Benzodiazepine and Z-Drug Quantitation Using an Agilent 6430 LC/MS/MS

Application Note

Forensics

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Abstract

A method was developed for the quantitation of benzodiazepines in biological samples with LC/MS/MS using an Agilent 6430 Triple Quadrupole LC/MS system. The method displays excellent accuracy and precision using a weighted-quadratic calibration model for the analysis of benzodiazepines and weighted-linear model for zolpidem, zopiclone, and zaleplon. Twenty-three compounds were evaluated to establish method feasibility. Sufficient resolution and peak shape can be achieved within a cycle time of 12 minutes. Additional validation studies confirmed that this method meets all criteria required for routine analysis of benzodiazepines and z-drugs in whole blood.

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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 of Driving Under the Influence of Drugs (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 [1]. 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 and this technique is becoming increasingly useful in routine toxicological analysis given its accuracy and sensitivity.

This application note addresses the development of an LC/MS/MS method for the quantitation of benzodiazepines, zolpidem, zopiclone, and zaleplon in whole blood. All benzodiazepines assessed in this method predicted a weighted-quadratic calibration model, whereas, zaleplon, zolpidem, and zopiclone predicted a weighted-linear fit model. Validation studies were conducted using the SWGTOX guidelines in conjunction with Virginia Department of Forensic Science validation guidelines [2,3]. This method was determined to meet all criteria for the qualitative and quantitative analysis of benzodiazepines and z-drugs. [4]

Experimental

Equipment and instrumentation

- Agilent 6430 LC/MS/MS system
- Agilent 1260 Infinity LC with Agilent Poroshell 120 EC-18 column, 2.1 × 75 mm, 2.7 µm
- Agilent 1260 Automatic Liquid Sampler
- Autosampler vials with inserts and caps
- Agilent MassHunter Optimizer Software
- Zymark TurboVap Evaporator
- Screw capped extraction tubes with 12 mL or greater capacity
- Kimble/Chase tapered glass tubes for evaporation and reconstitution (p/n 73785-5)
- Glass Pasteur pipets
- Pipets for accurate dispensing of volumes 10 µL to 250 µL, and 1 mL to 10 mL
- Test tube rocker or rotator
- Centrifuge capable of 2,000–3,000 rpm

Materials

- Water, Type I or HPLC grade
- Sodium carbonate, certified ACS powder
- 1-Chlorobutane, HPLC grade
- Acetonitrile, Fisher Optima grade or higher
- Formic acid, eluent additive ~ 98%

Mobile phase solutions

- 0.1% formic acid in water (mobile phase A)
- 0.1% formic acid in acetonitrile (mobile phase B)

The calibrators, controls, and internal standards for this method were purchased from Cerilliant and Grace-Alltech. The targets and associated internal standards are described in Table 1.

Table 1. Target Compounds and Corresponding Internal Standard

Target	Internal standard
7-aminoclonazepam	7-aminoclonazepam-d4
7-aminoflunitrazepam	
zopiclone	zopiclone-d4
zolpidem	zolpidem-d6
zaleplon	
chlordiazepoxide	diazepam-d5
flurazepam	
nordiazepam	
n-desalkylflurazepam	
phenazepam	
diazepam	
α-hydroxyalprazolam	α-hydroxyalprazolam-d5
α-hydroxymidazolam	
α-hydroxytriazolam	
midazolam	alprazolam-d5
alprazolam	
triazolam	
oxazepam	oxazepam-d5
lorazepam	
clonazepam	clonazepam-d4
flunitrazepam	
temazepam	temazepam-d5

Sample preparation

Samples were prepared according to procedures defined by the VA Department of Forensic Science (VDFS) [2]. Benzodiazepines, zolpidem, zopiclone, and zaleplon were extracted from biological samples by adding sodium carbonate buffer and extracting with 1-chlorobutane. The extract was quantitated and confirmed using an Agilent 6430 LC/MS/MS system with an Agilent 1260 Infinity LC with Poroshell 120 EC-18, 2.1 × 75 mm, 2.7 µM column.

Preparation of calibrators

Working standard solution (0.01 mg/mL): Pipette 100 µL 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 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 µL 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.

To prepare the calibration curve, pipette the following volumes of the 0.01 mg/mL or the 0.001 mg/mL working standard solutions into appropriately labeled 16 × 125 mm screw cap test tubes. To eliminate potential solvent effects, calibrators and controls may be dried under nitrogen prior to the addition of blank blood. Add 1.0 mL of blank blood to obtain the final concentration listed in Table 2.

Table 2. Preparation of Calibrators

Amount of 0.01 mg/mL stock solution (µL)	Amount of 0.001 mg/mL stock solution (µL)	Final concentration of benzodiazepines (mg/L)
200		2.0
100		1.0
50		0.5
	100	0.1
	50	0.05
	20	0.02
	10	0.01

The samples eluted using a gradient of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Once separated, the liquid phase was analyzed using an Agilent 6430 Triple Quadrupole LC/MS in positive ion mode. The chromatography was optimized and the quantifier and qualifier transitions were determined using the MassHunter Optimizer Software.

Procedure

1. Label clean 16 × 125 mL screw cap tubes appropriately for calibrators, controls and case samples.
2. Prepare calibrators and controls.
3. Add 1.0 mL case specimens to the appropriately labeled tubes.
4. Add 100 µL of the 0.001 mg/mL internal standard working solution to each tube and vortex.
5. Add 1 mL sodium carbonate and 6 mL 1-chlorobutane to each tube.
6. Cap and rotate tubes for 30 minutes.
7. Centrifuge at approximately 2,500 rpm for 15 minutes to achieve separation. Transfer organic (upper) layer to appropriately labeled tubes.
8. Evaporate samples to dryness at approximately 50 °C under nitrogen.
9. Reconstitute samples in 200 µL methanol. Transfer to autosampler vials with inserts for LC/MS/MS analysis.
10. Run the samples in a Worklist, using the LC/MS/MS method detailed in Tables 3 and 4.

Table 3. Instrumental Conditions

MSD parameters

Parameter	Value (+)
Ionization	ESI
Polarity	Positive
Gas temperature	350 °C
Gas flow	10 L/min
Nebulizer pressure	40 psi
Capillary	4,000 V
LC parameters	
Injection volume	3.0 µL
Column	Agilent Poroshell 120 EC-18, 2.1 × 75 mm, 2.7 µm
Column thermostat	35.0 °C
Needle wash	5.0 seconds
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Flow rate	0.5 mL/min
Gradient	Initial 10% B 4.0 minutes 30% B 8.0 minutes 40% B 8.5 minutes 95% B 10.5 minutes 95% B 11.0 minutes 10% B
Stop time	11.0 minutes
Post time	1.5 minutes

Table 4. Acquisition Method Information

Compound	ISTD?	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Frag (V)	CE (V)	Cell acc (V)	Retention time (min)
7-aminoclonazepam	No	286.1	Unit	121.1	Unit	147	34	7	1.79
7-aminoclonazepam	No	286.1	Unit	77.1	Unit	147	62	7	1.79
7-aminoclonazepam-d4	Yes	290.1	Unit	121.1	Unit	142	30	7	1.75
7-aminoclonazepam-d4	Yes	290.1	Unit	77.1	Unit	142	66	7	1.75
7-aminoflunitrazepam	No	284.1	Unit	135.1	Unit	147	26	7	2.3
7-aminoflunitrazepam	No	284.1	Unit	77.1	Unit	147	74	7	2.3
α-hydroxyalprazolam	No	325.1	Unit	216.1	Unit	148	42	7	5.44
α-hydroxyalprazolam	No	325.1	Unit	205.1	Unit	148	50	7	5.44
α-hydroxyalprazolam-d5	Yes	330.1	Unit	302.1	Unit	147	26	7	5.41
α-hydroxyalprazolam-d5	Yes	330.1	Unit	210.1	Unit	147	50	7	5.41
α-hydroxymidazolam	No	342.1	Unit	324.1	Unit	148	21	7	4.19
α-hydroxymidazolam	No	342.1	Unit	168.1	Unit	148	45	7	4.19
α-hydroxytriazolam	No	359.1	Unit	239	Unit	148	46	7	5.49
α-hydroxytriazolam	No	359.1	Unit	176	Unit	148	26	7	5.49
alprazolam	No	309.1	Unit	205.1	Unit	143	46	7	6.04
alprazolam	No	309.1	Unit	151.1	Unit	143	74	7	6.04
alprazolam-d5	Yes	314.1	Unit	286.1	Unit	148	26	7	5.99
alprazolam-d5	Yes	314.1	Unit	210.1	Unit	148	42	7	5.99
chlordiazepoxide	No	300.1	Unit	227	Unit	105	22	7	3.4
chlordiazepoxide	No	300.1	Unit	89.1	Unit	105	74	7	3.4
clonezapam	No	316.1	Unit	270	Unit	142	22	7	5.86
clonezapam	No	316.1	Unit	214	Unit	142	46	7	5.86
clonezapam-d4	Yes	320.1	Unit	274.1	Unit	135	24	7	5.82
clonezapam-d4	Yes	320.1	Unit	218.1	Unit	135	40	7	5.82
diazepam	No	285.1	Unit	193.1	Unit	130	32	7	7.22
diazepam	No	285.1	Unit	91.1	Unit	130	52	7	7.22
diazepam-d5	Yes	290.1	Unit	198.1	Unit	150	36	7	7.12
diazepam-d5	Yes	290.1	Unit	154	Unit	150	28	7	7.12
flunitrazepam	No	314.1	Unit	268.1	Unit	153	25	7	6.42
flunitrazepam	No	314.1	Unit	239.1	Unit	153	37	7	6.42
flurazepam	No	388.2	Unit	317.1	Unit	158	17	7	4.62
flurazepam	No	388.2	Unit	315	Unit	158	21	7	4.62
lorazepam	No	321	Unit	303	Unit	120	8	7	5.94
lorazepam	No	321	Unit	229	Unit	115	28	7	5.94
midazolam	No	326.1	Unit	291.1	Unit	194	29	7	4.35
midazolam	No	326.1	Unit	249.1	Unit	194	41	7	4.35
n-desalkylflurazepam	No	289.1	Unit	226.1	Unit	140	28	7	6.38
n-desalkylflurazepam	No	289.1	Unit	140	Unit	140	28	7	6.38
nordiazepam	No	271.1	Unit	165	Unit	153	26	7	5.69
nordiazepam	No	271.1	Unit	140	Unit	153	30	7	5.69

Table 4. Acquisition Method Information (continued)

Compound	ISTD?	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Frag (V)	CE (V)	Cell acc (V)	Retention time (min)
oxazepam	No	287.1	Unit	269	Unit	110	8	7	5.61
oxazepam	No	287.1	Unit	241	Unit	110	20	7	5.61
oxazepam-d5	Yes	292.1	Unit	274.1	Unit	120	8	7	5.56
oxazepam-d5	Yes	292.1	Unit	246.1	Unit	120	20	7	5.56
phenazepam	No	351	Unit	206	Unit	140	36	7	7.37
phenazepam	No	351	Unit	179	Unit	140	50	7	7.37
quetiapine	No	384.1	Unit	253.2	Unit	120	30	7	4.7
quetiapine	No	384.1	Unit	221.1	Unit	120	30	7	4.7
temazepam	No	301.1	Unit	283	Unit	116	10	7	6.69
temazepam	No	301.1	Unit	255.1	Unit	116	18	7	6.69
temazepam-d5	Yes	306.1	Unit	288.1	Unit	106	10	7	6.63
temazepam-d5	Yes	306.1	Unit	260.1	Unit	106	22	7	6.63
triazolam	No	343	Unit	315	Unit	170	28	7	6.4
triazolam	No	343	Unit	308	Unit	170	24	7	6.4
zaleplon	No	306.1	Unit	264.1	Unit	130	20	7	5.08
zaleplon	No	306.1	Unit	236.1	Unit	130	24	7	5.08
zolpidem	No	308.2	Unit	263.1	Unit	160	24	7	3.23
zolpidem	No	308.2	Unit	235.1	Unit	160	36	7	3.23
zolpidem-d6	Yes	314.2	Unit	263.1	Unit	168	24	7	3.19
zolpidem-d6	Yes	314.2	Unit	235.1	Unit	168	36	7	3.19
zopiclone	No	389.1	Unit	245	Unit	82	13	7	2.44
zopiclone	No	389.1	Unit	217	Unit	82	33	7	2.44
zopiclone-d4	Yes	393.1	Unit	245	Unit	72	12	7	2.43
zopiclone-d4	Yes	393.1	Unit	217	Unit	72	32	7	2.43

Results and Discussion

The method achieved satisfactory separation of target compounds in an overall cycle time of 12.5 minutes. Figures 1–3 illustrate the resolution and example MRM transitions achieved with this method. The method established good peak shape with no significant tailing or other chromatographic abnormalities.

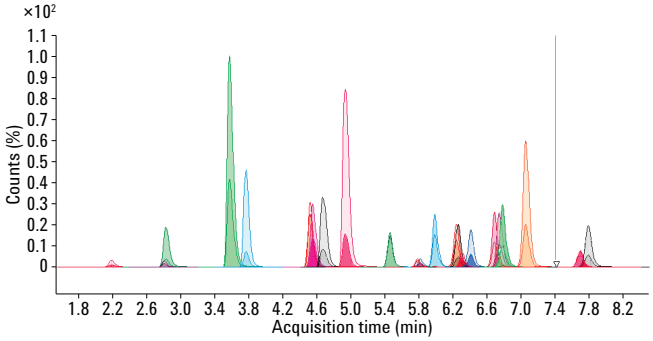

Figure 1. Qualitative chromatographic retention of analytes.

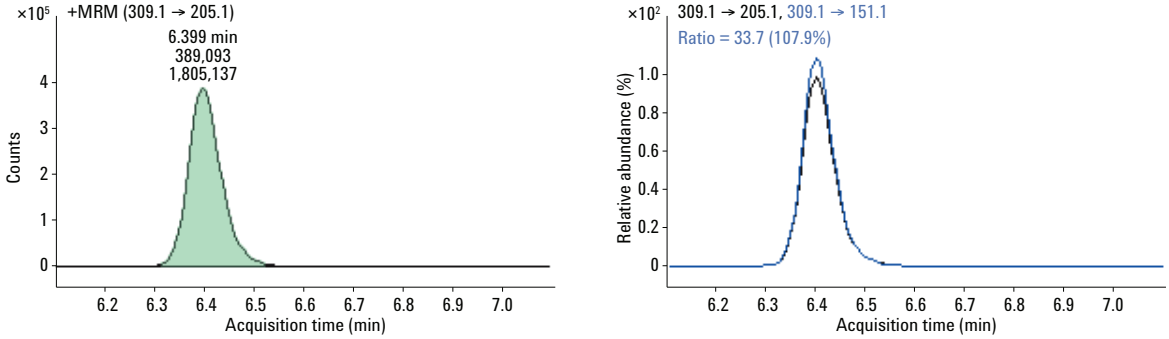

Figure 2. MRM of alprazolam.

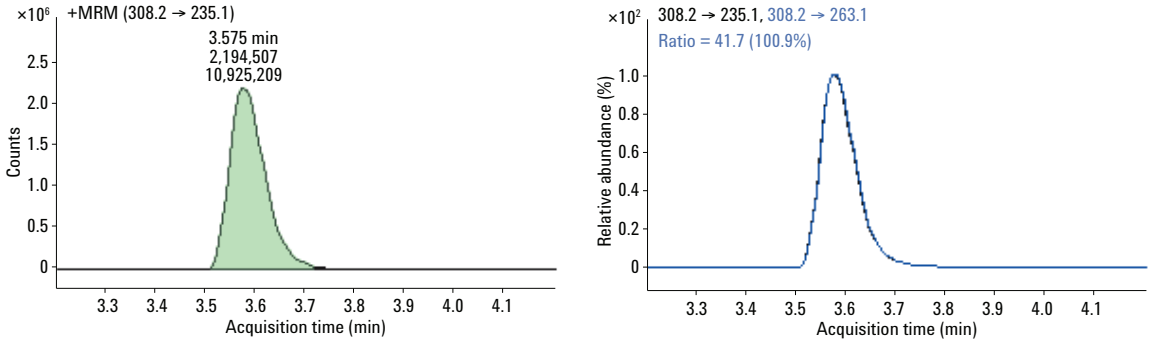

Figure 3. MRM of zolpidem.

A total of 16 calibration curves were analyzed to establish the calibration model for each target. The calibration model was established based on predetermined acceptance criteria of the R^2 value $\geq$ 0.985, the back calculated concentration of $\pm$ 20% for calibrators, and visual determination. The R^2 coefficients in this study were greater than 0.996 [3]. The dynamic range was established to be 0.01–2.0 mg/L.

Example calibration curves are shown in Figures 4–5. All benzodiazepines, which were 19 of the 22 targets, predicted a weighted-quadratic model. Alprazolam is shown in Figure 4 as an example of a weighted-quadratic calibration model.

The remaining 3 of the 22 target calibrations were weighted-linear models. Figure 5 shows the weighted-linear calibration model for zolpidem as an example.

Validation studies were conducted using the SWGTOX guidelines in conjunction with the Virginia Department of Forensic Science validation guidelines [3]. Items assessed during the validation study included linearity and calibration model fit, precision and accuracy using pooled and spiked whole blood samples, sensitivity (limits of detection (LOD) and limits of quantitation (LOQ)), interferences, robustness, carry-over, dilution integrity, stability, ion suppression/enhancement, and recovery. The LODs for the targets were less than or equal to 0.005 mg/L, with the exception of chlordiazepoxide and flunitrazepam, which were greater 0.01 mg/L. The LOQs for the targets were 0.01 mg/L, with the exception of flurazepam, midazolam, and zolpidem. A more comprehensive explanation of the results of this validation study can be found in "Validation of a Benzodiazepine and Z-Drug Method Using an Agilent 6430 LC/MS/MS " [4].

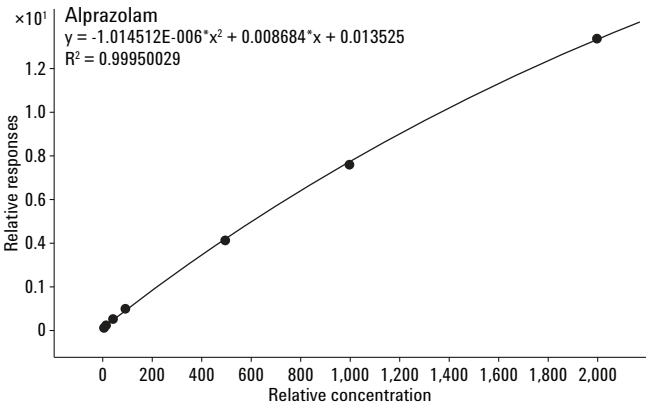

Figure 4. Weighted-quadratic calibration curve for alprazolam.

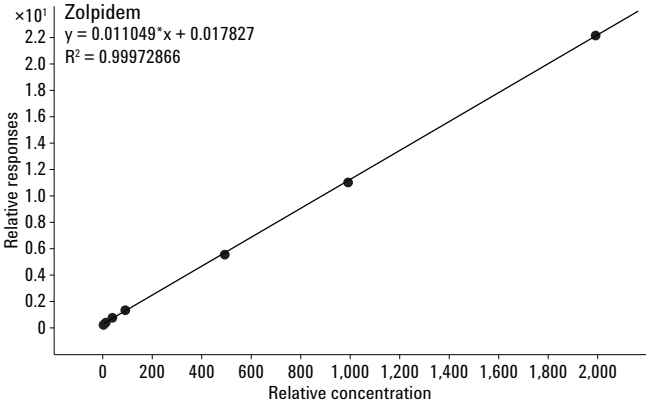

Figure 5. Linear calibration curve for zolpidem.

Conclusion

This method was developed to meet all criteria for the qualitative and quantitative analysis of benzodiazepines and z-drugs. Sample preparation does not require derivatization when compared to the detection using GC/MS/MS, providing a shorter run time of 12.5 minutes. The chromatography displays acceptable peak shape, with no abnormalities. The validation studies detailed in "Validation of a Benzodiazepine and Z-Drug Method Using an Agilent 6430 LC/MS/MS" indicates that the method meets all acceptance criteria for the qualitative and quantitative analysis of benzodiazepines and z-drugs in whole blood. Chlordiazepoxide is the only target which did not meet the requirements for quantitative analysis but is still able to be qualitatively assessed with this method.

References

1. O. Quintela, D. Crouch, "The determination of benzodiazepines using liquid chromatography with mass spectrometric detection", *J. Methods Mol Biol*., 2012;902:75-90.

2. "Standard Practices for Method Validation in Forensic Toxicology", SWGTOX, Doc 003, Revision 1, May 20, 2013.

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4. J. Hudson, Ph.D., J. Hutchings, Ph.D., C. Harper, Ph.D., R. Wagner, Ph.D., "Validation of a Benzodiazepine and Z-Drug Method Using an Agilent 6430 LC/MS/MS", Agilent Application Note 5991-2628EN, June 2013.

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