DEPARTMENT OF FORENSIC SCIENCE
METHOD VALIDATION SUMMARY FORM

Section: Toxicology

Method: Qualitative Validation of the Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method

Results recorded? ☑

Procedure documented? ☑

Method fit for use? ☑

Approved by: _____ Date: 10/24/25

Executive Validation Summary: "Qualitative Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method"

Summary:
The validation for the qualitative addition of nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam to the "Benzodiazepines, Zolpidem, Zopiclone, and Zaleplon Quantitation and Confirmation by LCMSMS" method. The biological matrices evaluated during the validation included blank blood, antemortem blood, postmortem blood, and urine.

Limitations:
Gidazepam showed poor chromatography at various concentrations, showed a large amount of carryover, and failed the LOD determination. Therefore, it was determined that gidazepam will not be added to the existing method.

Clobromazolam (phenazolam) showed instability in the extracted urine samples and multiple failures at the LOD in urine therefore clobromazolam will not be analyzed in urine samples.

4'-chloro deschloroalprazolam produced an instrumental response in the dynamic MRM window for alprazolam. Relative retention time should be used for the evaluation of the signal.

Studies Performed and Acceptance Criteria:

1. Immunoassay (ELISA) Cross-Reactivity (CR) – no acceptance criteria
2. Ion Optimization – no acceptance criteria
3. Sensitivity: Estimated Limit of Detection (LOD)
    a. >95% of samples meet RT, Qualifier Ratio, and S/N >3.3
4. Ionization Suppression/Enhancement
    a. >±25% indicate significant suppression/enhancement – must evaluate impact to sensitivity
5. Carryover
    a. No instrumental response greater than 10% of the threshold control
6. Interferences - No instrumental response greater than 10% of the threshold control
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
7. Stability - >±20% from Day 1 response indicates instability

Validation Results

Compound	ELISA CR (ave. across matrices and conc.)	LOD (mg/L)	Suppression/ Enhancement	Carryover (up to 4.0 mg/L)	Interferences	Stability (days)
Nitrazolam	105	0.005	Significant suppression	Passed	None	7 (5 days postmortem and urine)
4'-chloro deschloroalprazolam	53	0.005	Significant suppression	Passed	Potential response in alprazolam window	7
Clobromazolam (phenazolam)	112	0.005 (except urine)	Significant suppression	Passed	None	5
Clobazam	100	0.005	Significant suppression	Passed	None	5
N-desmethylclobazam	98	0.005	Significant suppression	Passed	None	5 (2 days for urine)
Gidazepam	116	Failed	Significant suppression	Failed	None	Not assessed
Desalkylgidazepam	104	0.005	Significant suppression	Passed	None	7

Memo To:	James Hutchings, Ph.D., Toxicology Program Manager
From:	Tiffany Leusby
CC:	Alka Lohmann, Director of Technical Services, Rebecca Wagner, Ph. D., Chemistry Research Section Supervisor, Carol O'Neal, Ph.D., Toxicology Section Supervisor
Date	September 25, 2025
RE:	Validation Summary Validation of Qualitative Addition of Seven Novel Benzodiazepines

Validation Summary- Qualitative Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method

The validation of "Qualitative Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method" was conducted pursuant to the validation plan. The compounds included in the qualitative validation are nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam. It should be noted that gidazepam showed poor chromatography at various concentrations, showed a significant carryover, and failed the LOD determination. Therefore, it was determined that gidazepam will not be added to the existing method.

The validation included the following:

1. Immunoassay (ELISA) Cross-Reactivity
2. Ion Optimization
3. Sensitivity: Estimated Limit of Detection (LOD)
4. Ionization Suppression/Enhancement
5. Carryover
6. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
7. Stability
8. Summary
9. References

An Agilent Technologies 1260 binary pump liquid chromatograph coupled independently to an Agilent Technologies 6470 tandem mass spectrometer as well as a Tecan Freedom Evo 75 and Tecan Sunrise Reader was used during validation. Validation experiments were performed in accordance with the approved validation plan. The biological matrices evaluated during the validation included blank blood, antemortem blood, postmortem blood, and urine.

1. Immunoassay (ELISA) Cross-Reactivity

The cross-reactivity for nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam was evaluated using the Immunalysis Direct Benzodiazepine ELISA kit at a series of concentrations (0.02 mg/L, 0.04 mg/L, and 4.0 mg/L). Three different blank matrix sources per matrix type (blank blood, postmortem blood, antemortem blood, urine) were fortified with these concentrations for each analyte independently. Each sample was evaluated with a single replicate except for phenazolam. Phenazolam was analyzed in triplicate for blank blood, postmortem blood and antemortem blood. The average was calculated and was used to determine the cross-reactivity. The B/B_0 value was calculated for each compound at each concentration.

In addition to the evaluation of the target compounds, a low positive control (0.02 mg/L), positive control (0.04 mg/L), and high positive control (4.0 mg/L) were evaluated in blank blood to compare the B/B_0 values and establish the cross-reactivity.

Equation 1 was used to determine the cross-reactivity for each compound of interest with the Immunalysis Direct Benzodiazepine ELISA kit.

Equation 1:

$$\text{Cross-reactivity (\%)} = \frac{(100 - B/B0\ Target\ Compound)}{(100 - B/B0\ Control\ Compouind)} \times 100$$

The cross-reactivity and standard deviation for all compounds at concentrations of 0.02 mg/L, 0.040 mg/L and 0.40 mg/L are shown in Table 1.

Table 1:

Clobazam - Cross-Reactivity (SD)	**0.02 mg/L**	**0.04 mg/L**	**0.40 mg/L**
Blank Blood	96 (9)	100 (3)	104 (2)
Antemortem Blood	89 (6)	98 (2)	105 (0.46)
Postmortem Blood	92 (11)	96 (6)	102 (3)
Urine	89 (57)	113 (4)	111 (1)

Clobromazolam (Phenazolam) - Cross-Reactivity (SD)	**0.02 mg/L**	**0.04 mg/L**	**0.40 mg/L**
Blank Blood	109 (7)	114 (1)	98 (1)
Antemortem Blood	97 (9)	112 (4)	109 (4)
Postmortem Blood	98 (2)	112 (4)	111 (1)
Urine	132 (3)	130 (1)	119 (0.14)

4'-Chloro Deschloroalprazolam - Cross-Reactivity (SD)	0.02 mg/L	0.04 mg/L	0.40 mg/L
Blank Blood	56 (28)	60 (29)	53 (9)
Antemortem Blood	26 (13)	19 (14)	64 (5)
Postmortem Blood	43 (39)	40 (11)	65 (9)
Urine	62 (27)	65 (20)	82 (7)

Desalkylgidazepam - Cross-Reactivity (SD)	0.02 mg/L	0.04 mg/L	0.40 mg/L
Blank Blood	97 (6)	101 (3)	109 (2)
Antemortem Blood	86 (14)	102 (5)	111 (1)
Postmortem Blood	90 (21)	105 (5)	111 (3)
Urine	107 (12)	115 (2)	116 (0.11)

Gidazepam - Cross-Reactivity (SD)	0.02 mg/L	0.04 mg/L	0.40 mg/L
Blank Blood	101 (23)	115 (4)	106 (1)
Antemortem Blood	123 (5)	114 (6)	109 (1)
Postmortem Blood	111 (7)	117 (3)	110 (0.43)
Urine	143 (8)	130 (2)	116 (0.34)

Nitrazolam - Cross-Reactivity (SD)	0.02 mg/L	0.04 mg/L	0.40 mg/L
Blank Blood	96 (1)	101 (2)	105 (4)
Antemortem Blood	97 (1)	95 (8)	103 (5)
Postmortem Blood	99 (2)	102 (2)	105 (2)
Urine	131 (2)	121 (2)	114 (0.06)

N-Desmethylclobazam (Norclobazam) - Cross-Reactivity (SD)	0.02 mg/L	0.04 mg/L	0.40 mg/L
Blank Blood	95 (5)	89 (2)	97 (2)
Antemortem Blood	91 (1)	85 (7)	101 (0.58)
Postmortem Blood	85 (10)	86 (4)	97 (3)
Urine	124 (7)	114 (5)	112 (1)

Nitrazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam all showed high cross-reactivity across all matrix types (from 85 to 143 % cross-reactivity). 4'-Chloro deschloroalprazolam' s cross-reactivity was the weakest for all matrix types (19-82 % cross-reactivity). Urine across the board showed consistently higher cross-reactivity than the blood matrix, particularly at 0.02 mg/L concentration.

2. Ion Optimization

The Agilent LCMSMS MassHunter Optimizer Software was used to determine the precursor ions, product ions, fragmentor energy and collision energy for the compounds of interest at a concentration of 1 ug/mL. It should be noted that gidazepam at 1 ug/mL did not optimize, therefore gidazepam was run again at a higher concentration of 1 mg/mL to get the appropriate number of transitions. After optimization, all seven drugs were run using MRM data acquisition method to determine the retention times. The method used all parameters from the current Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method. None of the drugs were shown to co-elute with each other. The bold product ions are the quantifier ions. The data is shown below in Table 2.

Table 2: Optimized instrumental parameters

Compound Name	Precursor Ion (m/z)	Product Ions (m/z)	Fragmentor (V)	Collison Energy (V)	Approximate Retention Time (min)
Gidazepam	387.05	**298.9**	135	24	4.91
		219		50	
Nitrazolam	320.12	**274**	135	28	5.09
		246.1		40	
N-Desmethylclobazam	287.1	**245**	80	20	5.89
		210.1		36	
4'-Chloro Deschloroalprazolam	309.09	**281**	135	28	5.98
		205		48	
Desalkylgidazepam	315.02	**208.1**	135	32	6.1
		183.9		32	
Clobromazolam (Phenazolam)	387	**308**	105	28	6.63
		358.9		32	
Clobazam	301.1	**259**	120	20	7.08
		224		36	

3. Sensitivity: Estimated Limit of Detection (LOD)

The estimated limit of detection (LOD) for this validation was defined as an administratively defined decision point (threshold concentration). The limit of detection is understood to be an estimate based on the condition of the instrument at the time of the evaluation. The administrative threshold was established to be 0.0050 mg/L for all qualitative target compounds in blank blood, antemortem blood, postmortem blood, and urine. In addition to the 0.0050 mg/L threshold control, a concentration of 0.0025 mg/L was evaluated. This is a deviation from the concentrations delineated in the validation plan. A Memorandum for Record was approved by the Toxicology Program Manager authorizing this change. Nine blank matrix sources, per matrix type were utilized in the determination of the estimated limit of detection

per matrix type. The matrix sources were fortified with the compounds of interest and analyzed in a single replicate over three batch analyses. The peak shape, retention time, qualifier ratio, and signal to noise ratio were evaluated for each compound at each concentration. The predetermined identification criteria included a retention time within ±3%, a qualifier ratio within ±20%, and a signal to noise ≥3.

When evaluating the estimated limit of detection in blank blood, antemortem blood, postmortem blood, and urine at 0.0025 mg/L and 0.0050 mg/L a variety of failures were observed. The observed results are described in Table 3 and 4.

Table 3: 0.0025 mg/L LOD results

Target Compound	Blank Blood	Antemortem Blood	Postmortem Blood	Urine
Nitrazolam	All Replicates Pass	All Replicates Pass	All Replicates Pass	3/27 Replicates Failed Qualifier Ratio
4'-Chloro Deschloroalprazolam	4/27 Replicates Failed Qualifier Ratio	8/27 Replicates Failed Qualifier Ratio	3/27 Replicates Failed Qualifier Ratio	3/27 Replicates Failed Qualifier Ratio
		1/27 Replicates Failed Peak Shape		
Clobromazolam (Phenazolam)	5/27 Replicates Failed Qualifier Ratio	4/27 Replicates Failed Qualifier Ratio	2/27 Replicates Failed Qualifier Ratio	4/27 Replicates Failed Qualifier Ratio
Clobazam	All Replicates Pass	All Replicates Pass	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio
N-Desmethylclobazam (Norclobazam)	2/27 Replicates Failed Qualifier Ratio	All Replicates Pass	2/27 Replicates Failed Qualifier Ratio	4/27 Replicates Failed Qualifier Ratio
				1/27 Replicates Failed Peak Shape
Desalkylgidazepam	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio	4/27 Replicates Failed Qualifier Ratio	5/27 Replicates Failed Qualifier Ratio
Gidazepam	19/27 Replicates Failed Qualifier Ratio	8/27 Replicates Failed Qualifier Ratio	5/27 Replicates Failed Qualifier Ratio	12/27 Replicates Failed Qualifier Ratio
	20/27 Replicates Failed Peak Shape	15/27 Replicates Failed Peak Shape	11/27 Replicates Failed Peak Shape	18/27 Replicates Failed Peak Shape
	6/27 Replicates Failed Retention Time			4/27 Replicates Failed Retention Time

Table 4: 0.0050 mg/L LOD results

Target Compound	Blank Blood	Antemortem Blood	Postmortem Blood	Urine
Nitrazolam	All Replicates Pass	All Replicates Pass	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio
4'-Chloro Deschloroalprazolam	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio
Clobromazolam (Phenazolam)	All Replicates Pass	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio	2/27 Replicates Failed Qualifier Ratio
Clobazam	All Replicates Pass	All Replicates Pass	All Replicates Pass	1/27 Replicates Failed Qualifier Ratio
N-Desmethylclobazam (Norclobazam)	1/27 Replicates Failed Qualifier Ratio	1/27 Replicates Failed Qualifier Ratio	1/27 Replicates Failed Qualifier Ratio	1/27 Replicates Failed Qualifier Ratio
Desalkylgidazepam	1/27 Replicates Failed Qualifier Ratio	All Replicates Pass	All Replicates Pass	All Replicates Pass
Gidazepam	9/27 Replicates Failed Qualifier Ratio	1/27 Replicates Failed Qualifier Ratio	9/27 Replicates Failed Qualifier Ratio	5/27 Replicates Failed Qualifier Ratio
	15/27 Replicates Failed Peak Shape	8/27 Replicates Failed Peak Shape	4/27 Replicates Failed Peak Shape	8/27 Replicates Failed Peak Shape

Some replicates did not meet all predetermined acceptance criteria for each matrix source. At 0.0050 mg/L, nitrazolam and clobazam had all replicates meet the predetermined acceptance criteria for blank blood, antemortem blood, and postmortem blood. Urine for nitrazolam and clobazam had one (1) replicate out of 27 replicates that did not meet the qualifier ratio acceptance criteria of ±20%.

4'-Chloro deschloroalprazolam met all the predetermined acceptance criteria for blank blood and postmortem blood. One (1) replicate out of 27 replicates for both antemortem blood and urine did not meet the qualifier ratio acceptance criteria of ±20%. Clobromazolam (phenazolam) had all replicates meet acceptance criteria for blank blood and antemortem blood. One (1) replicate for postmortem blood and two (2) replicates for urine failed to meet the qualifier ratio acceptance criteria of ±20%.

N-desmethylclobazam (norclobazam) had one (1) replicate out of 27 replicates for blank blood, antemortem blood, postmortem blood, and urine that did not meet the qualifier ratio acceptance criteria of ±20%. Desalkylgidazepam met all requirements for antemortem blood, postmortem blood, and urine. One (1) replicate for blank blood failed to meet the qualifier ratio acceptance criteria of ±20%.

In blank blood, gidazepam had nine (9) out of 27 replicates that did not meet the predetermined acceptance criteria for qualifier ratio (±20%), and fifteen (15) out of 27 replicates that failed the peak shape requirement. In antemortem blood, gidazepam had one (1) out of 27 replicates that did not meet

the qualifier ratio acceptance criteria of ±20%, and eight (8) out of 27 replicates that failed the peak shape requirement. In postmortem blood nine (9) out of 27 replicates did not meet the qualifier ratio acceptance criteria of ±20%, and four (4) out of 27 replicates failed the peak shape requirement. In urine there were five (5) out of 27 replicates that did not meet the qualifier ratio acceptance criteria of ±20%, and eight (8) out of 27 replicates failed the peak shape requirement.

Although acceptance criteria failures were observed, each compound in each matrix type (except for gidazepam and clobromazolam (phenazolam) in urine) had over 95% of the replicates meet the predetermined acceptance criteria. Therefore, the estimated limit of detection was determined to be 0.0050 mg/L for all compounds in all matrices except for clobromazolam (phenazolam) in urine and gidazepam for all matrix types. These compounds did not pass with a 95% acceptance rate. Therefore, clobromazolam will not be analyzed in urine and gidazepam will not be analyzed with this method.

4. Ionization Suppression/Enhancement

Ionization suppression and enhancement was evaluated by assessing the instrumental response of post-extraction fortified samples and neat standards. Post-extraction fortified samples were prepared from blank matrices that were subject to the liquid/liquid extraction protocol. After extraction, the blank samples were fortified with both target and internal standard. The neat samples were prepared by spiking an appropriate volume of the target analyte and internal standard, dried down, and then reconstituted in methanol.

Equation 2 was used to calculate the ionization suppression/enhancement for the target compounds and the internal standards. The ionization suppression/enhancement was assessed at two different concentrations: 0.02 mg/L and 1.0 mg/L.

Equation 2:

$$Ionization\ Suppression/Enhancement\ = \left(\frac{Average\ Post-Extracti\quad Fortified\ Sample}{Average\ Neat\ Sample}\right) \times 100$$

To fully evaluate the impact of ionization suppression/enhancement, triplicate determinations of each concentration for each matrix source were evaluated. A total of ten different sources per matrix type was used in the evaluation. The post-extraction fortified samples were compared to six replicate injections of neat standards. Table 5 shows data associated with ionization suppression and enhancement.

Table 5: Ionization Suppression and Enhancement

%Suppression/Enhancement ± Standard Deviation (%CV)

Target	Blank Blood	Antemortem Blood	Postmortem Blood	Urine
Compound	**n=60**	**n=60**	**n=60**	**n=60**
Nitrazolam	31± 5.8 (18)	60 ± 15.8 (33)	50 ± 8 (37)	29 ± 3.7 (40)
Clobromazolam (phenazolam)	35 ± 5.9 (17)	77 ± 11 (14)	52 ± 7.1 (36)	32 ± 3.4 (39)
Clobazam	49 ± 6.1 (12)	65 ± 13 (29)	60 ± 6.4 (34)	43 ± 3.4 (39)
4-Chloro Deschloroalprazolam	39 ± 5.9 (15)	66 ± 13 (24)	55 ± 7.1 (34)	34 ± 3.5 (35)
N-Desmethylclobazam	40 ± 5.4 (13)	71 ± 12 (17)	55 ± 7.3 (37)	34 ± 3.2 (39)
Desalkylgidazepam	56 ± 6.6 (15)	69 ± 12 (20)	57 ± 4.6 (31)	45 ± 3.5 (31)
Gidazepam	49 ± 13 (62)	44 ± 17 (49)	60 ± 19 (75)	43 ± 12 (74)
Alprazolam-D_5	44 ±6.7 (16)	89 ± 9.1 (14)	80 ± 8.7 (12)	51 ± 5.4 (11)
Diazepam-D_5	61 ± 6.1 (10)	91 ± 6.7 (11)	83 ± 6.1 (8)	65 ± 5.2 (8)

The values of 100% are indicative of no ionization suppression or enhancement in the samples. Values greater than 100% indicate ionization enhancement and values less than 100% indicate ionization suppression. Values greater than ±25% are indicative of significant ionization suppression or enhancement. The ionization enhancement did not exceed ±25% for any matrix type. Ionization suppression was noted in almost all instances. Blank blood, antemortem blood, postmortem blood, and urine all had indications of significant ionization suppression.

In addition to the average ionization suppression or enhancement, the variability between the matrices was also evaluated by assessing the %CV. The %CV was calculated for each matrix type and should not exceed 20%. Significant variability (%CV >20%) was noted for all matrix types for almost all compounds. Nitrazolam, clobazam, and 4'-chloro deschloroalprazolam failed to meet the predetermined acceptance criteria for %CV in antemortem blood, postmortem blood, and urine. Clobromazolam (phenazolam), N-desmethylclobazam (norclobazam), and desalkylgidazepam failed to meet the predetermined acceptance criteria for %CV in postmortem blood and urine. Gidazepam failed to meet the predetermined acceptance criteria for %CV in all matrix types.

Given the significant ionization suppression noted, and the variability between matrices exceeding a %CV of 20%, additional matrices were evaluated for the estimated limit of detection.

5. Carryover

Carryover was evaluated by analyzing blank matrix samples immediately following progressively higher concentrations of fortified matrix within the injection sequence. Two concentrations, 2.0 mg/L and 4.0 mg/L, were evaluated in three sources each of blank blood, antemortem blood, postmortem blood, and urine. The matrix sources were fortified with the compounds of interest over three batch analyses. The

blank sample immediately following the fortified matrix sample was evaluated for an instrumental response greater the 10% of the administratively established threshold (0.0050 mg/L). No blank matrix samples immediately following any fortified matrix sample had indications of carryover, except for gidazepam that showed significant carryover in all the blanks following both 2.0 mg/L and 4.0 mg/L concentrations.

6. Interferences

To assess for interference, the qualifier and quantifier ions for the target compounds and internal standards were monitored. If an instrumental response was noted and was less than 10% of the administratively established threshold response for the qualifier and quantifier ions, the impact of the instrumental response was deemed insignificant.

a. Endogenous Compounds

To evaluate samples for endogenous interferences, a total of ten matrix sources per matrix type (blank blood, antemortem blood, postmortem blood, and urine) were extracted and evaluated without the addition of internal standard. The samples were evaluated for the presence of instrumental response for the analyte and internal standard. No endogenous interferences were identified.

b. Internal Standard

To evaluate potential interferences of internal standard by a high concentration of analyte, samples were fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single matrix sample, per matrix type was evaluated. No interferences from a high concentration of analyte were detected.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, a single matrix sample, per matrix type was fortified with an appropriate concentration of internal standard (0.10 mg/L) without the analyte of interest and analyzed for the absence of response for the analyte. No interferences from the internal standard were detected.

c. Commonly Encountered Analytes

Interferences from commonly encountered compounds were evaluated by analyzing blank matrix fortified with high concentrations of commonly encountered drugs and metabolites. Table 6 depicts the compounds that were assessed for interference.

Table 6: Commonly encountered analytes

Drug Class	Drug	Concentration
Opioids and Cocaine	Oxymorphone, Hydromorphone, 6-Monoacetylmorphine, Acetylfentanyl, Fentanyl, Benzoylecgonine, Meperidine, Tramadol, Methadone, Morphine, Codeine, Oxycodone, Hydrocodone, Cocaethylene, Cocaine, Xylazine, Dexmedetomidine	0.2/2.0/1.0 mg/L
Anti-Epileptic Drugs	Gabapentin, Levetiracetam, Lamotrigine, Zonisamide, 10,11-dihydro-10-hydroxycarbamazepine, Oxcarbazepine, Topiramate, Carbamazepine, Phenytoin, Pregabalin, Lacosamide	0.004 mg/mL
NPS	Dibutylone, N-ethyl Pentylone, Tenocyclidine, Clonazolam, 4-Chloro-alpha-PVP, PV8, 6-MAPB, SDB-006, 3-Fluoro AMB, 4-Fluoro AMB, MMB-FUBINACA, MMB-CHMICA, 5F-AB-PINACA, MAB-CHMINICA, ADB-FUBICA, 4F-ADB, 4-APDB, 5-APDB, 6-APDB, MDMB-FUBINACA, 25I-NBOMe, 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOH, 25I-NBF, 25I-NBMD, Pentylone, 3-Methoxy-PCP, Methoxphenidine, Mitragynine, Methiopropamine, 5-DBFPV, 5F-PB-22, AB-FUBINACA, AB-PINACA, 3-Fluoro-phenmetrazine, PB-22	0.5 mg/L
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	0.01 mg/mL
Fentanyls	3-Fluorofentanyl, 4-Methoxybutyrylfentanyl, Acetylfentanyl, Acrylfentanyl, alpha-Methylacetylfentanyl, alpha-Methylfentanyl, Benzodioxolefentanyl, beta-Hydroxythiofentanyl, Butyrylfentanyl, Carfentanil, cis-3-Methylfentanyl, Cyclopropylfentanyl, Despropionylfentanyl, Fentanyl, Furanylfentanyl, Methoxyacetylfentanyl, Ocfentanil, ortho-Fluoroacrylfentanyl, ortho-Fluorobutyrylfentanyl, ortho-Fluorofentanyl, ortho-Fluoroisobutyrylfentanyl, para-Fluoroacrylfentanyl, para-Fluorobutyrylfentanyl, para-Fluorofentanyl, para-Fluoroisobutyrylfentanyl, Phenylfentanyl, Tetrahydrofuranfentanyl, trans-3-Methylfentanyl, U-47700, U-49900, Valerylfentanyl	0.025/0.05 mg/L
Acidic/Neutral Drugs	Acetaminophen, Carbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, Glutethimide, Ibuprofen, Levetiracetam, Oxcarbazepine, Phenytoin, Salicylic Acid	0.001 mg/mL
Basic Drugs	Amitriptyline, Citalopram, Cyclobenzaprine, Diphenhydramine, Nortriptyline, PCP, Trazodone, Dextromethorphan	10 mg/L
Amphetamines	Amphetamine, Methamphetamine, MDA, MDMA, Bupropion, Phentermine	0.002 mg/mL
Barbiturates	Butalbital, Phenobarbital, Butabarbital Pentobatbital, Secobarbital	0.004 mg/mL
Buprenorphine	Buprenorphine, Norbuprenorphine, Naloxone	0.04 mg/L
Cannabinoids	Δ9-THC, Δ9-OH-THC, Δ9-THC-COOH	0.1/0.50 mg/L

No interferences from commonly encountered compounds were noted. Individual benzodiazepines were extracted and evaluated for an instrumental response for the target compounds and internal standards within the analytical method. Table 7 lists the benzodiazepine interferences evaluated during the validation. In addition to the benzodiazepine interferences, each compound within the method was evaluated individually for interferences with other compounds within the method.

Table 7: Benzodiazepine interferent analysis

Benzodiazepines	
Oxazepam	Midazolam
Nordiazepam	Fluazepam
Clonazepam	Zaleplon
Lorazepam	α-Hydroxytriazolam
Alprazolam	Flualprazolam
Temazepam	Flubromazolam
Diazepam	Bromazolam
7-Aminoclonazepam	N-Desalkylflurazepam
7-Aminoflunitrazepam	Triazolam
Zopiclone	Flunitrazepam
8-Aminoclonazolam	Flubromazepam
Chlordiazepoxide	Etizolam
Pyrazolam	Clonazolam
Bromazepam	Phenazepam
α-Hydroxymidazolam	α-Hydroxyalprazolam

When evaluating the potential interferences for the existing method compounds 4'-chloro deschloroalprazolam produced an instrumental response for both the quantifier and qualifier transition within the Dynamic MRM window for alprazolam with acceptable retention time and qualifier ratios. This compound should employ relative retention time for retention time evaluation. No other potential interferences were identified from other compounds within the method.

7. Stability

The stability of extracted samples that were not analyzed immediately was evaluated at two concentrations for each matrix type (blank blood, antemortem blood, postmortem blood, and urine). The low concentration included was 0.0050 mg/L and the high concentration was 2.0 mg/L. The samples were extracted and injected in triplicate immediately to establish the Day 1 instrumental response. Both concentration levels were subsequently injected in triplicate every twenty-four hours over a seven-day period. The stability study was performed in a cooled autosampler that was maintained at approximately 4°C to minimize evaporation.

The instrumental response was compared for each time point. If the average instrumental response decreased below 80% or increased above 120% of the average Day 1 response, then the target was considered unstable after that time. Table 8 shows the stability for clobromazolam at low and high concentrations in each matrix type.

Table 8: Clobromazolam (Phenazolam) injection stability

Clobromazolam (Phenazolam)							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	115	111	94	101	86	99
Postmortem Blood	100	117	117	103	109	90	111
Antemortem Blood	100	114	113	102	102	82	112
Urine	100	126	129	103	106	88	129
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	105	106	91	94	79	97
Postmortem Blood	100	103	104	92	94	78	95
Antemortem Blood	100	103	106	93	97	80	96
Urine	100	105	107	94	97	79	97

When evaluating the stability of clobromazolam at 0.005 mg/L it was stable for one day in urine and seven days in blank blood, antemortem blood, and postmortem blood. At the high concentration, blank blood, postmortem blood, and urine were stable for five days while antemortem blood was stable for seven days. The stability of 4'-chloro deschloroalprazolam is shown in Table 9.

Table 9: 4’-Chloro deschloroalprazolam injection stability

4’-Chloro deschloroalprazolam							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	112	113	93	99	92	108
Postmortem Blood	100	109	110	103	99	83	103
Antemortem Blood	100	105	109	98	99	88	103
Urine	100	117	108	111	104	96	108
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	104	104	89	94	81	96
Postmortem Blood	100	103	103	91	95	81	95
Antemortem Blood	100	104	105	93	97	83	98
Urine	100	102	105	93	98	83	97

When evaluating the stability of 4’-chloro deschloroalprazolam, it was stable for seven days in all matrices for both low and high concentrations. The stability of nitrazolam is shown in Table 10.

Table 10: Nitrazolam injection stability

Nitrazolam							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	107	113	89	96	85	109
Postmortem Blood	100	101	110	98	94	80	99
Antemortem Blood	100	107	106	93	96	82	98
Urine	100	105	111	102	104	81	105
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	107	106	89	93	80	97
Postmortem Blood	100	105	106	90	94	79	95
Antemortem Blood	100	105	107	92	97	80	98
Urine	100	104	107	93	97	79	96

When evaluating the stability of nitrazolam, it was stable for seven days in all matrices at the low concentration. At the high concentration, postmortem blood and urine were stable for five days, blank blood and antemortem blood were stable for seven days. The stability of clobazam is shown in Table 11.

Table 11: Clobazam injection stability

Clobazam							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	92	93	82	98	76	83
Antemortem Blood	100	99	93	87	86	77	91
Postmortem Blood	100	97	93	88	90	77	90
Urine	100	92	91	84	95	74	88
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	94	96	85	87	75	88
Antemortem Blood	100	95	95	84	88	75	86
Postmortem Blood	100	95	96	85	90	77	88
Urine	100	95	95	85	89	77	89

The stability of clobazam was determined to be stable for five days in all matrices at both low and high concentrations. The stability of desalkylgidazepam is shown in Table 12.

Table 12: Desalkylgidazepam injection stability

Desalkylgidazepam							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	118	112	93	100	94	110
Postmortem Blood	100	116	108	101	91	86	107
Antemortem Blood	100	99	108	95	96	100	110
Urine	100	105	108	91	96	92	102
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	109	111	92	93	91	98
Postmortem Blood	100	106	108	93	93	89	97
Antemortem Blood	100	106	110	94	94	91	97
Urine	100	106	108	95	95	92	99

When evaluating the stability of desalkylgidazepam, it was stable for seven days in all matrices for both low and high concentrations. The stability of N-desmethylclobazam is shown in Table 13.

Table 13: N-Desmethylclobazam injection stability

N-Desmethylclobazam							
0.005 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	92	95	84	92	79	93
Antemortem Blood	100	96	97	96	86	87	93
Postmortem Blood	100	103	100	98	92	85	93
Urine	100	116	121	106	102	105	132
2.0 mg/L							
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Blank Blood	100	98	99	86	88	80	92
Antemortem Blood	100	98	98	86	90	81	91
Postmortem Blood	100	97	98	87	89	81	92
Urine	100	98	100	87	93	82	93

When evaluating the stability of N-desmethylclobazam at 0.005 mg/L it was stable for two days in urine, five days in blank blood, and seven days in antemortem blood and postmortem blood. At the high concentration, all matrices were stable for seven days.

The stability for gidazepam was not assessed given the large amount of carryover observed during the carryover evaluation.

8. Summary

The benzodiazepines evaluated within the qualitative validation were nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam. During validation it was noted that gidazepam showed poor chromatography at various low and high concentrations, significant carryover, and failed the LOD determination. Therefore, gidazepam was not fully validated within this method.

The cross-reactivity of nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam), clobazam, N-desmethylclobazam (norclobazam), gidazepam, and desalkylgidazepam were evaluated using the Immunalysis Direct Benzodiazepine ELISA kit. All compounds (except 4'-chloro deschloroalprazolam) showed at least an 85% cross-reactivity across all matrix types. 4'-Chloro deschloroalprazolam showed a significantly lower percent cross-reactivity response, however this cross-reactivity is still deemed acceptable as a screen with the knowledge that it is not as sensitive as the other compounds.

The estimated limit of detection was evaluated for each compound in blank blood, antemortem blood, postmortem blood, and urine. The estimated limit of detection for all matrices and all compounds was determined to be 0.0050 mg/L with the exception of gidazepam and clobromazolam (phenazolam) in urine.

Ionization suppression/enhancement was evaluated for the target compounds and internal standards within the method. Significant ionization suppression was noted when evaluating all compounds except for the internal standards. Although there was variability with the %CV, the instrument response for the fortified samples was still quite high and the peak shape was good. Even though the compounds were suppressed the data quality is sufficient to minimize concerns. Given the significant ionization suppression noted, additional matrix sources were evaluated when assessing the estimated limit of detection. Carryover was assessed during the validation, and no carryover was detected, except for gidazepam.

When evaluating interferences within the method, no endogenous interferences were noted. During the assessment of the contribution of analyte to internal standard response and internal standard response to analyte no interferences were detected. The assessment of interferences from commonly encountered compounds included an evaluation of benzodiazepines and other novel benzodiazepines currently within the analytical method.

When evaluating the stability of the compounds within the method, the raw instrumental response was stable for most compounds. All compounds, in all matrices were determined to be stable for five days apart from clobazam in urine that was stable for one day and N-desmethylclobazam in urine which was stable for two days. The comprehensive validation demonstrates that the liquid/liquid extraction method with analysis using LCMSMS is fit for the purpose of evaluating nitrazolam, 4'-chloro deschloroalprazolam, clobromazolam (phenazolam) (except urine), clobazam, N-desmethylclobazam (norclobazam), and desalkylgidazepam qualitatively.

All data from the validation has been stored on the Toxicology Northern Shared Drive.

9. References

Virginia Department of Forensic Science Quality Manual, Revision 32, 2025.

Virginia Department of Forensic Science Toxicology Procedures Manual, Revision 32, 2024.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology, 1st Edition, 2019.

Memo To:	James Hutchins, Ph.D., Toxicology Program Manager 6/3/25
From:	Tiffany Leusby, Forensic Scientist Senior
CC:	Rebecca Wagner, Ph.D., Research Section Supervisor, Alka Lohmann, Technical Services Director, Carol O'Neal, Ph.D., Toxicology Section Supervisor
Date	3/25/25
RE:	Validation Plan Validation for the Addition of 7 Novel Benzodiazepines

Validation Plan- **Validation Plan for the Qualitative Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method**

- It is proposed to validate the qualitative addition of nitrazolam, 4'-chloro deschloroalprazolam, phenazolam, clobazam, N-desmethylclobazam (Norclobazam), gidazepam, and desalkylgidazepam to the existing benzodiazepines quantitation and confirmation by liquid-liquid extraction using the LCMSMS method. Target analytes will be paired with the associated internal standard listed in Table 1.

Table 1 Target compounds and internal standard

Target	Internal Standard
Nitrazolam	Alprazolam-D_5
4'-chloro Deschloroalprazolam	Alprazolam-D_5
Phenazolam	Alprazolam-D_5
Clobazam	Diazepam-D_5
Norclobazam	Diazepam-D_5
Gidazepam	Diazepam-D_5
Desalkylgidazepam	Diazepam-D_5

A validation plan is outlined herein pursuant to the Quality Manual and Toxicology Procedures Manual. The validation plan is in accordance with ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019)

1. Immunoassay (ELISA) Cross-Reactivity
2. Ion Optimization
3. Sensitivity (Estimated limit of Detection)
4. Carryover
5. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
6. Stability
7. Ionization Suppression/Enhancement
8. References

1. Immunoassay (ELISA) Cross-Reactivity

In order to determine if the additional novel benzodiazepines will cross-react with the Immunalysis Direct Benzodiazepine ELISA kit a series of concentrations of nitrazolam, 4'-chloro deschloroalprazolam, phenazolam, clobazam, norclobazam, gidazepam, and desalkylgidazepam will be run.

Three different blank matrix sources per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine) will be fortified with a concentration of 0.40 mg/L (the concentration of the high positive control), the cut-off/positive control (0.040 mg/L), and ½ of the cut-off/low positive control (0.020 mg/L) for each analyte.

The analytes' B/B_0 values will be compared to the controls at the high positive control, positive control and low positive control concentrations and the cross-reactivity will be calculated using Equation 1.

Equation 1:

$$\text{Cross-reactivity}\,(\%) = \frac{(100 - B/B_0\ \text{Target Compound})}{(100 - B/B_0\ \text{Control Compound})} \times 100$$

2. Ion Optimization

The novel benzodiazepines will be added to the existing benzodiazepine method, however the Agilent LCMSMS MassHunter Optimizer Software will be used to determine the optimal precursor and product ions for nitrazolam, 4'-chloro deschloroalprazolam, phenazolam, clobazam, norclobazam, gidazepam, and desalkylgidazepam.

3. Sensitivity - Limit of Detection (LOD)

The estimated limit of detection for this validation shall be defined as an administratively-defined decision point (threshold concentration). The administratively-defined decision point shall be established using two concentrations. The concentrations to be evaluated are 0.010 mg/L and 0.0050 mg/L. These defined concentrations will be established as the decision point for reporting analytes within this method although a lower LOD may be analytically achievable.

The decision point shall be evaluated by fortifying, at minimum, three different blank matrix sources per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine). The three different blank matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met.

Predetermined identification criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥3.3

4. Carryover

Carryover will be evaluated by running injections of 2.0 mg/L and 4.0 mg/L of each individual novel benzodiazepine (nitrazolam, 4'-chloro deschloroalprazolam, phenazolam, clobazam, norclobazam, gidazepam, and desalkylgidazepam) followed by a matrix blank. For each concentration, there will be triplicate analyses with a minimum of three sources per matrix type. The matrix blanks will be evaluated for carryover. The highest analyte concentration at which no analyte carryover is observed, in the blank matrix, is determined to be the concentration at which the method is free from carryover. Analyte carryover is indicated by an instrumental response greater than 10% of the LOD.

5. Interferences

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method shall be monitored. Interferences below the limit of detection for the method may be deemed insignificant. If present, the impact on identification shall be evaluated. If the instrumental response is less than 10% of the threshold control or LOD response for the qualifier or quantifier ions, the impact is deemed insignificant.

a. Endogenous Compounds

A minimum of ten matrix samples for each matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine) within the validation should be evaluated, whenever possible.

b. Internal Standard

To evaluate potential interferences of the internal standards (alprazolam-D_5 and diazepam-D_5) by a high concentration of analyte, samples shall be fortified at 2.0 mg/L without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine) sample, per matrix type shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an internal standard concentration of 0.10 mg/L without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine) sample, per matrix type shall be evaluated.

c. Commonly Encountered Analytes

Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations.

All analytes that are already included in the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS method will be evaluated. Other potential interferents to be evaluated include:

Cannabinoids (0.1/0.5 mg/L THC, 11-Hydroxy-THC/Carboxy-THC)
Benzodiazepines (2.0 mg/L)
Anti-Epileptic Drugs (40 mg/L)
Barbiturates (30 mg/L)
Carisoprodol and meprobamate (100 mg/L)
Basic drugs from previously made mixes (5.0 mg/L)
Acid/neutral drugs from previously made mixes (10/5 mg/L)
Opioids and cocaine (0.2/2.0/1.0 mg/L from LCMSMS Method)
Fentanyl Analogs (0.10 mg/L)
Buprenorphine (0.020 mg/L)
NPS (1.0 mg/L)

Commonly encountered drugs (see above) will be fortified into a single blank matrix blank, per matrix type at the highest calibrator concentration without nitrazolam, 4'-chloro deschloroalprazolam, phenazolam, clobazam, norclobazam, gidazepam, and desalkylgidazepam added and then analyzed for the presence of the new benzodiazepines.

6. Stability

During the validation period, the stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. This enables the simulation of an abrupt abortion, delay, or interruption during instrumental analysis.

At minimum, a single blank matrix source, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine), will be extracted at two concentrations (2.0 and 0.0050 mg/L) and analyzed at minimum every twenty-four hours for a seven-day period with triplicate injections at each time point. For day one instrumental response, samples will be extracted and immediately analyzed. The responses will be averaged and all other responses from subsequent time points will be evaluated against the average response. The average instrumental responses for each time point will be compared to the day one instrumental response and plotted. Compounds are considered stable if the average signal response of the triplicate injections for a time point falls within the methods predefined acceptable bias (i.e., ±20%). For example, if the peak area increases above 120% or decrease below 80% of the original response the compound is no longer

deemed stable. Alternatively, the ratio of peak area of analyte to internal standard may be utilized in the stability evaluation as opposed to peak area.

7. Ionization Suppression/Enhancement

Ionization suppression and enhancement will be addressed with neat standards and post-extraction fortified samples. Two different sets of samples shall be prepared and their peak areas compared between sets. Neat standards, at low and high concentrations (0.0050 mg/L and 2.0 mg/L) will be prepared in neat extraction solvent and injected a minimum of six times each. The responses will be averaged for the two different concentrations. A minimum of ten duplicates of post-extraction fortified samples (matrix that is extracted and then fortified), per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine), will be prepared to compare to the neat standards. The responses will be averaged for the two concentrations. The ratio between the averages of the sets will then be used to assess ionization suppression or enhancement as shown in Equation 2.

Equation 2

$$Ion\ Suppression/Enhancement = \left(\frac{Average\ Post-Extraction\ Fortified\ Sample}{Average\ Neat\ Sample}\right) \times 100$$

The ionization suppression or enhancement will be evaluated for the qualifier and quantifier transitions for the analytes and internal standards within the method. If suppression or enhancement exceeds ±25% or the %CV exceeds 20%, an evaluation of the effect on limit of detection and bias shall be evaluated. The influence on the parameters shall be assessed by at least tripling the number of different sources of blank matrices used in the evaluation.

8. References

Virginia Department of Forensic Science Quality Manual, Revision 32, 2025.

Virginia Department of Forensic Science Toxicology Procedures Manual, Revision 32, 2024.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology, 1st Edition, 2019.

MEMORANDUM FOR RECORD

From: Tiffany Leusby Date: 06/18/2025

E-Signature:

Subject: Change of concentration values for BZ Research

DISTRIBUTION:

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The concentration values (0.0050 mg/L and 2.0 mg/L) listed in the Validation Plan for the Qualitative Addition of Seven Novel Benzodiazepines to the Benzodiazepines, Zolpidem, Zopiclone and Zaleplon Quantitation and Confirmation by LCMSMS Method for the ionization suppression/enhancement section will be changed to 0.020 mg/L and 1.0 mg/L.

The concentration values listed in the Sensitivity (Estimated limit of Detection) section will also be changed from the listed 0.010 mg/L and 0.0050 mg/L to 0.0050 mg/L and 0.0025 mg/L.

Both of these changes were approved by the research section supervisor, Dr. Becky Wagner.