Validation report (per T'nO 7a.3.3)

Benzodiazepine analysis using LC/MS/MS
Waters Acquity UPLC H-Class System/ Xevo TQD System LC/MS/MS
[Serial Numbers: J14CHA622G (column housing unit), K14SDl251G (Sample Manager-FTN), K14QSMS01A (Quaternary Solvent Manager), QCA863 (Xevo TQD)] [DOJ #31401]
Toxicology Unit-Milwaukee
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December 8, 2022

1. Scope and objective of the method.

This report describes the work that was conducted to validate the quantitation of diazepam, nordiazepam, oxazepam, temazepam, 7-aminoclonazepam, clonazepam, a-OH alprazolam, alprazolam, lorazepam, etizolam and zolpidem in blood and confirmation in urine. This report also describes the work that was completed to validate the confirmation of nitrazepam, 7-aminoflunitrazepam, norchlordiazepoxide, demoxepam, estazolam, nimetazepam, chlordiazepoxide, clobazam, delorazepam, zaleplon, flunitrazepam, bromazepam, diclazepam, 8-aminoclonazolam, midazolam, flualprazolam, OH-ethylflurazepam, flubromazepam, lormetazepam, triazolam, phenazepam, adinazolam, bromazolam, pyrazolam, clonazolam, a-OH-etizolam, a-OH-triazolam, a-OH-clonazolam, flubromazolam, a-OH-flubromazolam, flurazepam, and zopiclone/eszopiclone in blood and urine. Validation started in November of 2021 and completed in 2023. When referencing the ANSI/ASB standards it is assumed to be the first edition that was published unless stated otherwise.

2. Test method procedure including all chemicals, reagents, and reference standards that would be required for the method. Any instrumental parameters should also be included.

Chemicals:

- BGTurbo enzyme (β-Glucuronidase)
- Ethyl acetate
- Hexanes
- Instant buffer I
- Methanol
- Rapid hydrolysis buffer
- Methanol (LC/MS grade)
- Acetonitrile (LC/MS grade)
- Isopropanol (LC/MS grade)
- LC/MS/MS water
- Formic acid (LC/MS grade)

Reagents:

- 0.1M Phosphate buffer, pH 6.0 (LC/MS/MS)
- 1M acetic acid: acetonitrile
- EA with 2% Ammonium hydroxide
- LC/MS/MS mobile phase: Methanol with 0.1% formic acid
- LC/MS/MS mobile phase: Water with 0.1% formic acid
- LC/MS/MS mobile phase: Water with 2% formic acid
- LC/MS/MS needle wash
- LC/MS/MS seal wash

i. It is noted that zolpidem carboxylic acid was not detected at any concentration when it was extracted in blood or urine. After further investigation, it was determined that the zolpidem metabolite does not extract and does not elute with any of the wash or elution steps. Chemically, this metabolite is acidic whereas other benzodiazepines are typically neutral or basic. As a result of using a buffer at pH 6.0±0.1 to prepare samples and the UCT clean screen DAU column, it is suspected that the zolpidem metabolite remains unionized. Therefore, the zolpidem metabolite is not expected to be retained on the column once loaded onto the sorbent. As a result, the zolpidem metabolite will require a separate extraction for the detection and confirmation of it which will have to be validated at a later date.

ii. Adinazolam showed occasional issues at a concentration of 5 µg/L in blood and urine matrices. While the requirements for confirmation at this concentration were met (ion ratios within 20% of target, retention time ±2%, signal to noise ≥3, and acceptable chromatography), during casework analysis it is recommended that a higher concentration be extracted to ensure reliable and better reproducibility to detect adinazolam. The concentration shall not exceed 15 µg/L to meet ANSI/ASB Standard 054, Section 8.2.4.

iii. Alpha hydroxy triazolam showed similar LOD issues to adinazolam on the Xevo TQD mass spectrometer but not on the Xevo TQ-S micro. As such, the concentration to be extracted may be greater than 5 µg/L but shall not exceed 15 µg/L.

iv. All benzodiazepines are qualitative when extracting from a urine matrix. Some exceptions are noted in i-iii, but benzodiazepines that are quantitative in blood will use the lowest non-zero calibrator (10 µg/L and 5 µg/L for high dose and low dose drugs respectively) as the LOD. All other benzodiazepines will have a LOD of 5 µg/L.

- Limit of Quantitation (LOQ): This was assessed using a decision point concentration and simultaneously with calibration model, bias and precision. The LOQ will be the lowest concentration that achieves acceptable predefined detection, identification, bias, and precision criteria in all three spiked samples over the minimum of three analytical runs using blank blood as the matrix. The lower limit of quantitation (LLOQ) will be 10 µg/L for high dose benzodiazepines (i.e. lowest non-zero calibrator) and 5 µg/L for low dose benzodiazepines. This meets ANSI/ASB Standard 036, Sections 8.8.2 and 8.8.3 in addition to meeting or exceeding the minimal requirements set forth in ANSI/ASB Standards 119, 120 and 121 which list sensitivity requirements for some quantitative benzodiazepines. Table 6 in Supplementary Document One summarizes calibration, weighting, quantitative range and LOD/LLOQ of quantitative benzodiazepines.

- Carryover: It is important that carryover is addressed during validation because this can affect accuracy, bias, precision, and casework. Casework has the potential to have high analyte concentrations which could lead to carryover in subsequent analysis. For the LC/MS/MS, not only are solvent blanks required to check for carryover, but these blanks inherently wash the entire system. In addition, a post injection wash of 6 seconds is used to wash the needle between each injection. The solvent used is referred to as the needle wash and contains equal parts acetonitrile, methanol, isopropanol, and water (all LC/MS grade) with 0.1% formic acid. It is not expected that the injection wash time will need to be adjusted but it may be increased if carryover issues arise and are not resolved by other means (i.e. service visit)

    i. For high dose drugs, 5 µL stock standard (1 mg/mL) was added to 2 mL 40:60 MeOH: H_2O for an approximate concentration of 2500 µg/L for the carryover concentration. For low dose drugs, 2 µL stock standard (1 mg/mL) was added to 2 mL 40:60 MeOH: H_2O for an approximate concentration of 1000 µg/L. A-OH-etizolam, 7-aminoflunitrazepam and 7-amino clonazepam standards, whose stock concentrations were 100 µg/mL, were diluted by combining 10 µL of stock and added to 2 mL 40:60 MeOH: H_2O for an approximate concentration of 500 µg/L. These carryover samples were analyzed in triplicate with solvent blanks and blank blood following each carryover sample. No carryover was observed in the solvent blank, blank blood or blank urine. For novel benzodiazepines that have an LOD of 5 µg/L, the carryover concentration was 50 µg/L. No carryover was observed in the solvent blank or the blank matrices. Per TXPM 4.9.2.7, "Case sample results that are affected by carryover will not be reported until the carryover issue is resolved. If the carryover is detected in the preceding solvent blank, see TXPM 4.9.2.6 Solvent Blanks. If the potential carryover is detected in the case sample, the case sample will be reinjected with a preceding solvent blank. If the analyte is still present, the sample is inconclusive and will be re-extracted and reanalyzed if there is sufficient sample volume. The original injection and all subsequent injections will be included in the case record. The data ultimately used for reporting the substance must be clearly identified."

- Interferences:

    i. Matrix: Human blood, synthetic blood, human volunteer urine and synthetic urine were analyzed for the presence of interferences. Of the ten different unique sources of human blood or synthetic blood, no interferences were detected. Of the ten different unique sources of human volunteer urine and synthetic urine, no interferences were detected. The

human blood lots were: P210609, Q210609, O210609, N190730, R110317, S220317, T220317, and U220317
The synthetic blood lots were E45793 and E45351.
The human volunteer urine lots were A220816, B220816, C220816, D220817, E220818, F220818, G220817, H220817, I220818, and J220820.
The synthetic urine lots were D100621A, G090622A and T081822A.

ii. Stable isotope internal standards: Deuterated internal standards occasionally have nondeuterated analytes and therefore were evaluated at the minimum with one blank sample per matrix type. No un-deuterated analytes were integrated in the negative controls in blood and urine. Per TXPM 4.9.2.5, "For qualitative and quantitative methods, acceptable analytical results for negative controls are no analyte(s) of interest detected. If an analyte is integrated in the negative control at the default threshold area, the analyte's threshold area may be adjusted to be <50% of the LLOQ's analyte area response. If the analyte's area response is ≥50% of that seen in the LLOQ, then that calibrator must be dropped and a new LLOQ established. If analyte(s) are detected in negative controls, the unknowns must be re-analyzed."
iii. Other commonly encountered analytes: Fentanyl, norfentanyl, codeine, hydrocodone, oxycodone, oxymorphone, morphine, hydromorphone, 6-MAM, EME, COC, CE, BE, Amph, Meth, EPH, pseudoephedrine, MDA, MDMA, phentermine, amobarbital, butalbital, caffeine, carbamazepine, carisoprodol, glutethimide, meprobamate, pentobarbital, phenobarbital, phenytoin, secobarbital, topiramate, amitriptyline, chlorpheniramine, citalopram, cotinine, cyclobenzaprine, dextromethorphan, diphenhydramine, doxylamine, methadone metabolite (EDDP), fluoxetine, gabapentin, lamotrigine, lidocaine, meperidine, methadone, nicotine, PCP, sertraline, tramadol, trazodone, venlafaxine were extracted using this method and assessed as potential interferences for the analytes of interest (quantitative and qualitative) in blood and urine. No interferences from these other commonly encountered analytes were detected.
iv. High analyte concentrations: At times high analyte concentrations with relevant amounts of unlabeled analyte ions appear as isotopically labeled compound fragments which could impact quantitation or confirmation of analytes. The high positive control spiking solution (Lot# G031722B), which contains drug concentrations of 850 µg/L and 200 µg/L for high dose and low dose benzodiazepines respectively, showed that unlabeled analyte ions do not appear as isotopically labeled analytes except for clonazepam. For clonazepam, it is noted in this experiment that d4-clonazepam is integrated in the chromatogram window, has appropriate peak shape but has an ion ratio that is not acceptable. For the novel benzodiazepines, spiking solutions T031722C and T011022E, at a

concentration of 100 µg/L, were used to evaluate this potential interference. D5-estazolam, D5-oxazepam, and D7-zolpidem do not have interfering analyte ions present for these novel benzodiazepines at a concentration of 100 µg/L.

- Ion suppression/enhancement: Quantitative and qualitative analytes showed ion suppression and/or enhancement. Most analytes showed some ion suppression which has the greatest potential impact on LOD and LLOQ. Zopiclone, zaleplon, 7-aminoclonazepam, bromazepam, flunitrazepam, nimetazepam, norchlordiazepoxide, and pyrazolam showed ion suppression or enhancement greater than 25%. The LOD and/or LLOQ were evaluated for these analytes in nine sources of blank blood. Based on all the experiments performed in this validation, the LOD and LLOQ were largely unaffected. All other analytes did not exceed 25% ion suppression/enhancement and this further evaluation was not needed. Analytes extracted in urine are likely to be enhanced in the urine matrix. LC/MS/MS post column infusion of low and high analyte concentrations met the requirements for this experiment but post extraction addition of low and high analyte concentration for this assessment was also utilized. Both approaches are valid. Table 7 in Supplementary Document One summarizes this estimation of ion suppression/enhancement in the blood matrix. Post column infusion data is saved electronically within the unit quality files (all analytes in urine evaluated on 9/12/2022, 8-aminoclonazolam, a-OH-clonazolam, bromazolam, a-OH-flubromazolam, and adinazolam in blood performed on 9/23/2022).
- Stability: Circumstances may arise in which processed samples cannot be analyzed within a reasonable amount of time due to atypical events such as system check failures, or loss of power. Thus, analyte stability is important to evaluate so that samples are maintained before they undergo unacceptable changes. Analytes are considered unstable when the peak ratios (peak area of analyte to internal standard) exceed ±20% as represented by the error bars on the stability graphs in Supplementary Document One. Stability was evaluated for all analytes but is only required for quantitative analytes per ANSI/ASB standard 036 section 9.3.
    i. For blood processed samples, quantitative analytes had stability up to seven days post extraction. Several qualitative analytes had stability issues after day one. See the table below with all analytes' stability in blood.
        1. It is important to note some analytes had 1 replicate of 3 show poor peak response. This caused the average peak response to deviate greater than 20%. Analytes on the following day occasionally fell within the 20% again. The first time the average peak response exceeded 20%, the analyte was considered unstable.
        2. Qualitative analytes may be analyzed past their respective stability days. If the quality control requirements for confirmation are met, then the analyte may be reported. If the analyte is not detected after

the stability, it is up to analyst discretion to set up for re-analysis but is recommended or report as inconclusive.

ii. For urine processed samples, similar stability for all analytes was observed. Since blood stability of qualitative analytes were shorter than the quantitative analytes, this stability experiment was shortened to two days after extraction for time simplicity.
    1. See table 1 below for stabilities of analytes in urine.
    2. Since all analytes are qualitative only in urine, they may be analyzed after their respective stability days and confirmed if the quality control requirements are met. If the requirements are not met after the stability date, then report as INC or re-analyze if the sample allows.

iii. While the stability of qualitative analytes is an issue for this new method, the stability of the quantitative analytes met validation criteria.
    1. Quantitative analytes may be showing more stability because the peak response is calculated using a matching deuterated internal standard vs an un-deuterated internal standard.
    2. Qualitative analytes were assigned an internal standard so that the data is normalized to an extent. If qualitative analytes are to become quantitative at some point, they should be evaluated with their deuterated counterparts. Stability should be re-evaluated at that point.

Table 1. Analytes' stabilities in blood and urine

Drug/metabolite	Stability in Blood (days)	Stability in Urine (days)
7-Aminoclonazepam	7	1
Alprazolam	7	2
Clonazepam	7	2
Diazepam	7	2
Etizolam	7	2
Lorazepam	7	2
Nordiazepam	7	2
Oxazepam	7	2
Temazepam	7	2
Zolpidem	7	2
a-OH-alprazolam	7	2
2-OH-ethylflurazepam	6	2
Bromazepam	6	2

Bromazolam	1	2
Chlordiazepoxide	6	2
Norchlordiazepoxide	6	2
Clobazam	2	2
Clonazolam	2	2
8-aminoclonazolam	2	1
a-OH-clonazolam	1	2
Delorazepam	6	2
Demoxepam	6	2
Diclazepam	1	2
Estazolam	6	2
Flualprazolam	1	2
Flubromazepam	6	2
Flubromazolam	6	2
A-OH-flubromazolam	2	2
Flurazepam	1	2
Lormetazepam	3	2
Nimetazepam	6	2
Nitrazepam	6	2
Phenazepam	1	2
Pyrazolam	6	1
Zaleplon	6	2
Zopiclone	2	1
7-aminoflun	3	1
Flunitrazepam	7	2
Midazolam	7	2
Triazolam	7	2
A-OH-etizolam	7	2
A-OH-triazolam	7	2
Adinazolam	1	2

- Dilution integrity:
    i. Dilution of a sample is important because on occasion, high analyte concentrations above the calibration range can lead to inaccurate results. Additionally, low sample volume is a common occurrence and dilution of the sample allows for the analysis to continue. To assess dilution integrity, bias and within-run precision studies similar to those described in section 8.2 of ANSI/ASB standard 036 were performed at 1:1, 1:4, and 1:9 dilution ratios. Undiluted samples were prepared and analyzed concurrently so that the calculated concentrations of the diluted samples can be compared to undiluted samples. For low dose benzodiazepines, target concentrations were 15, 30, 75, and 150 µg/L. High dose benzodiazepine target concentrations were 50, 100, 250 and 500 µg/L. Bias was calculated for each analyte at the four different concentrations. Combined within-run precision was calculated using ANOVA as described in section 8.2.2.3.4 of ANSI/ASB standard 036. In addition, diluted concentrations were multiplied by their respective dilution factors so that these concentrations can be compared to undiluted sample concentrations. The acceptable concentration is ±20% of the target concentrations. The bias and within-run precision results are summarized in table 8 of Supplementary Document One. Undiluted and dilution results are summarized in tables 9 through 19 in Supplementary Document One.
        1. Quantitative results may not be reported for etizolam in diluted samples because bias exceeded ±20% at all three dilution ratios and diluted samples produced lower concentrations of etizolam when compared to the undiluted results. It is important to note that with-in and between-run bias met the ±20% requirement.
        2. At dilution ratios 1:1 and 1:4 for alprazolam, alpha-hydroxy alprazolam, 7-aminoclonazepam, clonazepam, diazepam, lorazepam, nordiazepam, oxazepam, temazepam, and zolpidem, the bias and within-run precision were less than 20%. In addition, when comparing diluted results to undiluted results, acceptable concentrations were obtained for these analytes. The diluted samples had acceptable bias and within-run precision calculations which meets the minimum requirements in section 8.2.2.3.4 of ANSI/ASB standard 036, but occasionally, the dilution concentration was below the acceptable target concentration. Therefore, its target concentration once multiplied by its respective dilution factor was below the acceptable range too. These values were still incorporated into bias and within-run statistical evaluations which justified the acceptance of these dilution ratios for those analytes but supports a recommendation to include dilution controls when casework is analyzed. The dilution control

will have to meet bias requirements before reporting diluted sample results.

3. At the dilution ratio 1:9 for alprazolam, alpha-hydroxy alprazolam, 7-amino clonazepam, diazepam, nordiazepam, oxazepam, and zolpidem, the bias and within-run precision requirements (±20% of target concentration) were met. Comparing diluted sample results to undiluted results met the acceptable criteria with few exceptions. However, a couple of the dilution concentrations exceeded the ±20% requirement (dilution target concentration and concentration once multiplied by the dilution factor). These results were still included in bias and precision calculations which met the requirement of ±20%. This supports that dilution controls should be analyzed concurrently if samples are diluted. Dilution controls must pass quality control requirements before reporting diluted sample results.
4. Dilution of clonazepam, lorazepam and temazepam using the highest dilution (1:9) produced similar results as stated above. Bias and within-run precision were within acceptable limits, often below 10%. However, several dilutions were below the acceptable range (±20%) for the dilution concentration and undiluted concentration. Therefore, this would be under-reporting the calculated concentration present in unknown samples. While not a requirement in dilution integrity experiments, between-run precision was evaluated too. Except for clonazepam which has a between-run precision of 21.35%, lorazepam and temazepam has a between-run precision less than 20%. Undiluted calculated results fell within the target concentrations ±20% and it is noted that analytical runs three, four and five had the unacceptable diluted results. Based on the historical values that are seen in casework and the calibration range of these analytes it is expected that a 1:9 dilution will be an infrequent occurrence. However, if a 1:9 dilution is required, it is recommended that an undiluted and 1:9 dilution control will be analyzed simultaneously which is allowable per section 9.5 of ANSI/ASB standard 054. Acceptable results of the undiluted and dilution control are those stated in TXPM 3.9 and TXPM 4. In addition, undiluted control results must be within ±20% of target concentration and the diluted control results must be within ±20% of target concentration of the undiluted control once multiplied by the dilution factor. Once these requirements are met, unknowns may be quantitatively evaluated at a dilution.

- Recovery:
    i. This experiment was originally called “extraction efficiency” but is routinely referred to as “recovery” in the toxicology literature. While this is not required per the ANSI/ASB 036 standard, it serves as a check to ensure that analytes of interest are extracted sufficiently for confirmation and quantitation. In addition, recovery need not be 100% to be considered sufficient. For this comparison, only quantitative analytes were considered and not qualitative analytes. Neat calibrators and positive controls were analyzed and compared to post extraction addition of calibrators and positive controls. Another comparison of neat calibrators and positive controls were compared to an average of six replicates of extracted calibrators and positive controls from the dilution integrity experiments. As seen in the graphs 258-260 in Supplementary Document One, extracted, neat, and post extraction addition of calibrators and positive controls, had recovery above 80%. Comparing them to each other, most have a percent difference below 10%. However, some calibrators or positive controls have a percent difference between 10% and 20%. Based on the totality of the experiments in this validation, this recovery is sufficient for confirmation and quantitation of these analytes.
- Parallel studies: This is not required for initial validation of a new method per ANSI/ASB 036 standard but is highly encouraged because it helps ensure repeatability, reproducibility and robustness of a method. These parallel studies were conducted concurrently with competency samples.
    i. The first parallel study was using two identical liquid chromatography columns of same length, inner diameter, particle size, and bonded phase but from different manufactured lots (BEH C18 2.1 x 100 mm, 1.7 µm). One column (lot # 0249343251) was primarily used for validation. The second column (lot #0427321461) was used for parallel studies.
    ii. The second set of parallel studies was using two different liquid chromatographs and mass spectrometers. A Waters Acquity H-Class UPLC coupled with a Xevo TQD mass spectrometer was primarily used for validation. This instrument uses a quaternary solvent manager that allows up to four different mobile phases to be run simultaneously. The second instrument was a Waters Acquity I-Class UPLC coupled with a Xevo TQs-micro mass spectrometer. This instrument uses a binary solvent manager that allows only two different mobile phases to run simultaneously. In addition, the two mass spectrometers are slightly different. It is reported that the Xevo TQ-s micro has increased sensitivity because the sampling cone is different and there is a step wave. The step wave is a series of lens that focuses ions better compared to the Xevo TQD.
    iii. A third set of parallel studies was inherently performed because of two different instruments is slightly different mobile phases. The TQD uses

pure methanol (LC/MS grade), water (LC/MS grade) and 2% formic acid in water for separation. The TQs-micro mobile phases are 0.1% formic acid in methanol (LC/MS grade) and 0.1% formic acid in water.

iv. A fourth set of parallel studies consisted of analyzing College of American Pathologists (CAP) samples from their Forensic Toxicology, Criminalistics program. These samples were analyzed on GC/MS using the current procedure and on LC/MS/MS using this new procedure. Both sets of results met acceptability according to CAP and both methods produced similar results. On GC/MS, CAP sample 2019 FTC-02 contained 7-amino clonazepam and clonazepam. 7-aminoclonazepam is qualitative only on GC/MS but a separate extraction for the quantitation of clonazepam was performed as required in our current procedure. As a result, clonazepam would have a reportable concentration of 23 µg/L. This sample extracted by this new method confirmed the presence of 7-amino clonazepam and clonazepam with reportable concentrations of 83 and 18, respectively. CAP acceptable results are 77.06 to 143.12 µg/L for 7-aminoclonazepam and 14.13 – 26.23 µg/L for clonazepam. For urine sample comparison, CAP sample 2019 FTC-04 was analyzed by GC/MS and LC/MS/MS using the current and new procedures respectively. In both procedures, 7-amino flunitrazepam was confirmed.

v. All parallel studies showed that repeatability, reproducibility, and robustness exist with the new method. Calibration, bias, precision, LOD, and LOQ are unaffected by changing liquid chromatography columns, solvent managers, mass spectrometers, and gradients.

vi. Madison Crime Lab had two Waters Acquity I-Class UPLC coupled with Xevo TQs-micro mass spectrometers installed in January of 2023. Additional parallel studies will be conducted with those instruments and will serve as the performance evaluation of these new instruments. The evaluation will serve as the demonstration of suitability of this new benzodiazepine method for use in that lab. Please see the supplementary validation summary containing these results.

- Competency samples:
    i. Analyzing proficiency samples from the College of American Pathologists (CAP) serves as an evaluation of the effects of this new method compared to a previously validated method. It ensures accurate results from the old method are comparable to the new method and serves as a competency test for toxicologists that were not part of the validation team. Seven different proficiency samples were tested by four different analysts. Some analysts had to analyze the same samples to ensure each analyst was tested for the extraction of blood, urine, and hydrolysis of urines. The benzodiazepines that were in the six different samples included zolpidem, 7-amino clonazepam, clonazepam, lorazepam, 7-amino flunitrazepam, alprazolam, and alpha-hydroxy alprazolam. The

seventh sample contained no benzodiazepines. A summary of each CAP sample, the analyst assigned, target concentrations of benzodiazepines, and acceptable results can be found in table 23 of Supplementary Document One. For an analyst to pass their competency test, their results had to include the correct confirmation of any benzodiazepines found within the sample and the calculated concentration of the unknown must have fallen between the acceptable range set by CAP. Tables 24 and 25 of Supplementary Document One is a summary of the competency assignments, the expected results, acceptable limits of benzodiazepine concentrations if any, and analyst results of their sample. All analysts that analyzed a competency sample passed and are deemed competent in this method for analysis of benzodiazepines. This shows the method is fit for its intended purpose and serves as a viable method for confirmation and/or quantitation of benzodiazepines that may be present in blood or urine.

- Enzyme Hydrolysis:
    i. Enzymatic hydrolysis of urine specimens should be performed to determine the total drug concentration prior to extraction. Hydrolysis is a process in which the conjugate bond is broken to allow the freed moiety (glucuronide) to be detected in urine specimens. Using Kura Biotech BGTurbo high efficiency Recombinant beta glucuronidase and an oxazepam glucuronide positive control, it was demonstrated that this enzyme hydrolysis procedure does free the glucuronide moiety from oxazepam and the unbound oxazepam is confirmed in the urine specimens. This confirmation allows an individual to assess unknown specimens for the presence of other unbound drugs that may be detected in urine.
- Recommendations:
    i. The purpose of this section is to include information that is useful to know when performing LC/MS/MS analysis. Ultra-high purity water is susceptible to spoilage and/or contamination. Routine maintenance on the water purification system is highly recommended so that contaminants do not build up in the instrument and cause issues. It is highly recommended that ultra-high purity water, that is freshly dispensed, be used for extraction and mobile phase preparations. At the minimum, 10% organic should be used to help reduce water spoilage. For example, the seal wash contains 10% acetonitrile and 90% water. Fresh preparations are typically better than using solvent that has been sitting out at room temperature. Water with an acid modifier (i.e. formic acid) helps prevent spoilage too.
    ii. Preparation of reagents to be used on the instrument (mobile phases, seal wash, and needle wash) should be prepared in dedicated liquid-

chromatograph glassware. This glassware shall not be washed with any detergent. It is only to be rinsed with LC grade organic solvents or ultra-high purity water. From time to time, it is good practice to rinse solvent bottles prior to preparing fresh solvents in the glassware to be used. The needle wash which contains equal parts water, IPA, acetonitrile, methanol and 0.1% formic acid is a good rinse solvent but several washes with fresh ultra-high purity water afterwards is recommended. It is also good practice to rinse the glassware with some of the freshly prepared solvent and dispose of that rinse; then proceed with the transfer of the remaining fresh solvent. It is good practice that serological glass pipets are used to prepare mobile phases since it is possible that concentrated acid may leach plasticizers from plastic pipette tips which would be transferred onto the instrument.

iii. Column Care and Use manuals provided by Waters Inc. are also available online and are filled with information including pH limits, temperature limits, column equilibration etc. It is important to note that column cleaning, regenerating and storage is crucial to maintain column performance. Changes in peak shape, peak splitting, shoulders on the peak, shifts in retention time, change in resolution, or increasing backpressure may indicate contamination of the column. Back flushing with neat organic solvent (i.e. methanol) is usually sufficient to remove the contaminant. Back flushing is performed by reversing the orientation of the column to not push the contaminant through the column and potentially into the mass spectrometer. Increasing column temperature increases cleaning efficiency. For periods longer than four days at room temperature, store reversed phase columns in 100% acetonitrile. For applications that use column temperatures above room temperature, store immediately after use in 100% acetonitrile for the best column lifetimes. Currently the H-class UPLC is flushed with ultra-high purity water for 10 minutes and acetonitrile for 10 minutes and is saved as the “shutdown” method to be run at the end of each sample batch when no other samples are to be run. The I-Class UPLC uses a shutdown method that utilizes 50:50 H_2O:ACN first because the water is not replaced as frequently as the H-class. Then the column is flushed with 100% acetonitrile for 10 minutes. Shutdown method runs for 20 minutes total.

iv. Check the column to ensure it is the correct column installed on the instrument for your analysis before starting the instrument up (priming, equilibration, gas flow, electronics). If you start up the instrument and notice you have the wrong column, prior to removing the column you should run a shutdown to flush the column of the mobile phase. See above about column care. It is encouraged that documentation near the instrument or computer includes the column basic information for each analysis type. Future validations may require more specialized columns to

achieve the desired sensitivity. However, the validation team should consider the benefits and drawbacks of the use of such specialized columns (cost benefit analysis).

v. Column eCord technology developed by Waters Inc. helps to track usage of the column. Be sure the metallic magnet end is snuggly fit on the column manager so that eCord information is downloaded and stored on the instrument software. It is unknown the maximum injections a column will handle before requiring replacement. Column lifetime is application specific but use of a guard column, storage in 100% acetonitrile and injecting clean samples aids in the column's longevity.

vi. When you need to shut down the mass spectrometer, double check the Waters Inc. procedure that you are reading. Often times, a reset of the mass spectrometer's electronics are sufficient and can be accomplished by pressing the tiny button with a paper clip or similar object located on the front of the MS above the ESI probe. The larger button (bigger than a thumbprint), located to the left of the reset button, is the power button. If the power button is pressed the MS vents to atmosphere as noticed by the sudden slowing down of the mechanical pump. If vented, it is highly recommended that the instrument be allowed to pump down and stay under vacuum for a minimum of one day. A performance check and sensitivity check should be conducted again before casework is to be analyzed.

vii. Check the solvent bottles before your analysis and ensure there is enough remaining solvent. Enough remaining solvent means the solvent line will remain above the solvent bottle filters. A quick and easy calculation to perform is take the flow rate of your method in mL/min and multiply by the gradient run time in minutes. Then multiply by the number of injections to get the total volume of solvent consumption that is to be expected. This is a conservative calculation since it is assumed that you are running just one solvent (i.e. 100%) when you rarely use just one solvent (i.e. an isocratic elution). This helps prevent solvent bottles from running "dry" which can cause pump head/seal damage which can cascade to other issues. In addition, the inlet pressure limits should be set up to have a low and high limit (500 psi and 15,000 psi, respectively)

    1. Example: Flow rate is 0.4 mL/min multiplied by a 9-minute gradient is 3.6 mL per injection. If you have 50 injections, you need approximately (50*3.6) 180 milliliters of each solvent.

viii. Changing solvent bottle filters helps reduce buildup of contamination and maintains optimal filtering capabilities. Annual replacement is recommended by Waters Inc. and is not part of annual preventative maintenance that is conducted by a Waters technician.

ix. There is no set time frame for how often sampling cones and gas nozzles should be switched to clean ones. Spare and clean ones should always be

on hand in case one needs to be replaced. In general, if you are seeing the sensitivity decrease in your performance check or sensitivity check, it may indicate sampling cone/gas nozzle are dirty. Other times, it may be sample issues.

1. To swap sample cones and gas nozzle for clean ones, be sure to close the isolation valve on the front part of the MS orifice before removing the sample cone and gas nozzle. If you swap without closing the isolation valve, atmosphere/air will be sucked into the MS. It is highly recommended the MS is cleaned by a Water's technician.

x. Caffeine has better stability when prepared in methanol as compared to pure water. It is unknown how long caffeine is stable in methanol for the system check but currently there is a one-year expiration date. Stability of caffeine in methanol in the sample manager which has a set point of 4 °C, seems to be similar to when stock solution is stored in the refrigerator.

xi. Guard columns or sometimes called pre-columns are useful and help protect your analytical column from matrix. Guard columns are relatively inexpensive compared to analytical columns. There is no set time frame for when to change guard columns and has not been determined. This will change over time as more analysis is executed on the LC/MS. However, if a higher backpressure on the solvent manager exists after equilibration of the column and guard column, you may need to backflush or clean the column or change the guard column. It is wise to have spare guard columns at the lab too. Inline filter units have been used in the past but only filter the mobile phase and do not really protect the analytical column as samples are injected.

xii. RMS noise calculation should be used for the system check and validations. RMS noise calculation was used for buprenorphine method, and it is assumed that is why the system check parameters for noise calculation is set where it is at. In addition, no ANSI/ASB standards specifically state how you should be calculating the noise, but it should be done consistently and if possible, through the software and not manually.

xiii. Phosphate buffer stored in a clear glass container at room temperature may be suitable for GC/MS analysis. However, phosphate buffer should be refrigerated for LC/MS/MS analysis. During the validation, it was documented that the phosphate buffer had some unknown solid material. Some possibilities include precipitates, dust particles, and microbial growth. This warranted further investigation and a brief root cause analysis was conducted. Historically, the expiration date of the phosphate buffer has been two years after the day of preparation. After discussion with others, it was determined that this expiration date was not experimentally documented to anyone's knowledge. In addition, phosphate buffer is used in multiple extractions and is consumed faster

with GC/MS analysis. The investigation moved to searching the literature to see if there were good practices to put in place. The most notable practice that was documented in the Colorado Bureau of Investigation (CBI) Toxicology operation manuals and United Chemical Technologies (UCT) Clinical and Forensic Applications manual was the use of refrigeration. CBI expiration dates of a prepared reagent was consistent with current practices, but UCT noted that storage should be at 5°C in glass with a stability of one month and a daily inspection for contamination. After discussion with this new information about storage conditions of phosphate buffer, a short study was conducted. Two lots of freshly prepared phosphate buffer were stored under refrigeration conditions (approx. 5°C). The buffer was stored in a glass container and capped with a plastic cap which is the standard practice that is currently used for this buffer. Approximately each day, one lot of the buffer was removed from the refrigerator and warmed to room temperature to simulate a real-world scenario. This buffer was inspected for each occurrence. The second lot was left in the refrigerator for the duration of this study and inspected daily. It was documented that after two weeks under refrigeration or removing the buffer from the refrigerator, solid material was observed. It is noted that inspection took place using the naked eye without additional light sources too. Based on this study it was best to set an expiration date at two weeks and refrigerate because fresh buffer is better. Smaller amounts of phosphate buffer should be prepared, stored in a glass container, and capped to limit waste. This two-week expiration date and storage conditions were set for the 1M acetic acid: acetonitrile as well, for simplicity. This made sense because acid and organic solvent helps prevent the spoilage of pure water. Through the validation, it demonstrated that the new expiration date and refrigeration was suitable for both solvents. Cold buffer and/or acid wash is discouraged because it will negatively affect analyte detection. These solvents should be brought close to room temperature prior to use.

6. Include data summaries to allow the validation study to be evaluated by competent analysts;

The data generated during this validation is stored in the Unit Quality Records in the Milwaukee Toxicology Unit. Due to the large number of pages, it is not attached to this report. If there are summary tables, graphs etc. associated with an experiment in question five, they are referenced in that paragraph appropriately and correspond to Supplementary Document One.

7. Comparisons of the results to appropriate performance characteristics (see T'nO 7a.3.6 below);

Performance characteristics are discussed in number five above.

8. Any measurement uncertainty results, as applicable;

Measurement uncertainty for quantitative benzodiazepines using this new method was calculated following TXPM 4.7 Measurement Uncertainty. Below is a summary of the measurement uncertainty at each positive control concentration. Those highlighted will be the reported measurement uncertainty.

Drug/metabolite	Low positive control	Medium positive control	High positive control
7-amino clonazepam	16.0	13.0	9.5
Alprazolam	13.0	11.0	11.0
Clonazepam	15.0	12.0	9.6
Diazepam	14.0	13.0	8.6
Etizolam	19.0	16.0	14.0
Lorazepam	16.0	12.0	11.0
Nordiazepam	8.9	8.9	6.8
Oxazepam	14.0	14.0	18.0
Temazepam	15.0	15.0	12.0
Zolpidem	16.0	11.0	11.0
Alpha-hydroxy alprazolam	17.0	13.0	13.0

9. Summary of results and a recommendation to accept or reject this new or modified procedure based upon the validation results. Refer to the performance characteristics and other observations to justify this recommendation;

Based on the validation results, it is recommended that this method should be accepted since it is meeting and/or exceeding new requirements set forth by the ANSI/ASB standards.

10. Include the date of the validation report and the names of the validation team

The validation report was completed on March 10, 2023. The members of the validation team are listed below

X Jonathan T. Tomko

Jonathan T. Tomko
Toxicologist Senior

X

Alison L. Goetz
Toxicologist Entry

X Amy Rutgers

Amy L. Rutgers
Toxicologist Advanced

X Ashley Wheel

Ashley N. Wheeler
Toxicologist Senior

X

Leah J. Macans
Toxicologist Technical Lead

X

Eric D. Westhafer
Toxicologist Senior

Additional members of the Toxicology unit at Wisconsin Division of Forensic Sciences include:

X

Kelsey M. Jungbluth
Toxicologist Advanced

X

Stephanny Restituyo
Toxicologist Senior

X

Cassidy L. Scheppa
Toxicologist Senior