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.