A RAPID AND COMPREHENSIVE UHPLC-MS/MS METHOD FOR THE ANALYSIS OF BENZODIAZEPINES IN URINE

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INTRODUCTION

- Benzodiazepines are important toxicology drugs which are widely prescribed and also highly abused.[1]
- Since the mid-2000s, new designer benzodiazepines have been reported and continue to emerge, these have unknown potencies and risks associated.[2]
- Counterfeit tablets containing designer benzodiazepines are of particular concern, as these could be unknowingly taken.[2]
- The aim of this study is to develop a comprehensive clinical research method for confirmatory analysis of traditional and designer benzodiazepines with UHPLC-MS/MS, which includes a rapid and simplified mixed-mode sample preparation method.

METHODS

Sample pretreatment

Samples were prepared by diluting Certified Reference Material (CRM) solutions into blank urine. Calibration samples were prepared with the concentration range of 5-1000 ng/mL for 26 benzodiazepines (Table 1). Quality control samples (QCs) at four concentrations covered by the calibration range were also prepared.

Sample extraction

Samples were extracted using mixed-mode solid phase extraction (SPE) in the wells of a Waters Oasis™ MCX µElution™ Plate. Enzymatic hydrolysis was performed in the wells of the extraction plate, minimizing transfer steps. The procedure was significantly simplified by eliminating the conditioning and equilibration steps and using a single wash step instead of two. Briefly, 100 µL of urine, 100 µL of hydrolysis enzyme mix and 20 µL of internal standard were added to the wells of a Waters Oasis MCX µElution Plate. Samples were incubated for 10 minutes and then loaded onto the SPE plate by vacuum. All samplles were then washed with 200 µL of 20:80 v/v MeOH:Water folowed by elution with 2 x 25 µL 50:50 v/v ACN:MeOH containing 5% strong ammonia. Samples were then diuted with 150 µL of 97:2:1 v/v/v Water:ACN:formic acid.

Sample analysis

- A UHPLC-MS/MS method was applied in conjunction with a Waters Xevo™ TQ-S micro Mass Spectrometer which was operated with electrospray positive mode.
- Two MRM transitions (quantifier and qualifier) were monitored for each of the 26 analytes and a single MRM transition for the deuterated internal standards.
- The chromatography conditions employed are displayed in Table 2. The initial starting condition was 2% mobile phase B, ramping to 90% over 3.5 minutes before re-equilibration.

Parameter	ACQUITY™ UPLC™ I-Class PLUS System
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Column	ACQUITY BEH™ C_{18} (2.1x100mm, 1.7µm) Column
Column temperature	40°C
Sample temperature	10°C
Injection volume	5 µL
Run time	4 minutes

Table 2. UHPLC conditions used for UPLC-MS/MS method

RAPID UHPLC-MS/MS METHOD FOR 26 BENZODIAZEPINES ANALYSIS IN URINE

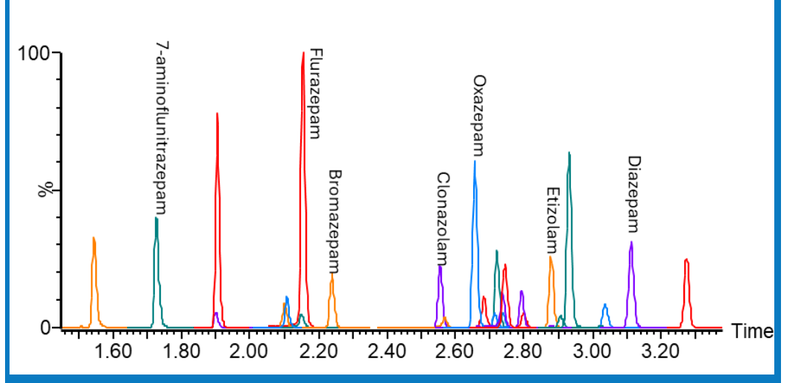

Figure 2. Chromatography of all 26 compounds included in the panel. The earliest eluting compound is 7-aminclonazepam at 1.55 minutes and the latest eluting compound is diclazepam at 3.28 minutes.

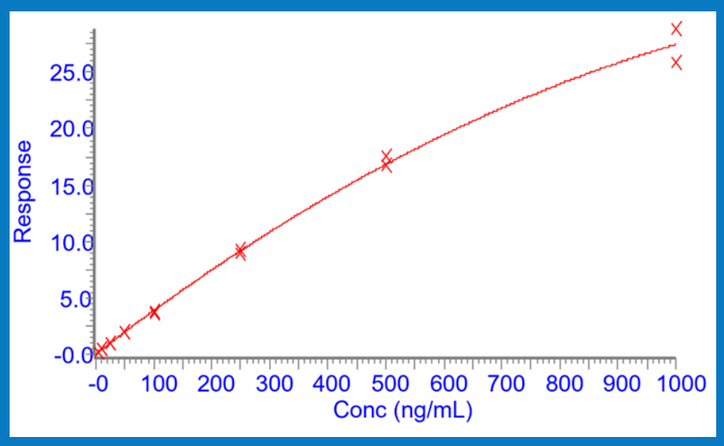

Figure 2. Calibration curve for etizolam. Response is quadratic (1/x weighting applied), R^2 = 0.998. All other compounds included in the panel $R^2 \geq 0.99$.

RESULTS

- Excellent separation and baseline resolution were achieved for most benzodiazepines. No interference was observed between analytes or internal standards. Figure 1 shows quantifier ion chromatograms from a urine sample spiked with 26 compounds at 50 ng/mL.
- Some internal standards interfered with quantitation e.g. clonazepam-d4 and lorazepam, in those cases, surrogate internal standards were used rather than the deuterated form.
- Extraction recovery and matrix effects were assessed from six lots of urine. The mean extraction recovery was 86% (range 79% to 102%) and all compounds had recoveries greater than 75%. Coefficients of variation (%CV) were less than 10% for all compounds.
- Ion suppression was observed for most analytes, ranging from 30% suppression (chlordiazepoxide) to 35% enhancement (α-hydroxyalprazolam). Except for 8-aminoclonazepam, matrix effects standard deviations were less than 15%. Using internal standards, matrix effects were less than 20% for all compounds, with the exception of pyrazolam and flubromazolam which were corrected using surrogate internal standards.
- Calibrators (5–1000 ng/mL) and QCs were analysed over five days. Quadratic response curves (1/x weighting) showed R^2 values from 0.990 to 0.999. Figure 2 displays the 8-point calibration curve for etizolam.
- Analysis of QCs spiked at four concentrations over five days resulted in coefficients of variation (CV%) less than 15% and all were within ± 15% of the nominal concentration (Table 1).
- To assess accuracy, a proficiency test sample from Axio Proficiency Testing, LGC Group was evaluated. The PT sample was found to contain both diazepam and nordiazepam with the performance scores for both analytes being deemed satisfactory ([z] ≤ 2). Ten replicates of the sample were analysed and all results had %RSD values < 5%.

	15 ng/mL		75ng/mL		200 ng/mL		750 ng/mL	
	Acc.	%CV	Acc.	%CV	Acc.	%CV	Acc.	%CV
7-aminoclonazepam	98.7	4.6	103.1	4.2	97.4	3.0	94.8	2.6
7-aminoflunitrazepam	95.9	3.9	105.1	5.2	98.6	3.0	93.6	3.7
8-aminoclonazolam	97.9	4.1	100.7	4.7	97.2	3.1	95.7	4.0
Chlordiazepoxide	102.8	3.6	100.1	5.1	96.8	2.8	96.1	3.0
a-hydroxyetizolam	102.7	6.0	98.9	8.1	94.9	4.3	95.7	4.2
Midazolam	103.1	3.3	100.7	5.5	97.0	2.7	96.0	3.1
Pyrazolam	97.5	6.0	102.1	5.5	96.7	3.8	93.5	5.7
Flurazepam	104.2	3.3	103.0	4.5	97.1	2.3	95.3	2.3
Bromazepam	104.5	4.1	103.4	4.0	97.0	3.1	93.6	2.6
Clonazolam	102.8	4.1	101.0	4.7	96.3	3.6	94.4	3.3
a-hydroxyalprazolam	98.6	3.9	95.6	5.9	96.0	3.0	96.0	5.3
Oxazepam	102.1	3.6	101.0	4.7	96.4	2.2	95.9	3.4
Flualprazolam	102.0	2.7	99.8	3.6	97.2	3.6	95.1	2.8
Clonazepam	105.3	2.2	108.5	3.9	101.5	3.9	101.0	6.7
Lorazepam	102.0	3.5	101.1	4.1	97.4	3.2	96.6	3.9
Flubromazolam	96.5	6.1	105.6	5.2	97.4	2.4	95.5	3.3
Alprazolam	105.2	3.6	102.7	5.8	96.9	3.4	93.6	3.0
Nordiazepam	104.6	4.3	104.3	4.1	96.8	3.0	94.6	2.6
Triazolam	104.0	4.3	101.5	4.9	96.3	3.1	94.2	3.7
Bromazolam	102.9	4.9	100.0	4.8	96.1	2.7	96.6	2.5
Etizolam	104.2	4.1	99.5	6.0	95.1	4.1	93.4	3.5
Flubromazepam	102.3	4.0	99.7	6.2	97.0	3.3	96.3	2.7
Temazepam	102.1	3.7	99.6	4.4	96.6	2.9	96.9	4.3
Phenazepam	102.8	3.4	101.1	5.1	96.4	3.2	95.4	3.2
Diazepam	104.8	4.4	104.2	4.9	97.6	2.6	95.2	4.8
Diclazepam	104.3	4.3	103.0	4.1	97.1	2.6	94.3	3.4

Table 1. Results for quality controls, which were blank urine samples spiked at four different concentrations (15, 75, 200, 750 ng/mL). Samples were analysed in replicates of six, over five days of testing.

CONCLUSION

- **The sample preparation method enables efficient extraction of a comprehensive panel of 26 benzodiazepines and designer benzodiazepines from urine samples.**
- **Chromatographic separation using the ACQUITY UPLC BEH C_{18} Column allows rapid analysis, with a total run time of just 4 minutes.**
- **Quantification using the Xevo TQ-S micro Mass Spectrometer offers accurate quantitation over a broad dynamic range.**
- **The combination of the sample preparation, fast chromatographic separation and MRM detection delivers a rapid, accurate and precise method for the comprehensive analysis of both traditional and emerging designer benzodiazepines in urine, for clinical research.**

References

1. Brunetti, et al. Designer Benzodiazepines: A Review of Toxicology and Public Health Risks. *Pharmaceuticals.* 14:560, 2021.
2. European Monitoring Centre for Drugs and Drug addiction (2021). *New benzodiazepines in Europe—a review* (June 2021),

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