (%CV)*	0.3, 20, 80 ng/mL	1.5, 100, 400 ng/mL
Within-run	Within ±8.3% (BL)	Within ±19.3% (BL)
Between-run	Within ±6.5% (BL)	Within ±14.1% (BL)
Grand Bias (%)*	Within ±8.4% (BL)	Within ±11.9% (BL)
Limit of Detection*	0.05 ng/mL (BL, TS)	0.5 ng/mL (BL), 1.0 ng/mL (TS)
Limit of Quantitation*	0.1 ng/mL (BL, TS)	1.0 ng/mL (BL), TS variable
Matrix Effects (%)	BL within ± 10.0%; TS within ± 25.0%	
Carryover (CO)	Acceptable following highest calibrator	
Interferences	No interferences from matrix, internal standard, commonly encountered drugs	
Processed sample stability (Relative peak area)*	Analytes stable up to 72 hours at 10°C in blood, except flubromazepam (stable up to 48 hours)	

**Tissue validation results are qualitative only; quantitative validation is in progress to verify LOQ, bias, and precision.*

References

1 Krotulski AJ, Walton SE, DeBord J, et al (2025) Trend Reports – NPS Benzodiazepines in the United States. Retrieved from https://www.cfsre.org/nps-discovery/trend-reports/nps-benzodiazepines

2 Hansen SL, Nielsen MKK, Linnet K, Rasmussen BS. (2021) Simple implementation of muscle tissue into routine workflow of blood analysis in forensic cases – A validated method for quantification of 29 drugs in postmortem blood and muscle samples by UHPLC–MS/MS. Forensic Science International; 325:110901.

MATERIALS & METHODS

Analysis was conducted on an Agilent 1290 Infinity II LC and Agilent 6470 triple quadrupole mass spectrometer. Positive electrospray ionization was used with optimized instrumental parameters.

Table 1: *Optimized instrumental parameters*

Parameter	Value
Column	Agilent Poroshell 120 Phenyl Hexyl (2.1 x 100 mm, 2.7 µm) with guard
Mobile Phases	A: 0.1% formic acid in DI water B: 0.1% formic acid in acetonitrile
Gradient	0-1.0 min (15-30% B) 1.0-5.0 min (30% B hold) 5.0-6.0 min (30-32% B) 6.0-8.0 min (32-90% B) 8.0-9.0 min (90% B hold)
Column temp	35°C
Flow rate	0.5 mL/min
Source parameters	350°C drying gas (7 L/min); 400°C sheath gas (12 L/min); 4000 V capillary; 40 psi nebulizer; 0 V nozzle

CONCLUSIONS

This method was validated in accordance with ANSI/ASB Standard 036 quantitatively for blood and qualitatively for tissue (bovine/chicken) matrices using LC-MS/MS. Quantitative validation for tissues is in progress. However, low limits of detection were still achieved and the ability to detect these analytes within tissue samples provides critical value in postmortem toxicology when traditional matrices are unavailable. This method provides a baseline for future method development assessing the detection of benzodope in human samples.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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