Validation of Reportable Qualitative Methods

Analyte	Novel Benzodiazepines (8-Aminoclonazolam, Clonazolam, Flualprazolam, Flubromazolam, Bromazolam, Etizolam, and Flubromazepam)
Units of Measure	ng/mL
Analyst Performing Validation Studies	P. Ke, C. Duvall, J. Reber
Responsible Supervisor	Jessica Lynn Ayala and Dayong Lee
Start Date	April 6, 2022
Completion Date	March 15, 2023
Primary Matrix	Urine
Secondary Matrix	N/A
Cut-off Calibrator	1 ng/mL
Equipment/Instrument	LCMS-3
Instrument Serial Number	SG2050G211
Method	NBZ.m

Validation Approval

Analyst: Cassandra Duvall — Digitally signed by Cassandra Duvall DN: cn=Cassandra Duvall, o=Houston Forensic Science Center, ou=Toxicology, email=cduvall@houstonforensicscience.org, c=US Date: 2023.04.07 08:53:07 -05'00' — 04/07/2023 (Date)

Analyst: Jami Reber — Digitally signed by Jami Reber Date: 2023.04.10 07:24:53 -05'00' — 04/10/2023 (Date)

Responsible Supervisor: Jessica Lynn Ayala — Digitally signed by Jessica Lynn Ayala Date: 2023.04.10 08:10:52 -05'00' — 04/10/2023 (Date)

Responsible Supervisor: Dayong Lee — Digitally signed by Dayong Lee Date: 2023.04.10 08:29:54 -05'00' — 04/10/2023 (Date)

Quality Division: Annalivia Harris — Digitally signed by Annalivia Harris Date: 2023.04.24 07:28:23 -05'00' — 04/24/2023 (Date)

Although Captain Pucheng Ke participated in urine studies, he departed HFSC prior to finalization of the validation. Therefore, his signature is not included in the Validation Approval above.

This method validation study, even though it may depict an approval date by both the Toxicology section and the Quality Division, will not be used in casework until Analytical Manual v3.9 has been issued.

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: 8-Aminoclonazolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 1239	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response was taken from the cutoff calibrator extracted in triplicate. IS interference was re-evaluated with a new deuterated IS, 8-aminoclonazolam-d4.
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	LOD response was taken from the cutoff calibrator extracted in triplicate.
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 168 hours.	Due to instrument maintenance, the stability was assessed after approximately 168 hours as compared to 120 hours for other NBZ analytes.
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 11% and 6% for the cutoff and high concentration (50 ng/mL).	Matrix interference studies (NBZ_20230308U_JR) conducted have shown that deuterated IS, 8-aminoclonazolam-d4, has no significant effect on d0. Therefore, matrix effects studies did not need to be re-evaluated with the addition of the new deuterated IS.
5	Extraction Efficiency	N/A	Extraction efficiency for the cutoff and high concentration (50 ng/mL) was -46% and -52%, respectively.	Extraction efficiency was deemed acceptable because 1) LOD acceptance criteria was consistently met, 2) deuterated internal standard was used for the analyte, and 3) additional studies for matrix and deuterated IS interference showed no significant interferences. Extraction efficiency is an additional experiment, not required to be evalutated by the Analytical Manual v3.9 and/or the ANSI/ASB standard 036.

Validation Study 1

Sensitivity (LOD)

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	795
	802
	495
NBZ_20220408U_PK	1034
	450
	886
NBZ_20220413U_PK	830
	541
	5315
Average Signal to Noise:	**1239**

Established LOD: 1 ng/mL
S:N at LOD: 1239

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: Signal to Noise ≥3.3
Acceptable detection and identification criteria

Validation Study 2 — CARRYOVER

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Average LOD Response*: 12559

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	1233663	1277492	1307634
Blank	36	53	37
%LOD Response	**0.29%**	**0.42%**	**0.29%**

Maximum Response in Blank: **0.4%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	12022	722745	1.00	NBZ_20220406U_PK
Run 2	12663	745516	1.00	NBZ_20220408U_PK
Run 3	12991	683068	1.00	NBZ_20220413U_PK
Average	**12559**	**717110**	**1.00**	

Matrix Interference Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	17	0.14%
2	13	0.10%
3	9	0.07%
4	9	0.07%
5	3	0.02%
6	9	0.07%
7	5	0.04%
8	0	0.00%
9	0	0.00%
10	15	0.12%

Interference from Stable-Isotope Internal Standards Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	1	0.01%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	8911	1.24%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)
Replicate 1	9824	206149	0.91
Replicate 2	9472	200819	0.90
Replicate 3	9204	193882	0.91
Average	**9500**	**200283**	**0.91**

Interference from Stable-Isotope Internal Standards Study Date: NBZ_20230308U_JR

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	6	0.06%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	2143	1.07%

Matrix Interference: No significant interference observed.

IS Interference: No significant interference observed.

Comments: LOD response was taken from the cutoff calibrator extracted in triplicate. IS interference was re-evaluated with a new deuterated IS, 8-aminoclonazolam-d4.

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Target Cutoff Concentration (ng/mL): 0.91
Control Acceptance: 20%
Run Date: NBZ_20230308U_JR

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	0.89	-2%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	0.91	0%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	0.89	-2%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	0.87	-4%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	0.90	-1%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	0.90	-1%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	0.90	-1%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.88	-3%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000	0.89	-2%	No significant interference

Conclusions: No significant interference observed

Comments: LOD response was taken from the cutoff calibrator extracted in triplicate.

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4 **AUTOSAMPLER STABILITY**

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			168 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (168 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20230308U_JR Re-injected 3/15/2023	Calibrator	1.00	0.05				1.01	1.00	0.05	1%

Stability of Punctured Controls

		Time Zero		24 Hours			168 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (168 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20230308U_JR Re-injected 3/15/2023	Cutoff	0.91	0.05				0.88	0.87	0.05	-3%
		0.90	0.05				0.89	0.89	0.05	-1%
		0.91	0.05				0.91	0.90	0.05	0%
	Positive	1.88	0.10				1.88	1.86	0.10	0%
		1.65	0.09				1.65	1.64	0.09	0%
		1.57	0.08				1.55	1.54	0.08	-1%

Stability of Unpunctured Controls

		Time Zero		24 Hours			168 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (168 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20230308U_JR	Cutoff	0.91	0.05				0.87	0.87	0.05	-4%
		0.90	0.05				0.89	0.88	0.05	-1%
		0.91	0.05				0.89	0.88	0.05	-2%
	Positive	1.88	0.10				1.86	1.85	0.10	-1%
		1.65	0.09				1.66	1.64	0.09	0%
		1.57	0.08				1.58	1.56	0.08	1%

Results: Punctured and unpunctured samples were shown to be stable for 168 hours.
Comments: Due to instrument maintenance, the stability was assessed after approximately 168 hours as compared to 120 hours for other NBZ analytes.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 5

MATRIX EFFECTS

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1	14094	722803	
2	14002	678937	
3	13744	686362	
4	13916	686863	
5	14099	688830	
6	14639	679803	
Mean	**14082**	**690600**	
SD	303	16277	
%CV	2%	2%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
795083	728911	
791584	717475	
796416	731039	
784909	726792	
793832	727618	
802926	716442	
794125	**724713**	
5918	6184	
1%	1%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	13002	-8%	732154	6%
2	12867	-9%	602088	-13%
3	10249	-27%	556128	-19%
4	13078	-7%	683159	-1%
5	13303	-6%	693357	0%
6	13032	-7%	674281	-2%
7	10252	-27%	569731	-18%
8	13003	-8%	641445	-7%
9	12833	-9%	672964	-3%
10	13317	-5%	687141	-1%
Mean	12494		651245	
SD	1193		57575	
%CV	10%		9%	
% suppression/ enhancement	-11%		-6%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	740705	-7%	648788	-10%
2	752119	-5%	705660	-3%
3	722580	-9%	710111	-2%
4	712417	-10%	714021	-1%
5	734068	-8%	703110	-3%
6	716052	-10%	706342	-3%
7	720745	-9%	698533	-4%
8	728987	-8%	701521	-3%
9	730445	-8%	705084	-3%
10	738558	-7%	687594	-5%
Mean	729668		698077	
SD	12194		18710	
%CV	2%		3%	
% suppression/ enhancement	-8%		-4%	

Results: Average percent signal suppression was 11% and 6% for the cutoff and high concentration (50 ng/mL).

Comments: Matrix interference studies (NBZ_20230308U_JR) conducted have shown that deuterated IS, 8-aminoclonazolam-d4, has no significant effect on d0. Therefore, matrix effects studies did not need to be re-evaluated with the addition of the new deuterated IS.

Acceptance Criteria: **Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement**

Validation Study 5

EXTRACTION EFFICIENCY

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20230308U_CD
1	27721	1099147	
2	27890	1091725	
3	28464	1096230	
4	28464	1097598	
5	28277	1091164	
6	27910	1086600	
Mean	**28121**	**1093744**	
SD	322	4733	
%CV	1%	0%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20230308U_CD
624413	543589	
621678	542396	
624789	542551	
622881	540641	
623860	543923	
625571	539972	
623865	**542179**	
1401	1578	
0%	0%	

Matrix Effect Study Date: NBZ_20230308U_CD

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% Extraction Efficiency	Peak Response at High	% Extraction Efficiency
1	15815	-44%	507038	-54%
2	15082	-46%	549940	-50%
3	20136	-28%	664997	-39%
4	9919	-65%	340394	-69%
5	10066	-64%	315777	-71%
6	11805	-58%	419236	-62%
7	12126	-57%	382531	-65%
8	17451	-38%	588457	-46%
9	17338	-38%	643314	-41%
10	22471	-20%	853218	-22%
Mean	15221		526490	
SD	4249		168526	
%CV	28%		32%	
% Average Extraction Efficiency	-46%		-52%	

Matrix Effect Study Date: NBZ_20230308U_CD

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% Extraction Efficiency	Peak Response at High	% Extraction Efficiency
1	321541	-48%	252753	-53%
2	294963	-53%	262888	-52%
3	400609	-36%	330041	-39%
4	217758	-65%	177422	-67%
5	223016	-64%	167153	-69%
6	264244	-58%	225557	-58%
7	276343	-56%	216381	-60%
8	372375	-40%	317670	-41%
9	316119	-49%	282218	-48%
10	430345	-31%	388344	-28%
Mean	311731		262043	
SD	71687		69718	
%CV	23%		27%	
% Average Extraction Efficiency	-50%		-52%	

Results: Extraction efficiency values for the cutoff and high concentration (50 ng/mL) were -46% and -52%, respectively.

Comments: Extraction efficiency was deemed acceptable because 1) LOD acceptance criteria was consistently met, 2) deuterated internal standard was used for the analyte, and 3) additional studies for matrix and deuterated IS interference showed no significant interferences. Extraction efficiency is an additional experiment, not required to be evalutated by the Analytical Manual v3.9 and/or the ANSI/ASB standard 036.

Acceptance Criteria: N/A

SUMMARY OF VALIDATION PERFORMANCE

Analyte: 8-Aminoclonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK, CD, JR
Study Dates: 4/6/2022 to 3/15/2023
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01; 220713C-C-0.01
Controls: 220303K-Q-0.1; 220826K-Q-0.1
Internal Standard: 220404C-IS-1; 230118C-15-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for 8-aminoclonazolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Clonazolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 505	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 8% at the cutoff concentration and 3% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	730
	343
	893
NBZ_20220408U_PK	425
	420
	347
NBZ_20220413U_PK	373
	655
	355
Average Signal to Noise:	**505**

Established LOD: 1 ng/mL
S:N at LOD: 505

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2

CARRYOVER

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 16539

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	1610423	1563465	1521354
Blank	103	82	138
%LOD Response	**0.62%**	**0.50%**	**0.84%**

Maximum Response in Blank: **0.8%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	17013	722745	1.00	NBZ_20220406U_PK
Run 2	16601	745516	1.00	NBZ_20220408U_PK
Run 3	16003	683068	1.00	NBZ_20220413U_PK
Average	**16539**	**717110**	**1.00**	

Matrix Interference — Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	25	0.15%
2	20	0.12%
3	43	0.26%
4	0	0.00%
5	42	0.26%
6	23	0.14%
7	32	0.19%
8	53	0.32%
9	0	0.00%
10	53	0.32%

Interference from Stable-Isotope Internal Standards — Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	0	0.00%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	8911	1.24%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	1.01	1%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	1.00	0%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	1.02	2%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	1.01	1%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	1.00	0%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	1.00	0%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	0.99	-1%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.98	-2%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000.0	0.98	-2%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

AUTOSAMPLER STABILITY

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.02				1.01	0.96	0.02	1%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.01	0.02				1.01	0.96	0.02	0%
		1.02	0.02				1.03	0.98	0.02	1%
		1.03	0.02				1.01	0.96	0.02	-1%
	Positive	2.00	0.04				2.06	1.96	0.05	3%
		2.04	0.05				2.00	1.90	0.04	-2%
		2.05	0.05				2.03	1.93	0.05	-1%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.01	0.02				1.02	0.97	0.02	1%
		1.02	0.02				1.04	0.99	0.02	3%
		1.03	0.02				1.02	0.97	0.02	-1%
	Positive	2.00	0.04				2.01	1.91	0.04	0%
		2.04	0.05				2.02	1.92	0.05	-1%
		2.05	0.05				2.03	1.93	0.05	-1%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%

Validation Study 5

MATRIX EFFECTS

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1	18976	876073	
2	18475	836379	
3	18376	840959	
4	19061	849731	
5	18332	839747	
6	18988	836736	
Mean	**18701**	**846604**	
SD	341	15225	
%CV	2%	2%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
795083	728911	
791584	717475	
796416	731039	
784909	726792	
793832	727618	
802926	716442	
794125	**724713**	
5918	6184	
1%	1%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	17352	-7%	774930	-8%
2	17865	-4%	824681	-3%
3	17092	-9%	837582	-1%
4	17277	-8%	842124	-1%
5	17516	-6%	825759	-2%
6	17036	-9%	821457	-3%
7	16890	-10%	817001	-3%
8	17244	-8%	834970	-1%
9	17087	-9%	821627	-3%
10	17460	-7%	825600	-2%
Mean	17282		822573	
SD	283		18523	
%CV	2%		2%	
% suppression/ enhancement	-8%		-3%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	740705	-7%	648788	-10%
2	752119	-5%	705660	-3%
3	722580	-9%	710111	-2%
4	712417	-10%	714021	-1%
5	734068	-8%	703110	-3%
6	716052	-10%	706342	-3%
7	720745	-9%	698533	-4%
8	728987	-8%	701521	-3%
9	730445	-8%	705084	-3%
10	738558	-7%	687594	-5%
Mean	729668		698077	
SD	12194		18710	
%CV	2%		3%	
% suppression/ enhancement	-8%		-4%	

Results: Average percent signal suppression was 8% at the cutoff concentration and 3% at high concentration (50 ng/mL).
Comments: N/A

Acceptance Criteria: **Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement**

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Clonazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for clonazolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Flualprazolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 754	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 1% at the cutoff concentration, and signal enhancement was 3% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	118
	190
	2964
NBZ_20220408U_PK	162
	361
	131
NBZ_20220413U_PK	1753
	485
	626
Average Signal to Noise:	**754**

Established LOD: 1 ng/mL
S:N at LOD: 754

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2

CARRYOVER

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 29567

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	2318836	2279052	2291735
Blank	256	145	245
%LOD Response	**0.87%**	**0.49%**	**0.83%**

Maximum Response in Blank: **0.9%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	29708	1249536	1.00	NBZ_20220406U_PK
Run 2	29480	1264671	1.00	NBZ_20220408U_PK
Run 3	29511	1229238	1.00	NBZ_20220413U_PK
Average	**29567**	**1247815**	**1.00**	

Matrix Interference Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	55	0.19%
2	53	0.18%
3	113	0.38%
4	54	0.18%
5	0	0.00%
6	30	0.10%
7	46	0.15%
8	29	0.10%
9	65	0.22%
10	22	0.07%

Interference from Stable-Isotope Internal Standards Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	11.71	0.04%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	146.81	0.01%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	1.02	2%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	1.02	2%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	1.00	0%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	1.03	3%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	1.04	4%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	1.03	3%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	0.99	-1%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	1.02	2%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000	1.04	4%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4 — **AUTOSAMPLER STABILITY**

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.02				1.02	0.99	0.02	2%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.02	0.02				0.97	0.94	0.02	-6%
		1.01	0.02				0.99	0.96	0.02	-2%
		1.02	0.02				1.02	0.99	0.02	0%
	Positive	2.06	0.05				2.03	1.97	0.05	-2%
		2.12	0.05				2.01	1.96	0.05	-5%
		2.04	0.05				2.05	1.99	0.05	1%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.02	0.02				1.03	1.00	0.02	1%
		1.01	0.02				1.02	0.99	0.02	1%
		1.02	0.02				1.01	0.98	0.02	-1%
	Positive	2.06	0.05				1.99	1.94	0.05	-3%
		2.12	0.05				2.00	1.94	0.05	-5%
		2.04	0.05				2.05	1.99	0.05	0%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%

Validation Study 5

MATRIX EFFECTS

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1	30971	1295393	
2	31370	1232292	
3	30779	1260622	
4	31226	1242640	
5	30591	1250181	
6	31310	1227765	
Mean	**31041**	**1251482**	
SD	313	24586	
%CV	1%	2%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1279833	1139652	
1280942	1099855	
1285608	1132345	
1282877	1114916	
1272551	1108994	
1279353	1088704	
1280194	**1114078**	
4389	19289	
0%	2%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	31094	0%	1321394	6%
2	30172	-3%	1253007	0%
3	30683	-1%	1281380	2%
4	30380	-2%	1292251	3%
5	30285	-2%	1315910	5%
6	30287	-2%	1296713	4%
7	31211	1%	1278747	2%
8	30350	-2%	1275635	2%
9	32120	3%	1278080	2%
10	30715	-1%	1285645	3%
Mean	30729		1287876	
SD	603		19997	
%CV	2%		2%	
% suppression/ enhancement	-1%		3%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	1261352	-1%	1175678	6%
2	1236121	-3%	1128838	1%
3	1261248	-1%	1130484	1%
4	1263949	-1%	1131671	2%
5	1260285	-2%	1143570	3%
6	1256583	-2%	1133917	2%
7	1275105	0%	1143059	3%
8	1248588	-2%	1118706	0%
9	1273390	-1%	1133363	2%
10	1289726	1%	1133684	2%
Mean	1262635		1137297	
SD	14731		15206	
%CV	1%		1%	
% suppression/ enhancement	-1%		2%	

Results: Average percent signal suppression was 1% at the cutoff concentration, and signal enhancement was 3% at high concentration (50 ng/mL).

Comments: N/A

Acceptance Criteria: **Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement**

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Flualprazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for flualprazolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Flubromazolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 326	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 1% at cutoff concentration, and signal enhancement was 3% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	252
	521
	424
NBZ_20220408U_PK	122
	292
	75
NBZ_20220413U_PK	559
	460
	230
Average Signal to Noise:	**326**

Established LOD: 1 ng/mL
S:N at LOD: 326

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2

CARRYOVER

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 17204

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	1335332	1286856	1283504
Blank	180	80	134
%LOD Response	**1.05%**	**0.47%**	**0.78%**

Maximum Response in Blank: **1.0%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	17662	878330	1.00	NBZ_20220406U_PK
Run 2	17318	902830	1.00	NBZ_20220408U_PK
Run 3	16634	844738	1.00	NBZ_20220413U_PK
Average	**17204**	**875299**	**1.00**	

Matrix Interference — Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	51	0.29%
2	34	0.20%
3	103	0.60%
4	27	0.16%
5	97	0.57%
6	68	0.40%
7	27	0.16%
8	0	0.00%
9	53	0.31%
10	39	0.23%

Interference from Stable-Isotope Internal Standards — Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	25.33	0.15%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	129.50	0.01%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	0.95	-5%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	0.98	-2%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	0.96	-4%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	0.98	-2%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	0.96	-4%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	1.00	0%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	0.97	-3%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.94	-6%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000	1.02	2%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4

AUTOSAMPLER STABILITY

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.02				0.98	0.96	0.02	-2%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	0.97	0.02				0.99	0.96	0.02	2%
		1.01	0.02				0.97	0.95	0.02	-4%
		0.99	0.02				1.00	0.98	0.02	2%
	Positive	1.99	0.04				1.97	1.92	0.04	-1%
		1.99	0.04				1.99	1.94	0.04	0%
		1.98	0.04				1.96	1.91	0.04	-1%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	0.97	0.02				0.99	0.97	0.02	2%
		1.01	0.02				1.00	0.97	0.02	-1%
		0.99	0.02				0.98	0.96	0.02	-1%
	Positive	1.99	0.04				2.05	2.00	0.04	3%
		1.99	0.04				1.96	1.91	0.04	-1%
		1.98	0.04				1.96	1.91	0.04	-1%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%

Validation Study 5

MATRIX EFFECTS

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1	17570	721731	
2	17175	693070	
3	17736	697982	
4	18017	710018	
5	17697	700747	
6	18300	690015	
Mean	**17749**	**702260**	
SD	385	11784	
%CV	2%	2%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
887226	803401	
889998	771636	
887238	787351	
880051	787233	
881117	785637	
903376	782791	
888168	**786342**	
8390	10219	
1%	1%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	17868	1%	751704	7%
2	17361	-2%	694282	-1%
3	17700	0%	732527	4%
4	17467	-2%	731144	4%
5	17158	-3%	733870	5%
6	17413	-2%	725474	3%
7	18014	1%	723750	3%
8	17248	-3%	720402	3%
9	17720	0%	726921	4%
10	17565	-1%	727049	4%
Mean	17551		726712	
SD	274		14264	
%CV	2%		2%	
% suppression/ enhancement	-1%		3%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	898174	1%	823247	5%
2	873648	-2%	788371	0%
3	903958	2%	809279	3%
4	886693	0%	804585	2%
5	901782	2%	807672	3%
6	891794	0%	800171	2%
7	904003	2%	816348	4%
8	884646	0%	796966	1%
9	907840	2%	799109	2%
10	898565	1%	806527	3%
Mean	895110		805228	
SD	10735		9935	
%CV	1%		1%	
% suppression/ enhancement	1%		2%	

Results: Average percent signal suppression was 1% at cutoff concentration, and signal enhancement was 3% at high concentration (50 ng/mL).

Comments: N/A

Acceptance Criteria: **Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement**

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Flubromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for flubromazolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Bromazolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 320	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/06/2020 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 3% at the cutoff concentration, and signal enhancement was 2% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	299
	318
	217
NBZ_20220408U_PK	256
	240
	202
NBZ_20220413U_PK	94
	630
	621
Average Signal to Noise:	**320**

Established LOD: 1 ng/mL
S:N at LOD: 320

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2

CARRYOVER

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 19651

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	1524932	1449147	1461689
Blank	114	130	104
%LOD Response	**0.58%**	**0.66%**	**0.53%**

Maximum Response in Blank: **0.7%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	20560	878330	1.00	NBZ_20220406U_PK
Run 2	19653	902830	1.00	NBZ_20220408U_PK
Run 3	18739	844738	1.00	NBZ_20220413U_PK
Average	**19651**	**875299**	**1.00**	

Matrix Interference — Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	33	0.17%
2	34	0.17%
3	33	0.17%
4	15	0.08%
5	10	0.05%
6	25	0.13%
7	0	0.00%
8	73	0.37%
9	0	0.00%
10	0	0.00%

Interference from Stable-Isotope Internal Standards — Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	11	0.06%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	130	0.01%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/06/2020 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	1.04	4%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	0.99	-1%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	1.00	0%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	1.01	1%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	1.01	1%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	1.04	4%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	0.95	-5%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.99	-1%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000.0	1.02	2%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4 **AUTOSAMPLER STABILITY**

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.02				1.00	0.98	0.02	0%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.01	0.02				0.96	0.94	0.02	-5%
		1.03	0.02				0.99	0.97	0.02	-4%
		1.01	0.02				0.96	0.94	0.02	-5%
	Positive	2.03	0.04				2.04	2.00	0.04	1%
		2.02	0.04				1.98	1.94	0.04	-2%
		2.06	0.04				2.05	2.01	0.04	-1%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.01	0.02				1.03	1.01	0.02	1%
		1.03	0.02				1.02	1.00	0.02	-1%
		1.01	0.02				0.98	0.97	0.02	-2%
	Positive	2.03	0.04				2.02	1.98	0.04	-1%
		2.02	0.04				2.02	1.98	0.04	0%
		2.06	0.04				1.98	1.94	0.04	-4%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 5

MATRIX EFFECTS

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)
1	21137	838623
2	20729	787519
3	20966	812296
4	21429	807837
5	20984	803371
6	20951	798527
Mean	**21033**	**808029**
SD	234	17244
%CV	1%	2%

NBZ_20220406U_PK

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)
887226	803401
889998	771636
887238	787351
880051	787233
881117	785637
903376	782791
888168	**786342**
8390	10219
1%	1%

NBZ_20220406U_PK

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	20427	-3%	861954	7%
2	19733	-6%	790203	-2%
3	20735	-1%	824776	2%
4	20061	-5%	819954	1%
5	20552	-2%	829230	3%
6	19789	-6%	819015	1%
7	20001	-5%	820597	2%
8	20338	-3%	820521	2%
9	21111	0%	825575	2%
10	20221	-4%	816976	1%
Mean	20297		822880	
SD	430		17368	
%CV	2%		2%	
% suppression/ enhancement	-3%		2%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	898174	1%	823247	5%
2	873648	-2%	788371	0%
3	903958	2%	809279	3%
4	886693	0%	804585	2%
5	901782	2%	807672	3%
6	891794	0%	800171	2%
7	904003	2%	816348	4%
8	884646	0%	796966	1%
9	907840	2%	799109	2%
10	898565	1%	806527	3%
Mean	895110		805228	
SD	10735		9935	
%CV	1%		1%	
% suppression/ enhancement	1%		2%	

Results: Average percent signal suppression was 3% at the cutoff concentration, and signal enhancement was 2% at high concentration (50 ng/mL).

Comments: N/A

Acceptance Criteria: Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Bromazolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for bromazolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Etizolam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 366	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 3% at the cutoff concentration, and signal enhancement was 2% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	246
	334
	747
NBZ_20220408U_PK	336
	304
	88
NBZ_20220413U_PK	832
	248
	162
Average Signal to Noise:	**366**

Established LOD: 1 ng/mL
S:N at LOD: 366

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2

CARRYOVER

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 9783

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	723582	695423	710510
Blank	74	50	78
%LOD Response	**0.76%**	**0.52%**	**0.80%**

Maximum Response in Blank: **0.8%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	10417	2146276	1.00	NBZ_20220406U_PK
Run 2	9378	2172077	1.00	NBZ_20220408U_PK
Run 3	9553	2044765	1.00	NBZ_20220413U_PK
Average	**9783**	**2121039**	**1.00**	

Matrix Interference — Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	23	0.24%
2	44	0.45%
3	35	0.36%
4	41	0.42%
5	17	0.18%
6	9	0.09%
7	25	0.25%
8	5	0.05%
9	0	0.00%
10	9	0.09%

Interference from Stable-Isotope Internal Standards — Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	46.56	0.48%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	264719.69	12.48%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	0.99	-1%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	0.95	-5%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	0.98	-2%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	0.98	-2%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	1.00	0%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	0.95	-5%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	1.01	1%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.92	-8%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000.0	0.97	-3%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4 — **AUTOSAMPLER STABILITY**

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.00				1.05	0.97	0.00	5%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.06	0.00				1.07	0.99	0.00	1%
		1.03	0.00				1.07	0.99	0.00	4%
		1.06	0.00				1.09	1.00	0.00	3%
	Positive	2.10	0.01				2.11	1.95	0.01	1%
		2.07	0.01				2.00	1.85	0.01	-3%
		2.10	0.01				2.09	1.93	0.01	0%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.06	0.00				1.07	0.99	0.00	1%
		1.03	0.00				1.06	0.98	0.00	3%
		1.06	0.00				1.08	1.00	0.00	2%
	Positive	2.10	0.01				2.11	1.95	0.01	0%
		2.07	0.01				2.17	2.00	0.01	5%
		2.10	0.01				2.12	1.96	0.01	1%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%

Validation Study 5

MATRIX EFFECTS

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
1	10757	422941	
2	10005	397349	
3	10687	402487	
4	10443	400929	
5	10395	403943	
6	10564	396223	
Mean	**10475**	**403978**	
SD	269	9749	
%CV	3%	2%	

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)	NBZ_20220406U_PK
2177623	1989828	
2154181	1924627	
2173106	1962782	
2163694	1935609	
2151796	1959826	
2176410	1939711	
2166135	**1952064**	
11319	23569	
1%	1%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	10081	-4%	423814	5%
2	10192	-3%	399445	-1%
3	10252	-2%	410936	2%
4	10172	-3%	414266	3%
5	10001	-5%	408500	1%
6	10163	-3%	409725	1%
7	10224	-2%	412201	2%
8	10201	-3%	409047	1%
9	10321	-1%	409567	1%
10	10141	-3%	413894	2%
Mean	10175		411139	
SD	89		6071	
%CV	1%		1%	
% suppression/ enhancement	-3%		2%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	2182318	1%	2035737	4%
2	2107878	-3%	1950689	0%
3	2173842	0%	1971276	1%
4	2149265	-1%	1980751	1%
5	2187224	1%	2018630	3%
6	2146153	-1%	1982113	2%
7	2175134	0%	1980772	1%
8	2186154	1%	1953813	0%
9	2168288	0%	1960728	0%
10	2206055	2%	1990713	2%
Mean	2168231		1982522	
SD	27648		27179	
%CV	1%		1%	
% suppression/ enhancement	0%		2%	

Results: Average percent signal suppression was 3% at the cutoff concentration, and signal enhancement was 2% at high concentration (50 ng/mL).

Comments: N/A

Acceptance Criteria: Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Etizolam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for etizolam confirmation analysis in urine.**

METHOD VALIDATION PROTOCOL AND RESULTS

Analyte: Flubromazepam
Units: ng/mL
Method: NBZ.m
Instrument: LCMS-3
SOP Reference: **Toxicology Analytical Manual v3.9**

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	VALIDATION EXPERIMENT	SOP CRITERIA	RESULTS	COMMENTS
1	Limit of Detection (LOD)	Signal to Noise ≥3.3 Acceptable detection and identification criteria	LOD = 1 ng/mL S:N = 50	The decision point concentration was defined as the LOD.
2	Carryover	No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.	No significant carryover observed following samples containing analyte at up to 100 ng/mL	N/A
3	Matrix Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022
3	Labeled IS Interference	Response of blank matrix must be <20% of the average response of LOD	No significant interference observed.	
3	Exogenous Substances Interferences	Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.	No significant interference observed	N/A
4	Processed Sample Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	N/A	Study not performed. Sample preparation will be completed once started without prolonged interruptions.
4	Autosampler Stability	Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%	Punctured and unpunctured samples were shown to be stable for 120 hours.	N/A
5	Matrix Effects	Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement	Average percent signal suppression was 3% at cutoff concentration, and signal enhancement was 4% at high concentration (50 ng/mL).	N/A

Validation Study 1

Sensitivity (LOD)

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

	Signal to Noise Ratio
Concentration	*1.0*
NBZ_20220406U_PK	30
	55
	32
NBZ_20220408U_PK	53
	50
	58
NBZ_20220413U_PK	42
	95
	37
Average Signal to Noise:	**50**

Established LOD: 1 ng/mL
S:N at LOD: 50

Comments: The decision point concentration was defined as the LOD.

Acceptance Criteria: **Signal to Noise ≥3.3**
Acceptable detection and identification criteria

Validation Study 2 — CARRYOVER

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Average LOD Response*: 2104

	Response		
Study Date:	**NBZ_20220406U_PK**	**NBZ_20220408U_PK**	**NBZ_20220413U_PK**
Concentrated Sample (100 ng/mL)	158636	154995	158363
Blank	16	20	34
%LOD Response	**0.74%**	**0.97%**	**1.61%**

Maximum Response in Blank: **1.6%**

Results: No significant carryover observed following samples containing analyte at up to 100 ng/mL
Comments: N/A

Acceptance Criteria: **No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.**

Validation Study 3

MATRIX & IS INTERFERENCE

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

LOD Response

	Analyte	IS	Concentration (ng/mL)	
Run 1	2188	99323	1.00	NBZ_20220406U_PK
Run 2	1935	101624	1.00	NBZ_20220408U_PK
Run 3	2189	99801	1.00	NBZ_20220413U_PK
Average	**2104**	**100249**	**1.00**	

Matrix Interference — Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT	
	Peak Response	Percent of LOD Response
1	12	0.58%
2	0	0.00%
3	13	0.62%
4	1	0.03%
5	7	0.33%
6	3	0.14%
7	21	0.99%
8	14	0.66%
9	4	0.17%
10	5	0.24%

Interference from Stable-Isotope Internal Standards — Study Date: NBZ_20220406U_PK

Experiment	Peak at Analyte RT		Peak at IS RT	
	Peak Response	Percent of LOD Response	Peak Response	Percent of LOD Response
Matrix with IS but no D0 (IS = 50 ng/mL)	22.29	1.06%	N/A	N/A
Matrix with D0 but no IS (D0 = 100 ng/mL)	N/A	N/A	1873.84	1.87%

Matrix Interference: No significant interference observed.
IS Interference: No significant interference observed.
Comments: LOD response per run was taken from the cutoff calibrator in analytical runs between 4/6/2022 and 4/13/2022

Acceptance Criteria: **Response of blank matrix must be <20% of the average response of LOD**

Validation Study 3

EXOGENOUS SUBSTANCE INTERFERENCE

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Target Cutoff Concentration (ng/mL): 1.00
Control Acceptance: 20%
Run Date: NBZ_20220406U_PK

Group	Compound	Compound Concentration (ng/mL)	Calculated Cutoff Concentration (ng/mL)	% Difference from Target	Comment
Amphetamines (AMP)	Amphetamine	500	1.02	2%	No significant interference
	Methamphetamine				
	MDMA				
	MDEA				
Opioids (OPI)	Morphine	1000	1.05	5%	No significant interference
	Hydrocodone				
	Codeine				
	Norbuprenorphine	200			
	Buprenorphine				
	Fentanyl				
	Norfentanyl Oxalate				
	Hydromorphone	1000			
	Methadone				
	EDDP				
	Oxycodone				
	Oxymorphone				
	Tramadol				
	o-Desmethyltramadol				
Cocaine and Metabolites (COC)	Benzoylecgonine	500	0.99	-1%	No significant interference
	Cocaine				
	Cocaethylene				
Cannabinoids (THC)	Δ9-THC	500	1.02	2%	No significant interference
	11-Hydroxy-Δ9-THC				
	Δ9-THC-COOH				
	Δ8-THC				
	Cannabinol				
	Cannabinolic Acid				
Basic and Neutral Mix (BSD)	Amitriptyline	500	0.93	-7%	No significant interference
	Benzylpiperizine				
	Chlorpheniramine				
	Cyclobenzaprine				
	Dextromethorphan				
	Diphenhydramine				
	Doxylamine				
	Fluoxetine				
	Imipramine				
	Ketamine				
	Norketamine				
	Meperidine				
	Nortriptyline				
	Propoxyphene				
	Sertraline				
	Trazodone				
	Venlafaxine				
	Zopiclone				
Carisoprodol/Meprobamate (CAR)	Carisoprodol	500	1.01	1%	No significant interference
	Meprobamate				
Over-the-Counter Drugs	Acetaminophen	1000	1.03	3%	No significant interference
	Caffeine				
	Ibuprofen				
	Naproxen				
	Pseudoephedrine				
	Theobromine				
	Acetaminophen				
Traditional Benzodiazepines and Zolpidem (BNZ)	7-Aminoclonazepam	1000	0.99	-1%	No significant interference
	Zolpidem				
	Alpha-hydroxyalprazolam				
	Oxazepam				
	Nordiazepam				
	Clonazepam				
	Lorazepam				
	Alprazolam				
	Tempazepam				
	Diazepam				
PCP	Phencyclidine	1000	0.97	-3%	No significant interference

Conclusions: No significant interference observed

Comments: N/A

Acceptance Criteria: Concentrations of analytes of interest must be within ±20% of the average concentration of LOD.

Validation Study 4

PROCESSED SAMPLE STABILITY

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Run Date	Sample	Time Zero Response	24H Response	48H Response	72H Response	Acceptable Range		Pass/Unstable
						Low	High	
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A	#DIV/0!	#DIV/0!	N/A
		N/A	N/A	N/A	N/A			
		N/A	N/A	N/A	N/A			

Results: N/A
Comments: Study not performed. Sample preparation will be completed once started without prolonged interruptions.

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 4 **AUTOSAMPLER STABILITY**

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Stability of Punctured Calibrators

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Calibrator	1.00	0.02				1.06	0.92	0.02	6%

Stability of Punctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.10	0.02				1.06	0.92	0.02	-3%
		1.10	0.02				1.00	0.86	0.02	-9%
		1.08	0.02				1.02	0.89	0.02	-5%
	Positive	2.09	0.04				2.11	1.83	0.04	1%
		2.08	0.04				2.14	1.86	0.04	3%
		2.16	0.04				2.20	1.91	0.04	2%

Stability of Unpunctured Controls

		Time Zero		24 Hours			120 Hours			
Run Date	Sample	Concentration (Time Zero Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (24 h Curve)	Response Ratio	Concentration (Time Zero Curve)	Concentration (120 h Curve)	Response Ratio	% Difference from Time Zero (Response Ratio)
NBZ_20220408U_PK; NBZ_20220413U_PK	Cutoff	1.10	0.02				1.11	0.96	0.02	1%
		1.10	0.02				1.09	0.95	0.02	-1%
		1.08	0.02				1.07	0.93	0.02	-2%
	Positive	2.09	0.04				2.22	1.93	0.04	7%
		2.08	0.04				2.07	1.80	0.04	0%
		2.16	0.04				2.15	1.87	0.04	0%

Results: Punctured and unpunctured samples were shown to be stable for 120 hours.
Comments: N/A

Acceptance Criteria: **Average signal (peak area, or ratio of peak area analyte/IS) compared to time 0 is within 20%**

Validation Study 5

MATRIX EFFECTS

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

Neat Response at Analyte RT

	Cut-off	High Conc' (50 ng/mL)
1	2159	88226
2	2039	84288
3	2307	84975
4	2216	85619
5	2300	84930
6	2178	83923
Mean	**2200**	**85327**
SD	100	1538
%CV	5%	2%

NBZ_20220406U_PK

Neat Response at IS RT

Cut-off	High Conc' (50 ng/mL)
98017	86600
99177	82623
99285	84941
97305	84390
99375	85514
99513	83881
98779	**84658**
901	1371
1%	2%

NBZ_20220406U_PK

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at Analyte RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	2149	-2%	91663	7%
2	2083	-5%	87111	2%
3	2155	-2%	87212	2%
4	2018	-8%	89049	4%
5	2180	-1%	88666	4%
6	2131	-3%	87956	3%
7	2073	-6%	90345	6%
8	2240	2%	88251	3%
9	2181	-1%	87099	2%
10	2203	0%	88263	3%
Mean	2141		88562	
SD	67		1478	
%CV	3%		2%	
% suppression/ enhancement	-3%		4%	

Matrix Effect Study Date: NBZ_20220406U_PK

Matrix Source	Peak at IS RT			
	Peak Response at Cutoff	% suppression/ enhancement	Peak Response at High	% suppression/ enhancement
1	100177	1%	87957	4%
2	96095	-3%	86680	2%
3	99308	1%	87249	3%
4	98670	0%	87239	3%
5	99399	1%	86425	2%
6	97459	-1%	86260	2%
7	100325	2%	87963	4%
8	98988	0%	84917	0%
9	99092	0%	86880	3%
10	100108	1%	86386	2%
Mean	98962		86796	
SD	1314		898	
%CV	1%		1%	
% suppression/ enhancement	0%		3%	

Results: Average percent signal suppression was 3% at cutoff concentration, and signal enhancement was 4% at high concentration (50 ng/mL).

Comments: N/A

Acceptance Criteria: **Average suppression/enhancement ≤25% or CV ≤20% of the suppression/enhancement**

SUMMARY OF VALIDATION PERFORMANCE

Analyte: Flubromazepam
Units: ng/mL
Instrument: LCMS-3

Analyst: PK
Study Dates: 4/6/2022 to 6/1/2022
Matrix: Urine

The intent of this summary is to capture and document important information about the performance of this method outside the required measurements for validation.

Failed Runs (include dates/reasons): N/A

Date	Reason

Deviations from SOP: N/A

Other Observations:

Working Standards Verified in Validation:
Calibrators: 220404C-C-0.01
Controls: 220303K-Q-0.1
Internal Standard: 220404C-IS-1

Sample Preparation Steps: Refer to Toxicology Analytical Manual v3.9, "Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry" section titled "Extraction Procedure".

Location of Raw Data: Toxicology section shared electronic storage.

Recommended Maximum Run Length (Unknown Samples): 30

Conclusion: **This method is fit for use on casework for flubromazepam confirmation analysis in urine.**