

# Validation of a Benzodiazepine and Z-Drug Method Using an Agilent 6430 LC/MS/MS

## Application Note

Forensics

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### Abstract

A method was developed and validated for the quantitation of benzodiazepines in biological samples with LC/MS/MS using an Agilent 6430 Triple Quadrupole LC/MS system. Validation studies showed that the LC/MS/MS method provides reliable results for the analysis of benzodiazepines and the z-drugs zolpidem, zopiclone, and zaleplon that meet acceptable criteria set for this application. The concentration range of target compounds used in this validation was chosen to fit the commonly encountered range of analyte concentrations seen in casework. The method displays good accuracy and precision for the detection of benzodiazepines and z-drugs in blood. Other aspects evaluated during validation include interference, stability, dilution integrity, suppression/enhancement, and recovery for all target compounds.



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## Introduction

Benzodiazepines are analyzed in urine, oral fluid, and blood in many forensic toxicology laboratories. Quantitative analysis of benzodiazepines in blood is performed in the investigation of Driving Under the Influence of Drug (DUID) cases and constitutes a significant portion of the workload for many forensic toxicology laboratories worldwide. Standard GC/MS and GC/MS/MS analysis requires time consuming sample preparation involving derivatization prior to analysis. HPLC quantitation has the advantage over GC/MS sample preparation, which does not require derivatization, but is limited in the number of target compounds analyzed due to resolution constraints. HPLC also requires confirmation using another analytical instrument such as LC/MS or GC/MS. However, the developing role of liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) in forensic and clinical toxicology has been assessed by leading experts in the field. This technique is becoming increasingly useful in routine toxicological analysis given its accuracy and sensitivity [1].

This application note addresses the development and validation of an LC/MS/MS method on an Agilent 6430 Triple Quadrupole LC/MS system for the quantitation of benzodiazepines and the z-drugs zolpidem, zopiclone, and zaleplon. Validation studies included calibration model fits,

precision and accuracy of the method, sensitivity measured by the limit of detection (LOD) and the limit of quantitation (LOQ), stability, robustness, dilution integrity, carryover, and ion suppression. Validation studies were conducted using the SWGTOX guidelines in conjunction with the Virginia Department of Forensic Science validation guidelines [2,3]. As a result, the method met all criteria for data integrity, and was found to be a reliable method for routine benzodiazepine and z-drug analysis in whole blood.

## Experimental

The method includes an alkaline liquid-liquid extraction with quantitation and confirmation by an Agilent 6430 Triple Quadrupole LC/MS system, using Agilent MassHunter Quantitative Analysis (B.0.4) software for data acquisition and analysis. Benzodiazepines, zolpidem, zopiclone, and zaleplon were extracted from biological samples with sodium carbonate buffer and 1-chlorobutane in accordance with the Virginia Department of Forensic Science's Procedures Manual [3]. The method was validated for the target compounds shown in Table 1. A more comprehensive explanation of the method, including sample preparation and instrument parameters is detailed in "Benzodiazepine and Z-Drug Quantitation Using an Agilent 6430 LC/MS/MS" [4].

Table 1. Target Compounds and Internal Standards

Target	Internal standard	Target	Internal standard
7-aminoclonazepam	7-aminoclonazepam-d4	$\alpha$ -hydroxyalprazolam	$\alpha$ -hydroxyalprazolam-d5
7-aminoflunitrazepam	7-aminoclonazepam-d4	$\alpha$ -hydroxymidazolam	$\alpha$ -hydroxyalprazolam-d5
zopiclone	zopiclone-d4	$\alpha$ -hydroxytriazolam	$\alpha$ -hydroxyalprazolam-d5
zolpidem	zolpidem-d6	midazolam	alprazolam-d5
zaleplon	zolpidem-d6	alprazolam	alprazolam-d5
chlordiazepoxide	diazepam-d5	oxazepam	oxazepam-d5
flurazepam	diazepam-d5	lorazepam	oxazepam-d5
nordiazepam	diazepam-d5	clonazepam	clonazepam-d4
<i>n</i> -desalkylflurazepam	diazepam-d5	flunitrazepam	clonazepam-d4
phenazepam	diazepam-d5	temazepam	temazepam-d5
diazepam	diazepam-d5		

## Sample preparation

Validation studies were performed using pooled and spiked standards. Samples were extracted using the procedure outlined in "Benzodiazepine and Z-Drug Quantitation Using an Agilent 6430 LC/MS" [4]. Pooled standards were prepared by spiking a large volume of blank blood with respective concentrations of target compounds. One-milliliter aliquots were taken from the pooled samples and extracted prior to quantitative analysis by LC/MS/MS. Spiked standards were prepared by pipetting appropriate volumes of working standard solutions into clean test tubes with 1.0 mL of blank blood.

**Working standard solution (0.01 mg/mL):** Pipette 100  $\mu$ L of the 1 mg/mL (or 1 mL of the 0.1 mg/mL) stock solution into a 10-mL volumetric flask and qs to volume with methanol.

**Working standard solution (0.001 mg/mL):** Pipette 1.0 mL of 0.01 mg/mL working standard solution into a 10-mL volumetric flask and qs to volume with methanol.

**Stock internal standard solution (0.01 mg/mL):** Pipette 100  $\mu$ L of the 1 mg/mL (or 1 mL of 0.1 mg/mL) stock solution of deuterated standards into a 10-mL volumetric flask and qs to volume with methanol.

**Working internal standard solution (0.001 mg/mL):** Pipette 1.0 mL of the 0.1 mg/mL stock internal standard solution into a 10-mL volumetric flask and qs to volume with methanol.

## Results and Discussion

### Chromatography

Figure 1 shows an example chromatogram of an extracted sample illustrating the chromatographic separation achieved with this method. As demonstrated in Figure 1, separation of the 22 targets is excellent with a run time of less than 12 minutes. Peak shape is good with no significant tailing or other chromatographic abnormalities.

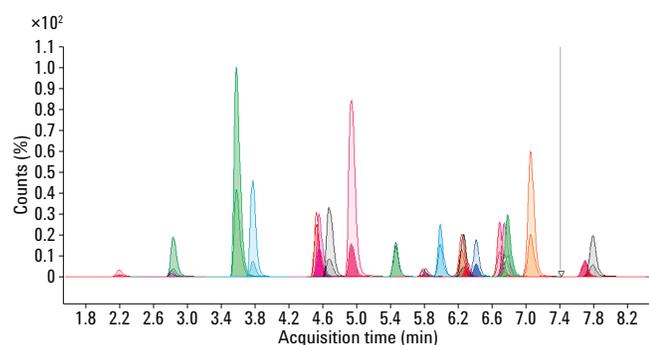


Figure 1. Qualitative chromatographic retention of analytes.

## Linearity and calibration model

Seven calibrators were run with every batch and used to assess the instrument response for each target compound. To establish the calibration model, the origin is ignored and the correlation coefficient ( $R^2$ ) should be  $\geq 0.985$ . The back calculated concentration should be within  $\pm 20\%$  of the target concentration.

All benzodiazepines assessed in this method predicted a weighted-quadratic calibration model, whereas, zaleplon, zolpidem, and zopiclone predicted a weighted-linear fit model. The best fit calibration model as well as the dynamic range and average  $R^2$  value are tabulated in Table 2. A total of sixteen calibration curves were used to assess the calibration model.

The table shows that the  $R^2$  values for each target are  $> 0.997$ , which is within the predetermined acceptance criteria of  $\geq 0.985$ .

Table 2. Calibration Reproducibility of Benzodiazepines and Z-Drugs Using LC/MS/MS

Target	Dynamic range (ng/mL)	Calibration model	Average $R^2$
7-aminoclonazepam	10–2,000	Quadratic	0.998 $\pm$ 0.0009
7-aminoflunitrazepam	10–2,000	Quadratic	0.998 $\pm$ 0.0008
$\alpha$ -hydroxyalprazolam	10–2,000	Quadratic	0.999 $\pm$ 0.0004
$\alpha$ -hydroxymidazolam	10–2,000	Quadratic	0.999 $\pm$ 0.0009
$\alpha$ -hydroxytriazolam	10–2,000	Quadratic	0.999 $\pm$ 0.0010
<i>n</i> -desalkylflurazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0007
alprazolam	10–2,000	Quadratic	0.999 $\pm$ 0.0007
chlordiazepoxide	10–2,000	Quadratic	0.998 $\pm$ 0.0010
clonazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0003
diazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0006
flunitrazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0002
flurazepam	10–2,000	Quadratic	0.998 $\pm$ 0.0012
lorazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0013
midazolam	10–2,000	Quadratic	0.998 $\pm$ 0.0013
nordiazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0005
oxazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0015
phenazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0009
temazepam	10–2,000	Quadratic	0.999 $\pm$ 0.0005
triazolam	10–2,000	Quadratic	0.999 $\pm$ 0.0003
zaleplon	10–2,000	Linear	0.999 $\pm$ 0.0019
zolpidem	5.0–2,000	Linear	0.999 $\pm$ 0.0002
zopiclone	10–2,000	Linear	0.997 $\pm$ 0.0013

## Accuracy

Accuracy studies were conducted with both spiked and pooled samples. Blank blood samples were spiked with 22 target compounds at three different concentrations (0.05, 0.25, 0.75 mg/L) in triplicate with each batch for a total of six batches. The samples were extracted, then quantitated by LC/MS/MS. Pooled samples were fortified into 50.0 mL of blank blood and a 1-mL aliquot was taken and extracted. A total of five different concentrations (0.01, 0.03, 0.70, 0.14, 0.22 mg/L) was assessed with triplicate analysis for each batch for a total of five batches.

The acceptance criterion for the spiked accuracy was  $\pm 20\%$  for all three concentrations levels. The pooled accuracy acceptance criterion was also  $\pm 20\%$  for all concentrations except at the LOQ. The acceptance criterion at the LOQ was  $\pm 30\%$ . All extractions were used to determine the overall accuracy for the method.

Table 3 represents the accuracy of the spiked blood samples. The percent accuracy also demonstrates bias within the measurements. The *n* was 18 for all three concentration levels.

The spiked accuracy ranged from  $122 \pm 6\%$  to  $86 \pm 11\%$ . All targets were within the acceptance criteria of  $\pm 20\%$  except for midazolam at 250 ng/mL, which had an accuracy of  $122 \pm 6\%$  at 250 ng/mL.

Table 3. Percent Accuracy/Bias for Spiked Benzodiazepines Quantitated by LC/MS/MS

Spiked accuracy			
% Accuracy (SD); n = 18			
	50 ng/mL	250 ng/mL	750 ng/mL
7-aminoclonazepam	96 (9)	103 (14)	103 (10)
7-aminoflunitrazepam	108 (11)	109 (16)	105 (9)
zopiclone	88 (12)	112 (15)	99 (7)
zolpidem	109 (5)	112 (9)	104 (3)
zaleplon	99 (3)	105 (9)	100 (4)
chlordiazepoxide	86 (11)	97 (15)	93 (7)
flurazepam	114 (7)	114 (7)	101 (5)
nordiazepam	103 (5)	111 (11)	106 (5)
<i>n</i> -desalkylflurazepam	106 (5)	111 (11)	104 (8)
phenazepam	105 (5)	113 (12)	109 (8)
diazepam	108 (4)	113 (9)	106 (4)
$\alpha$ -hydroxyalprazolam	98 (3)	105 (8)	101 (3)
$\alpha$ -hydroxymidazolam	106 (4)	103 (6)	96 (4)
$\alpha$ -hydroxytriazolam	103 (5)	107 (7)	100 (5)
midazolam	114 (14)	122 (6)	112 (4)
alprazolam	102 (7)	111 (8)	106 (5)
triazolam	102 (4)	111 (9)	106 (7)
oxazepam	98 (13)	115 (7)	109 (3)
lorazepam	103 (6)	106 (6)	101 (4)
clonazepam	104 (4)	109 (8)	103 (4)
flunitrazepam	103 (6)	105 (6)	99 (4)
temazepam	102 (2)	107 (8)	103 (4)

Table 4 represents the accuracy of the pooled blood samples. The percent accuracy also demonstrates bias in the measurement. All target compounds were assessed at five different pooled concentrations. All extractions were used to determine the accuracy to establish an *n* of 15.

The pooled accuracy ranged from 76 ± 5% to 178 ± 9%. All targets were within the predetermined acceptance criterion with the exception of chlordiazepoxide, midazolam, and  $\alpha$ -hydroxymidazolam. The accuracy of chlordiazepoxide ranged from 139 ± 16% to 178 ± 9%. Also, the accuracy of  $\alpha$ -hydroxymidazolam was beyond the acceptance criteria at 1,400 ng/mL with an accuracy of 76 ± 5%. Midazolam at 30 ng/mL was slightly higher than the acceptance criteria with an accuracy of 123 ± 7%. Overall, this method was proven to be accurate for all the targets evaluated with the exception of three compounds.

Table 4. Percent Accuracy/Bias for Pooled Benzodiazepines Quantitated by LC/MS/MS

**Pooled accuracy**

**% Accuracy (SD); n = 15**

	10 ng/mL	30 ng/mL	700 ng/mL	1,400 ng/mL	2,200 ng/mL
7-aminoclonazepam	100 (9)	99 (4)	101 (3)	97 (7)	103 (4)
7-aminoflunitrazepam	95 (11)	102 (6)	99 (6)	95 (11)	103 (5)
zopiclone	118 (17)	103 (7)	101 (6)	91 (10)	90 (7)
zolpidem	105 (4)	114 (4)	109 (4)	105 (7)	110 (5)
chlordiazepoxide	178 (9)	154 (18)	139 (16)	145 (52)	140 (13)
$\alpha$ -Hydroxymidazolam	86 (8)	100 (5)	83 (4)	76 (5)	83 (4)
midazolam	110 (10)	123 (7)	117 (5)	111 (8)	118 (5)
flurazepam	96 (10)	106 (7)	97 (3)	93 (7)	98 (4)
zaleplon	104 (9)	109 (12)	106 (8)	102 (9)	108 (8)
$\alpha$ -hydroxyalprazolam	–	–	–	–	–
$\alpha$ -hydroxotriazolam	89 (10)	106 (6)	87 (13)	80 (21)	91 (6)
nordiazepam	92 (6)	103 (6)	100 (4)	97 (9)	102 (5)
oxazepam	101 (10)	114 (8)	103 (7)	99 (8)	105 (7)
clonazepam	98 (6)	106 (6)	97 (13)	96 (10)	100 (7)
lorazepam	94 (10)	120 (11)	95 (14)	90 (16)	95 (16)
alprazolam	87 (8)	102 (5)	102 (6)	98 (8)	103 (6)
<i>n</i> -desalkylflurazepam	86 (6)	116 (27)	97 (5)	92 (8)	99 (11)
triazolam	94 (7)	107 (6)	101 (6)	96 (9)	101 (8)
flunitrazepam	99 (7)	109 (4)	98 (3)	93 (7)	98 (6)
temazepam	89 (5)	107 (4)	99 (3)	95 (7)	96 (14)
diazepam	90 (8)	105 (4)	98 (4)	94 (7)	99 (6)
phenazepam	100 (7)	111 (8)	104 (9)	100 (14)	104 (10)

## Precision

The inter-run and intra-run precision was also assessed for both spiked and pooled blood samples. Samples were fortified at three concentrations (0.05, 0.25, 0.75 mg/L). The inter-run precision was assessed by analyzing triplicate analyses of the targets over five concentrations for a total of five batches. The intra-run precision was established by analyzing the triplicate analyses of the five batches and reporting the highest imprecision per individual batch.

Table 5. Inter-run Precision of Spiked Benzodiazepines Quantitated by LC/MS/MS

### Spiked inter-run precision

Mean  $\pm$  SD (% CV) n = 18

	50 ng/mL	250 ng/mL	750 ng/mL
7-aminoclonazepam	48 $\pm$ 5 (10)	258 $\pm$ 34 (13)	773 $\pm$ 71 (9)
7-aminoflunitrazepam	54 $\pm$ 6 (10)	273 $\pm$ 40 (15)	785 $\pm$ 68 (9)
zopiclone	44 $\pm$ 6 (14)	281 $\pm$ 39 (14)	744 $\pm$ 51 (7)
zolpidem	54 $\pm$ 3 (5)	281 $\pm$ 23 (8)	778 $\pm$ 22 (3)
zaleplon	49 $\pm$ 1 (3)	262 $\pm$ 22 (9)	751 $\pm$ 30 (4)
Chlordiazepoxide	43 $\pm$ 6 (13)	244 $\pm$ 37 (15)	698 $\pm$ 53 (8)
flurazepam	57 $\pm$ 3 (6)	284 $\pm$ 16 (6)	756 $\pm$ 34 (5)
nordiazepam	51 $\pm$ 2 (5)	278 $\pm$ 28 (10)	792 $\pm$ 41 (5)
n-desalkylflurazepam	53 $\pm$ 3 (5)	278 $\pm$ 29 (10)	778 $\pm$ 57 (7)
phenazepam	53 $\pm$ 3 (5)	284 $\pm$ 50 (5)	815 $\pm$ 58 (7)
diazepam	54 $\pm$ 2 (4)	283 $\pm$ 22 (8)	791 $\pm$ 27 (4)
$\alpha$ -hydroxyalprazolam	49 $\pm$ 1 (3)	263 $\pm$ 20 (8)	754 $\pm$ 25 (3)
$\alpha$ -hydroxymidazolam	53 $\pm$ 2 (4)	256 $\pm$ 14 (5)	720 $\pm$ 27 (4)
$\alpha$ -hydroxytriazolam	52 $\pm$ 3 (5)	268 $\pm$ 18 (7)	751 $\pm$ 36 (5)
midazolam	57 $\pm$ 7 (12)	305 $\pm$ 15 (5)	842 $\pm$ 30 (4)
alprazolam	51 $\pm$ 4 (7)	278 $\pm$ 19 (7)	794 $\pm$ 37 (5)
triazolam	51 $\pm$ 2 (4)	276 $\pm$ 23 (8)	796 $\pm$ 51 (6)
oxazepam	49 $\pm$ 6 (13)	287 $\pm$ 18 (6)	818 $\pm$ 22 (3)
lorazepam	52 $\pm$ 3 (6)	266 $\pm$ 16 (6)	758 $\pm$ 29 (4)
clonazepam	52 $\pm$ 2 (3)	273 $\pm$ 20 (7)	774 $\pm$ 29 (4)
flunitrazepam	51 $\pm$ 3 (6)	262 $\pm$ 14 (5)	742 $\pm$ 29 (4)
temazepam	51 $\pm$ 1 (2)	268 $\pm$ 21 (8)	772 $\pm$ 29 (4)

The precision of the samples was measured as the coefficient of variance (% CV) for the inter-run and intra-run analyses. The predetermined acceptance criterion for inter-run and intra-run precision is within  $\pm$  20 of the target concentration. The spiked and pooled concentrations were evaluated with these predetermined criteria. Results of these analyses are shown in Tables 5–8.

As shown in Table 5, all targets met the required acceptance criteria of within  $\pm$  20% for the % CV. The % CV ranged from 15% to 2% for the benzodiazepine targets. The *n* was 18 for all targets analyzed.

Table 6. Intra-run Precision of Spiked Benzodiazepines Quantitated by LC/MS/MS

### Spiked intra-run precision

Mean  $\pm$  SD (% CV) n = 3

	50 ng/mL	250 ng/mL	750 ng/mL
7-aminoclonazepam	47 $\pm$ 1 (3)	284 $\pm$ 12 (1)	834 $\pm$ 3 (0.4)
7-aminoflunitrazepam	50 $\pm$ 1 (1)	289 $\pm$ 2 (0.7)	825 $\pm$ 12 (1)
zopiclone	39 $\pm$ 5 (14)	307 $\pm$ 7 (2)	768 $\pm$ 34 (4)
zolpidem	51 $\pm$ 1 (2)	284 $\pm$ 2 (0.5)	768 $\pm$ 2 (0.3)
zaleplon	49 $\pm$ 1 (2)	280 $\pm$ 5 (2)	751 $\pm$ 14 (2)
chlordiazepoxide	50 $\pm$ 3 (7)	259 $\pm$ 11 (4)	751 $\pm$ 19 (3)
flurazepam	57 $\pm$ 3 (5)	292 $\pm$ 1 (0.2)	773 $\pm$ 3 (0.3)
nordiazepam	49 $\pm$ 4 (8)	261 $\pm$ 3 (1)	744 $\pm$ 18 (2)
n-desalkylflurazepam	56 $\pm$ 5 (9)	271 $\pm$ 8 (3)	726 $\pm$ 14 (2)
phenazepam	50 $\pm$ 3 (6)	267 $\pm$ 3 (1)	753 $\pm$ 9 (1)
diazepam	52 $\pm$ 2 (3)	280 $\pm$ 2 (1)	760 $\pm$ 1 (0.1)
$\alpha$ -hydroxyalprazolam	50 $\pm$ 0 (0)	270 $\pm$ 6 (2)	728 $\pm$ 11 (2)
$\alpha$ -hydroxymidazolam	55 $\pm$ 1 (1)	246 $\pm$ 6 (3)	694 $\pm$ 14 (2)
$\alpha$ -hydroxytriazolam	56 $\pm$ 1 (1)	269 $\pm$ 8 (3)	684 $\pm$ 3 (0.4)
midazolam	53 $\pm$ 1 (1)	5*4 $\pm$ 4 (1)	803 $\pm$ 18 (2)
alprazolam	47 $\pm$ 1 (2)	270 $\pm$ 3 (1)	733 $\pm$ 4 (0.5)
triazolam	50 $\pm$ 1 (2)	272 $\pm$ 3 (1)	774 $\pm$ 12 (2)
oxazepam	36 $\pm$ 2 (6)	313 $\pm$ 8 (2)	825 $\pm$ 11 (1)
lorazepam	57 $\pm$ 0 (0)	226 $\pm$ 8 (3)	762 $\pm$ 24 (3)
clonazepam	50 $\pm$ 1 (1)	267 $\pm$ 1 (0.2)	770 $\pm$ 20 (3)
flunitrazepam	49 $\pm$ 1 (2)	258 $\pm$ 2 (1)	725 $\pm$ 20 (3)
temazepam	50 $\pm$ 0 (0)	260 $\pm$ 2 (0.6)	733 $\pm$ 4 (0.6)

The intra-run precision for the spiked blank blood samples is described in Table 6. All targets were within the predetermined acceptance criteria of having the % CV within  $\pm 20\%$ . The range of the % CV for the spiked samples was between 14–0.1%.

Inter-run precision for pooled samples is shown in Table 7. The % CV ranged from 3 to 35% for inter-run precision. All targets with the exception of chloridazepoxide, alpha-hydroxytriazolam, and n-desalkylflurazepam met the predetermined acceptance criteria at every concentration.

Table 7. Inter-run Precision of Pooled Benzodiazepine Blood Samples Quantitated by LC/MS/MS

**Pooled inter-run precision**

**Mean  $\pm$  SD ng/mL (% CV); n = 15**

	10 ng/mL	30 ng/mL	700 ng/mL	1,400 ng/mL	2,200 ng/mL
7-aminoclonazepam	9.97 $\pm$ 0.88 (9)	29.76 $\pm$ 1.29 (4)	707 $\pm$ 23 (3)	1,358 $\pm$ 92 (7)	2,263 $\pm$ 99 (4)
7-aminoflunitrazepam	9.47 $\pm$ 1.08 (11)	30.75 $\pm$ 1.65 (5)	694 $\pm$ 40 (6)	1,335 $\pm$ 154 (12)	2,256 $\pm$ 115 (5)
zopiclone	11.76 $\pm$ 1.69 (14)	30.94 $\pm$ 2.06 (7)	704 $\pm$ 39 (6)	1,271 $\pm$ 133 (11)	1,985 $\pm$ 164 (8)
zolpidem	10.55 $\pm$ 0.38 (4)	34.10 $\pm$ 1.31 (4)	764 $\pm$ 31 (4)	1,467 $\pm$ 103 (7)	2,430 $\pm$ 111 (5)
chlordiazepoxide	17.81 $\pm$ 0.88 (5)	46.22 $\pm$ 5.49 (12)	973 $\pm$ 112 (12)	2,040 $\pm$ 723 (35)	3,078 $\pm$ 284 (9)
$\alpha$ -hydroxymidazolam	8.56 $\pm$ 0.83 (10)	29.99 $\pm$ 1.52 (5)	580 $\pm$ 28 (5)	1,068 $\pm$ 73 (7)	1,832 $\pm$ 78 (4)
midazolam	11.01 $\pm$ 104 (9)	36.87 $\pm$ 1.98 (5)	818 $\pm$ 33 (4)	1,559 $\pm$ 107 (7)	2,589 $\pm$ 111 (4)
flurazepam	9.62 $\pm$ 1.00 (10)	31.89 $\pm$ 2.06 (6)	678 $\pm$ 24 (4)	1,302 $\pm$ 93 (7)	2,149 $\pm$ 85 (4)
zaleplon	10.40 $\pm$ 0.91 (9)	32.63 $\pm$ 3.56 (11)	743 $\pm$ 56 (8)	1,430 $\pm$ 130 (9)	2,371 $\pm$ 169 (7)
$\alpha$ -hydroxyalprazolam	–	–	–	–	–
$\alpha$ -hydroxytriazolam	8.91 $\pm$ 1.00 (11)	31.92 $\pm$ 1.66 (5)	609 $\pm$ 89 (15)	1,125 $\pm$ 290 (26)	2,001 $\pm$ 123 (6)
nordiazepam	9.15 $\pm$ 0.58 (6)	31.03 $\pm$ 1.93 (6)	701 $\pm$ 29 (4)	1,360 $\pm$ 120 (9)	2,238 $\pm$ 107 (5)
oxazepam	10.10 $\pm$ 1.01 (10)	34.13 $\pm$ 2.37 (7)	718 $\pm$ 48 (7)	1,388 $\pm$ 119 (9)	2,305 $\pm$ 157 (7)
clonazepam	9.76 $\pm$ 0.65 (7)	31.67 $\pm$ 1.89 (6)	678 $\pm$ 93 (14)	1,339 $\pm$ 138 (10)	2,210 $\pm$ 164 (7)
lorazepam	9.40 $\pm$ 1.02 (11)	35.86 $\pm$ 3.39 (9)	666 $\pm$ 96 (14)	1,261 $\pm$ 222 (18)	2,090 $\pm$ 342 (16)
alprazolam	8.74 $\pm$ 0.83 (10)	30.75 $\pm$ 1.56 (5)	717 $\pm$ 39 (5)	1,369 $\pm$ 108 (8)	2,255 $\pm$ 138 (6)
n-desalkylflurazepam	8.65 $\pm$ 0.63 (7)	34.78 $\pm$ 8.05 (23)	676 $\pm$ 33 (5)	1,286 $\pm$ 119 (9)	2,167 $\pm$ 250 (12)
triazolam	9.42 $\pm$ 0.74 (8)	31.99 $\pm$ 1.70 (5)	705 $\pm$ 43 (6)	1,345 $\pm$ 131 (10)	2,219 $\pm$ 185 (8)
flunitrazepam	9.93 $\pm$ 0.74 (7)	32.76 $\pm$ 1.14 (3)	685 $\pm$ 22 (3)	1,301 $\pm$ 100 (8)	2,159 $\pm$ 138 (6)
temazepam	8.92 $\pm$ 0.52 (6)	32.15 $\pm$ 1.21 (4)	691 $\pm$ 20 (3)	1,325 $\pm$ 93 (7)	2,119 $\pm$ 311 (15)
diazepam	9.04 $\pm$ 0.79 (9)	31.61 $\pm$ 1.33 (4)	689 $\pm$ 28 (4)	1,311 $\pm$ 103 (8)	2,170 $\pm$ 128 (6)
phenazepam	10.01 $\pm$ 0.69 (7)	33.25 $\pm$ 2.26 (7)	728 $\pm$ 60 (8)	1,403 $\pm$ 195 (14)	2,279 $\pm$ 218 (10)

The intra-run precision for the pooled samples is shown in Table 8. All targets were within the  $\pm 20\%$  CV predetermined acceptance criterion. The % CV ranged between 1 to 18%. Overall, the interpretation of the accuracy and precision for both spiked and pooled samples indicates that the method is both accurate and precise for the targets with the exception of chlordiazepoxide, alpha-hydroxytriazolam, and *n*-desalkylflurazepam in the inter-run precision analysis.

Table 8. Intra-run Precision of Pooled Benzodiazepine Blood Samples Quantitated Using LC/MS/MS

**Pooled intra-run precision**

**Mean  $\pm$  SD ng/mL (% CV); n = 3**

	10 ng/mL	30 ng/mL	700 ng/mL	1,400 ng/mL	2,200 ng/mL
7-aminoclonazepam	8.36 $\pm$ 0.11 (1)	27.69 $\pm$ 0.39 (1)	676 $\pm$ 21 (3)	1,205 $\pm$ 95 (8)	2,311 $\pm$ 132 (6)
7-aminoflunitrazepam	8.14 $\pm$ 0.32 (4)	28.53 $\pm$ 1.04 (4)	636 $\pm$ 25 (4)	1,076 $\pm$ 29 (10)	2,188 $\pm$ 136 (6)
zopiclone	10.03 $\pm$ 0.38 (4)	28.30 $\pm$ 1.07 (4)	734 $\pm$ 6 (1)	1,330 $\pm$ 243 (18)	2,185 $\pm$ 121 (6)
zolpidem	10.57 $\pm$ 0.23 (2)	31.75 $\pm$ 0.48 (2)	715 $\pm$ 21 (3)	1,302 $\pm$ 115 (9)	2,334 $\pm$ 153 (7)
chlordiazepoxide	16.77 $\pm$ 0.06 (1)	38.43 $\pm$ 0.29 (1)	816 $\pm$ 27 (3)	1,456 $\pm$ 106 (7)	2,720 $\pm$ 175 (6)
$\alpha$ -Hydroxmidazolam	7.20 $\pm$ 0.31 (4)	28.00 $\pm$ 0.84 (3)	567 $\pm$ 23 (4)	978 $\pm$ 111 (11)	1,884 $\pm$ 103 (5)
midazolam	9.73 $\pm$ 0.63 (7)	33.92 $\pm$ 1.73 (5)	790 $\pm$ 28 (4)	1,382 $\pm$ 108 (8)	2,524 $\pm$ 120 (5)
flurazepam	9.30 $\pm$ 0.32 (3)	29.48 $\pm$ 0.91 (3)	646 $\pm$ 19 (3)	1,157 $\pm$ 97 (8)	2,104 $\pm$ 150 (7)
zaleplon	9.59 $\pm$ 0.41 (4)	26.79 $\pm$ 0.28 (1)	664 $\pm$ 19 (3)	1,231 $\pm$ 111 (9)	2,189 $\pm$ 154 (7)
$\alpha$ -Hydroxyalprazolam	–	–	–	–	–
$\alpha$ -Hydroxytriazolam	7.71 $\pm$ 0.65 (8)	31.22 $\pm$ 0.93 (3)	637 $\pm$ 24 (4)	1,126 $\pm$ 93 (8)	2,056 $\pm$ 101 (5)
nordiazepam	8.73 $\pm$ 0.42 (5)	27.99 $\pm$ 0.34 (1)	710 $\pm$ 17 (3)	1,177 $\pm$ 85 (7)	2,124 $\pm$ 146 (7)
oxazepam	8.64 $\pm$ 0.09 (1)	31.76 $\pm$ 1.00 (3)	697 $\pm$ 19 (3)	1,257 $\pm$ 108 (9)	2,273 $\pm$ 156 (7)
clonazepam	9.33 $\pm$ 0.08 (1)	28.52 $\pm$ 0.44 (2)	671 $\pm$ 7 (1)	1,199 $\pm$ 100 (8)	2,154 $\pm$ 114 (5)
lorazepam	7.84 $\pm$ 0.53 (7)	35.37 $\pm$ 0.57 (2)	661 $\pm$ 19 (3)	1,168 $\pm$ 91 (8)	2,130 $\pm$ 135 (6)
alprazolam	8.46 $\pm$ 0.19 (2)	28.30 $\pm$ 0.68 (3)	665 $\pm$ 18 (3)	1,197 $\pm$ 92 (8)	2,121 $\pm$ 108 (5)
<i>n</i> -desalkylflurazepam	7.65 $\pm$ 0.33 (4)	28.65 $\pm$ 0.35 (2)	648 $\pm$ 15 (2)	1,157 $\pm$ 77 (7)	2,108 $\pm$ 162 (8)
triazolam	8.40 $\pm$ 0.35 (4)	29.30 $\pm$ 0.61 (2)	667 $\pm$ 13 (2)	1,179 $\pm$ 78 (7)	2,121 $\pm$ 122 (6)
flunitrazepam	10.35 $\pm$ 0.59 (6)	31.70 $\pm$ 1.09 (3)	697 $\pm$ 24 (3)	1,260 $\pm$ 101 (8)	2,251 $\pm$ 119 (5)
temazepam	8.12 $\pm$ 0.43 (5)	29.99 $\pm$ 0.57 (2)	667 $\pm$ 17 (3)	1,195 $\pm$ 92 (8)	2,142 $\pm$ 127 (6)
diazepam	8.44 $\pm$ 0.16 (2)	29.22 $\pm$ 0.50 (2)	658 $\pm$ 21 (3)	1,183 $\pm$ 94 (8)	2,120 $\pm$ 144 (7)
phenazepam	9.35 $\pm$ 0.31 (3)	29.47 $\pm$ 0.41 (1)	674 $\pm$ 17 (2)	1,213 $\pm$ 81 (7)	2,196 $\pm$ 148 (7)

## Sensitivity (LOD, LOQ)

The LOD and lower limit of quantitation (LOQ) were evaluated with samples spiked at 1.0, 2.5, and 5.0 ng/mL along with the calibrators at 10.0 – 2,000 ng/mL. Standard identification criteria for LOD were  $\pm 5\%$  for retention time,  $\pm 20\%$  for qualifier ion ratio, and a signal-to-noise ratio of 3:1 at a minimum. The retention time and qualifier ion ratios were compared to the average of the calibrators. Standard identification criteria for LOQ were  $\pm 5\%$  for retention time,  $\pm 20\%$  for qualifier ion ratio, and a back calculated concentration within  $\pm 20\%$  of the target concentration. The signal-to-noise ratio should be 10:1 at a minimum. The retention time and qualifier ion ratio were compared to the average of the calibrators. Samples were required to meet the acceptance criteria in  $\geq 75\%$  of the samples to be established as the target LOD and LOQ. Results are shown in Table 9.

All targets satisfied the predetermined acceptance criteria for the LOD at 5.0 ng/mL or lower. The LOQ was satisfied at a concentration of 10.0 ng/mL for most of the targets.

Table 9. LOD and LOQ for Benzodiazepines Using LC/MS/MS

Target compound	LOD (ng/mL)	LOQ (ng/mL)
7-aminoclonazepam	5.0	10.0
7-aminoflunitrazepam	2.5	10.0
$\alpha$ -hydroxyalprazolam	5.0	10.0
$\alpha$ -hydroxymidazolam	1.0	10.0
$\alpha$ -hydroxytriazolam	5.0	10.0
<i>n</i> -desalkylflurazepam	2.5	10.0
alprazolam	2.5	10.0
chlordiazepoxide	1.0	10.0
clonazepam	2.5	10.0
diazepam	2.5	10.0
flunitrazepam	1.0	10.0
flurazepam	1.0	10.0
lorazepam	5.0	10.0
midazolam	1.0	10.0
nordiazepam	2.5	10.0
oxazepam	2.5	10.0
phenazepam	2.5	10.0
temazepam	1.0	10.0
triazolam	1.0	10.0
zaleplon	2.5	10.0
zolpidem	1.0	5.0
zopiclone	1.0	10.0

## Recovery

Recovery was assessed with three different concentrations over a period of four batches. The high and low recoveries were averaged for an overall recovery for the process over the concentration range. The extracted control response was compared to double blank blood samples that were spiked with both internal standard and control after extraction. The raw instrumental response was used to calculate the average recovery for each concentration.

Table 10 represents the average percent recovery for all targets at 100, 250, and 500 ng/mL.

Table 10. Average Percent Recovery for Benzodiazepines Using a Liquid/Liquid Extraction

% Recovery (SD); n = 24	
	Average recovery
7-aminoclonazepam	63 (22)
7-aminoflunitrazepam	104 (8)
zolpidem	97 (8)
chlordiazepoxide	75 (10)
$\alpha$ -hydroxymidazolam	85 (7)
midazolam	92 (10)
flurazepam	167 (76)
zaleplon	93 (9)
$\alpha$ -hydroxyalprazolam	60 (19)
$\alpha$ -hydroxytriazolam	64 (17)
nordiazepam	90 (9)
oxazepam	72 (20)
clonazepam	85 (7)
lorazepam	76 (10)
alprazolam	82 (17)
<i>n</i> -desalkylflurazepam	87 (18)
triazolam	88 (7)
flunitrazepam	85 (7)
temazepam	94 (10)
diazepam	94 (8)
phenazepam	89 (4)

## Interference studies

Interferences from endogenous compounds, internal standards, target analytes and commonly encountered analytes were evaluated. There should be no source of interference for the method to be accepted. Ten negative matrix samples were analyzed over five batches to test for any interference to the target analytes from endogenous compounds. To test for interferences from internal standard, or target to internal standard, two samples were analyzed. One was fortified with only internal standard (10 ng/mL) and one with only the targets of interest (2,000 ng/mL). Finally, ten blank blood samples were fortified with the commonly encountered analytes listed in Table 11 to test for analyte interference. The results of these studies show that no interferences were detected for all targets.

## Ion suppression and enhancement

Potential interference from ion suppression and enhancement was evaluated. Neat standards and double blank samples were prepared at three different concentrations and spiked post-extraction. The responses were used to determine the extent of ion suppression or enhancement. Results of this study are shown in Table 12. A value of 100% indicates no ion suppression or enhancement. Values greater than 100% indicate ion enhancement, while values less than 100% indicate ion suppression. The ion suppression/enhancement ranges from 84 to 164%.

Table 12. Ion Suppression/Enhancement for Benzodiazepines Using LC/MS/MS

% Suppression/enhancement (SD); n = 24	
	Average
7-aminoclonazepam	111 (9)
7-aminoflunitrazepam	113 (10)
zolpidem	106 (10)
chlordiazepoxide	84 (17)
$\alpha$ -hydroxymidazolam	85 (5)
midazolam	99 (7)
flurazepam	164 (73)
zaleplon	98 (20)
$\alpha$ -hydroxyalprazolam	95 (4)
$\alpha$ -hydroxytriazolam	96 (5)
nordiazepam	97 (8)
oxazepam	95 (21)
clonazepam	96 (6)
lorazepam	97 (6)
alprazolam	95 (5)
<i>n</i> -desalkylflurazepam	97 (6)
triazolam	96 (6)
flunitrazepam	96 (4)
temazepam	95 (18)
diazepam	99 (6)
phenazepam	96 (4)

Table 11. Interferents and Concentration of Commonly Encountered Analytes

Drug class	Drug	Concentration ( $\mu\text{g/mL}$ )
opioids	codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone	10
	6-monoacetylmorphine	2.5
cocaine	cocaine, cocaethylene, benzoylecgonine	10
amphetamine	amphetamine, methamphetamine, phentermine, MDA, MDMA	10
cannabinoids	THC, carboxy-THC	0.1/2
barbiturates	butalbital, secobarbital, phenobarbital	100
carisoprodol and meprobamate	carisoprodol, meprobamate	100
fentanyl	fentanyl	1
acetaminophen, salicylic acid	acetaminophen, salicylic acid	500
base drugs	chlorpheniramine, imipramine, desipramine, paroxetine, trazodone	10
acid/neutral drugs	ibuprofen, butalbital, acetaminophen, meprobamate, caffeine, gluethemide, naproxen, metaxalone, carbamazepine, diazepam	10

## Carryover

Carryover was addressed by injecting progressively higher concentrations of target analytes followed by solvent blanks. The solvent blanks were monitored for signs of carryover, such as contribution to the quantitation transition. The highest concentration of analytes injected was 7,000 mg/L and no carryover was detected at this concentration.

## Stability

### Processed/extracted sample stability

Processed/extracted sample stability was addressed through the daily injection of three control samples over a period of seven days. The response was averaged over the three samples and compared over the seven-day period. If the average response decreased below 80% or increased above 120%, then the target was considered unstable after that time period.

Most of the targets were stable up to seven days. Diazepam and triazolam exceeded the  $\pm 20\%$  limit at day seven. Therefore, diazepam and triazolam are only considered stable up to six days after extraction.

### Bench top stability

The stability of a sample at standard operating conditions was assessed by storing five concentrations of pooled blood samples on the bench top for over 24 hours. After 24 hours, the pooled blood samples were extracted in triplicate and analyzed. The concentration was compared to the mean calculated value of the pooled samples from the pooled accuracy and precision data. The calculated concentrations were compared for deviance from the previously established mean value.

After a 24-hour incubation period, triplicate extractions were completed with 1.0 mL of pooled blood sample. The average concentration was compared to the calculated pooled accuracy mean. All targets were within the predetermined acceptance criteria of  $\pm 20\%$  with the exception of zopiclone at 2,200 ng/mL. This indicates that the targets are stable in blood after being exposed to normal laboratory conditions for 24 hours. Samples with concentrations above 1,400 ng/mL of zopiclone should be re-sampled and re-extracted if they have remained at room temperature for extended periods of time prior to extraction.

## Dilution integrity

To address dilution integrity, a large volume of sample was fortified at approximately 1,000 ng/mL, and samples were taken as undiluted (1.0 mL sample), 1:2 (0.5 mL sample), 1:4 (0.25 mL sample), and 1:5 (0.20 mL sample). The acceptable criteria for accuracy was  $\pm 20\%$  of the back calculated concentration.

This study showed that only the 0.5 mL (1:2) dilution volume was able to meet the acceptance criteria of the undiluted back calculated concentration. The smaller volumes did not meet that criteria. Therefore, casework samples can be diluted by no more than 1:2 to still maintain the predetermined acceptance criteria. The dilution integrity was also assessed by preparing a large volume of sample that was fortified at 1000 ng/mL. The sample was extracted undiluted as well as at a 1:2, 1:4, and 1:5 diluted. To prepare the dilutions, 1.0 mL of 1,000 ng/mL was diluted with the appropriate amount of blank blood. For example, the 1:2 dilution was prepared by diluting 1.0 mL of 1,000 ng/mL in 1.0 mL of blank blood. From that diluted sample, 1.0 mL was used for extraction. Each dilution was assessed in triplicate analysis over a total of four batches for an  $n$  of 12. This evaluation showed that a 1:4 large volume dilution was still within the predetermined acceptance criteria.

## Previously analyzed or non-probative casework samples

Non-probative DUID casework samples were reanalyzed with the newly developed method. The results were then compared and used as an accuracy assessment. Most of the benzodiazepine targets passed the previously analyzed casework sample analysis. Minimal weight is placed on these results due to the age difference of the samples and the limited scope of testing when comparing previous methods to the newly developed method.

## Conclusion

This method development and validation provides a rapid and sensitive technique for the detection and quantitation of benzodiazepines and z-drugs by LC/MS/MS. The range of target compounds used in this validation was chosen to fit the commonly encountered range of analyte concentrations seen in casework. Most of the target compounds passed the comprehensive validation, which proves that this method provides reliable quantitative results. During validation, it was noted that zopiclone has limited stability in basic conditions and time in this environment should be limited. It has been determined that this method is a valid means of analyzing benzodiazepines and z-drugs in for routine drug tests, providing quick, accurate, and reproducible results.

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