A Validated Method for the Quantitative Determination of Zolpidem, Zopiclone, and Zaleplon in Blood, Stomach Contents, and Liver by LC-MS/MS

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ABSTRACT

After attending this poster session, attendees will have been introduced to a validated method for the quantitation of zolpidem, zopiclone, and zaleplon (ZZZ drugs) in blood, stomach contents, and liver by basic liquid-liquid extraction (LLE) and LC-MS/MS. This presentation will impact the forensic science community by addressing the lack of a simple validated method able to rapidly and simultaneously confirm all three ZZZ drugs with matching deuterated internal standards (zolpidem-D6, zopiclone-D4, zaleplon-D4) rather than characteristically similar benzodiazepine internal standards. The method described meets the requirements of SWGTOX guidelines.

INTRODUCTION

- Zolpidem, zopiclone, and zaleplon (ZZZ drugs) are sedative hypnotics prescribed to treat onset and maintenance forms of insomnia.[1,2]
- These GABA receptor agonists cause central nervous system (CNS) depressant activity with a faster onset of action and shorter half-life than benzodiazepines.[3]
- Residual effects include eating, drinking, driving, or having sex while sleeping[2]
- Often found in driving under the influence of drugs, post-mortem, and drug-facilitated sexual assault cases in combination with benzodiazepines, ethanol, or other CNS depressants.[4]

Table 1: ZZZ Drug Pharmacology[2]

	Therapeutic C_{max} (ng/mL)*	Postmortem levels (ng/mL)*	$t_{1/2}$ (h)	pKa
Zolpidem	100-200 (10 mg)	>4000	2.5-3	6.2
Zopiclone	60-90 (7.5 mg)	>600	5-6	5.4
Zaleplon	20-30 (10 mg)	>1000	~1	-

*Z-drug blood concentration (dose)

MATERIALS & METHODS

Materials

ZZZ drug analytes and deuterated internal standards were obtained as 1 mg/mL solutions from Cerilliant (Round Rock, TX). Negative sheep's blood was obtained from Colorado Serum Company (Denver, CO). Human urine, stomach contents, and liver matrices from drug-free individuals were used for the preparation of quality controls (QCs).

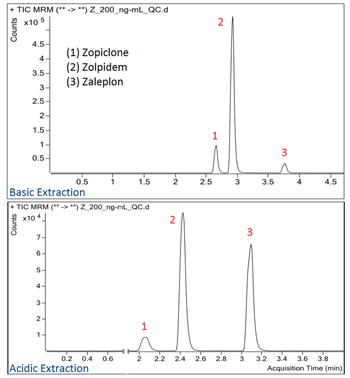
Sample Preparation & Extraction

The ZZZ drugs were prepared in combined working solutions of 5 and 0.5 µg/mL. Seven calibrators (10, 25, 50, 100, 250, 500, and 1000 ng/mL) were prepared in blood as the representative matrix. Three quality control samples (25, 400, 750 ng/mL) were prepared in 500 µL blood, stomach contents (SC), and liver (LV). The deuterated internal standard was added in 10 µL aliquots from a combined working standard of 1.0 µg/mL. The basic liquid-liquid extraction method utilized 200 µL saturated sodium borate (pH 12) and 2 mL ethyl acetate. Samples were mixed for five minutes and centrifuged for ten minutes at 3500 rpm. The organic layer was isolated and dried under a nitrogen flow. Samples were reconstituted in 2 mL 80:20 0.1% formic acid (FA) in H_2O:0.1% FA in CH_3CN.

Validation

Method validation was carried out according to SWGTOX guidelines.[7] The within-run and between-run bias/precision was calculated according to equations found in the SWGTOX Standard Practices for Method Validation. The calibration models were determined using standard residual plots. Dilution integrity was assessed in blood at x4, x10, and x25 in triplicate. Internal standard interference was determined by monitoring blank matrix samples fortified with analyte at the upper limit of calibration range. Ion suppression was assessed by post-extraction spike of mobile phase and negative matrix with 25 and 750 ng/mL QCs.

RESULTS



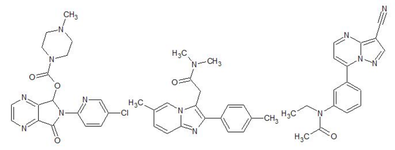


Figure 1 (left): Total Ion Chromatograms of Basic (top) and Acidic (bottom) fractions

Figure 2 (above): ZZZ Drug Structures

Table 2: LC Method

Time (min)	A%*	B%*
0	80	20
0.8	80	20
1.9	55	45
2.9	5	95
4.4	5	95
4.7	80	20
Post Time: 2.5 min	Flow: 0.6 mL/min	

*A = 0.1% FA in H_2O *B = 0.1% FA in CH_3CN

Table 3: Tandem Mass Spectrometry Method

Compound	Retention Time (min)	MRM Transitions (m/z)*	Collison Energy (eV)
Zopiclone	2.07	389.1 > 245.0	16
		389.1 > 217.0	36
Zolpidem	2.43	308.2 > 263. 1	24
		308.2 > 235.1	36
Zaleplon	3.10	306.1 > 264.0	20
		306.2 > 236.0	24
Zopiclone-D4	2.05	393.1 > 349.2	4
		393.1 > 245.0	16
Zolpidem-D6	2.42	314.2 > 263.1	28
		314.2 > 235.1	36
Zaleplon-D4	3.09	310.2 > 268.5	4
		310.2 > 240.2	24

*Transitions used for quantitation are in ***bold italics***

Table 4: Method Validation Summary

Parameter	SWGTOX Criteria	Experimental Value
Bias (low, mid, high)	≤20%	<15% all analytes
Precision	≤20%	<15% all analytes*
Calibration Model	Method Specific	-
Zolpidem/Zaleplon	-	Quadratic
Zopiclone	-	Linear
LOD	3x S/N + qualitative	-
Zolpidem/Zopiclone	-	0.5 ng/mL
Zaleplon	-	1.0 ng/mL
LOQ	Lowest non-zero calibrator	10 ng/mL
Carryover	≤30% of LOQ	-
Zolpidem	-	0.35%
Zopiclone	-	0.23%
Zaleplon	-	1.52%
Interferences	Matrix, ISTD, other analytes	N/A
Ionization Suppression/Enhancement (%CV)	≤25% (15%)	-
Zolpidem	-	-12.90% (20.54%)
Zopiclone	-	12.99% (64.25%)
Zaleplon	-	-41.69% (-10.34%)
Dilution Integrity Precision and Bias	≤20%	<20%

*Exception – zopiclone in SC = 16.3% CV

MATERIALS & METHODS

Alternative analytes monitored for interference by a neat spike in reconstitution solvent included fentanyl, opiates (7), cocaine, diphenhydramine, buspirone, trazodone, PCP, dextromethorphan, ketamine, 3-MeO-PCP, amitriptyline, nortriptyline, venlafaxine, norvenlafaxine, clozapine, tramadol, nortramadol, doxepin, nordoxepin, olanzapine, duloxetine, fluoxetine, norfluoxetine, quetiapine, and norquetiapine.

Liquid-Chromatography-Tandem Mass Spectrometry

Analysis was performed on the Agilent® 1290 Infinity II Stack and 6460 QqQ/MS system equipped with an Agilent® InfinityLab Poroshell 120 EC-C18 column (3mm x 100mm, 2.7µm) and guard EC-18 guard column (5mm x 3mm, 2.7µm). Multiple reaction monitoring (MRM) and positive-ion mode electrospray ionization (ESI) was used. The capillary voltage was 3.5 kV.

DISCUSSION

- An existing LLE was modified for the quantitation of zolpidem, zopiclone, and zaleplon.
- Bias and precision (%CV) results were generally less than 10% for the majority of matrix/analyte/concentration combinations.
- Consistently higher bias and precision were calculated for the SC samples.
- The limit of quantitation was set at the lowest non-zero calibrator as ZZZ drugs tend to be found, in blood, at concentrations exceeding the 10 ng/mL.[2,6]
- Although the post-extraction addition indicated that substantially high values of ion suppression and enhancement were present at values exceeding SWGTOX guidelines, no apparent impact was observed on the bias or precision of quantitation.
- No interferences were detected from the matrices (blood, SC, LV, urine), deuterated ISTDs, or other commonly encountered analytes.

CONCLUSION

- Advantages of the method include the rapid extraction, use of deuterated internal standards, and the short run time (4.7 min).
- The method is capable of detecting ZZZ drugs at forensically relevant concentrations (*Table 1*).
- Using matrix matched controls, quantitative analysis was performed using a blood calibration curve.
- The method adheres to SWGTOX validation guidelines.

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