Validation Protocol for the Quantitation of Pain Management Medications in Human Urine using LC-MS/MS

July 25, 2021

Ver 1.9

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COBALT LAB SOLUTIONS

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Purpose: The purpose of this document is to describe the experiments and acceptance criteria involved in the bioanalytical assay validation using liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS).

Our intent is to describe how we perform assay validations. Since one of the overarching goals of Cobalt Lab Solutions is to provide you with tools to help make you more successful with your testing, we invite you to use this protocol or modify it in a way that suits your testing needs.

Background: LC-MS/MS has proven to be a powerful method for the direct quantitation of small molecules, such as medications) in biological matrices. In the backdrop of pain management, medication monitoring is a very important and powerful tool for detecting and measuring the amount of medication present in biological matrix, such as urine.

Pain management physicians use medication monitoring to monitor their patient's compliance with their prescribed medication list since pain management patients fall into a category of high-risk for prescription-drug abuse and it is a magnet for individuals demonstrating recreational drug-seeking behavior. Two problems predominate this high-risk group. First is the issue of addiction, where patients take medications that are not prescribed specifically for them, or they may also engage in consuming illicit medications. The second, more subtle issue is the potential for diversion of prescription medications. Diversion is revealed by urine drug testing as the repeated absence of a prescription medication that is still being sought by the patient. This problem occurs due to the high street-value of these medications. Patients procure the medication from a prescribing physician under false pretenses and then sell the medication, rather than taking it for therapeutic purposes.

Urine drug testing assists pain management doctors in clearly assessing their patient's drug-taking behavior and permitting the doctors to make informed decisions. Urine is the biological matrix of choice because these medications are often eliminated in urine at detection levels easily achievable by modern clinical tests. The most powerful detection method available is LC-MS/MS, providing both sensitivity and specificity that is not achievable with other available technologies, such as the enzyme immunoassay screen. LC-MS/MS has surpassed gas

chromatography combined with tandem mass spectrometry as the gold standard for urine drug testing.

In order for a bioanalytical assay to be deemed appropriate for use in a clinical laboratory, it must pass assay validation standards set forth by the FDA, CLIA, COLA, and CAP standards. This document describes the validation of a bioanalytical assay for the detection and quantitation of exogenous substances in human urine. During assay validation, assays are evaluated for the following parameters: assay dynamic range, limit of detection, sensitivity, specificity, matrix effects, matrix lot-to-lot, inter-day precision and accuracy, intra-day precision and accuracy, interfering substances/concomitant medications, linearity of dilution, and stability. Specificity is addressed during assay development.

Procedure Descriptions and Acceptance criteria

Matrix effects, Linearity, and ion suppression description: A biological matrix refers to the raw materials collected from a subject for the purpose off testing. Biological matrices are complex fluids or homogenized solids from which the drug targets are extracted prior to analysis. The most common methods for removing impurities are solid-phase extraction, liquid-liquid extraction, or dilution of matrix.

The purpose of sample preparation is to remove components of the biological matrix that are not relevant to the analytical method being developed. Mass spectrometers work by ionizing molecules introduced into the ionization source via the interface with the liquid chromatography instrument. Optimally, the only molecules introduced into the source would come from the analytes of interest, but sample preparation techniques inevitably also carry matrix components along with the analytical targets. These matrix components may be derived from the matrix themselves, such as salts, sugars, lipids, creatinine, protein, and other biological waste products in urine, or other drugs that have been eliminated along with the drug of interest in the patient's urine that are carried throughout the sample preparation procedure. These matrix components can interfere with the ionization of target molecules (drugs) in the source, resulting in ion suppression. Ion suppression manifests itself as a suppressed signal for the target analyte relative to the signal of that analyte in pure solvent (typically water).

When matrix effects are present, it is the best practice to use an isotopically-labeled internal standard that is structurally identical to the analytical target. Isotopically-labeled internal standards are considered the best control because they co-elute with the target analyte and are structurally identical, save for some isotopic changes (typically substitution of deuterium for hydrogen ions). Generally, a matrix effect that interferes with the ionization of a target analyte will also interfere with the ionization of the internal standard. The internal standard acts as a normalizing component for the signal and corrects for ion suppression caused by matrix effects.

Experimental design and acceptance criteria for the determination of Matrix effects: There are multiple ways to determine if matrix effects exist for a bioanalytical assay using LC-MS/MS. For our purposes, we either prepare

calibration curves or QC standards in both Optima-Grade water and in human matrix and run replicate calibration curves *or* QCs prepared and run in both water and urine.

For our purposes, we will prepare calibration curves in matrix and in water. Both calibration curves will be subjected to the normal specimen preparation process and they will both be acquired by the acquisition method that is being tested. Plots are created using the nominal value of the standards versus the area counts for each standard level, typically for three replicate calibration curves. If the slopes of the calibration curves created by the water standards deviated by less than ± 15% of the slope of the matrix curves, then no matrix effects were encountered. However, if the slopes of the curves deviated by greater than ± 15%, then matrix effects were deemed to exist.

In order to determine if the internal standard compensated for any matrix effects, internal standard-normalized area ratios (Area of analyte/Area of IS) of the standards prepared in matrix were compared to those prepared in water. Calibration curves are created from the determined concentration of the standards versus the nominal concentration of the standards. If the internal standard-normalized peak area ratios deviated by less than ± 15%, then internal standard compensated for the ion suppression. If the slopes of the curves deviated by greater than 1 ± 15% ($0.85 \leq m \leq 1.15$), then internal standard did not correct the problem of matrix effects, and the method will fail.

Matrix lot-to-lot comparison: Individual lots of urine matrix differ according to a person's overall health, hydration status, and renal health. Although drug-free human urine is used during validation, a single lot of urine is not enough to demonstrate the ruggedness of the assay system when such variability in the matrix exists. Due to this, and in accordance with current CAP standards, a minimum of 10 lots of human matrix were collected from donors who verify that they are not taking the analytes that are being validated.

Experimental design and acceptance criteria matrix lot-to-lot comparison: These donor urine samples were blinded as to the donor and were spiked at a

low-to-mid-level with each analyte. These samples were prepared and run according to the SOP. Acceptance criteria are that the quantitative value for each analyte must be within the precision requirement for the assay, ±15% of the analytical target concentration. Analytes falling outside of this range will not pass validation.

Recovery: It is common for patient samples to be subjected to extraction procedures such as solid-phase extraction or liquid-liquid extraction, prior to analysis by LC-MS/MS. When patient samples are subjected to a pre-analytical extraction process, the amount of analyte recovered from the preparative process should be assessed. In order to do this, samples of spiked biological matrix are extracted, or processed alongside blank, or unspiked biological matrix. The blank, extracted biological matrix is then spiked with analyte at a level equivalent to the pre-preparative spiked standards. The specimens are run and the recovery is expressed as a percentage as follows:

$$\left[\frac{Cpre}{Cpost}\right] \times 100\%$$

Where C_{pre} refers to the concentration recovered from the pre=preparative spike solution and C_{post} is the concentration recovered from the post-extraction spike solution.

In the case of dilution assays, the percentage recovery is considered to be 100% since no separation or extraction steps were performed.

Analytical measurement range: The analytical measurement range (AMR) of the assay refers to the concentration range that the assay is validated within. The AMR is determined by running a series of calibration curve standards covering a concentration range that encompass the concentration of analyte that one expects to find in patient samples; it must be relevant to the analyte target for the bioanalytical method. The limits of the AMR are bounded by the lower limit of

quantitation (LLOQ) and the upper limit of quantitation (ULOQ). The dynamic range may be described by a linear or quadratic fit.

Experimental design and acceptance criteria for the determination of analytical measurement range and linear range: Calibration curves were created using a minimum of six non-zero calibration points. Three replicates of each curve were created in matrix and subjected to the same preparative procedure that will be used for the final bioanalytical method. In order to be accepted as the AMR, all of the points describing the calibration curve must pass within ± 15% of the nominal concentration, except at the LLOQ where ± 20% is acceptable. Furthermore, the correlation coefficient R^2 for the calibration curve must be ≥ 0.99, or R should be ≥0.98 in order to be acceptable.

Verification of linear range: Once the assay dynamic range has been established, the range can be verified by running a series of three calibration curves in biological matrix. All three calibration curves will be prepared as per the sample preparation protocol. During the course of data acquisition, one calibration curve will be designated as the 'standard' curve and the other two will be acquired as analytes.

Experimental Design and acceptance criteria for verification of linear range:

Standard assay acceptance criteria will apply to the 'standard' calibration curve. In order to verify the linear range, the percent deviation of the two calibration curves designated as 'analytes' must be within ± 15% of the nominal value, except at the LLOQ where ±20% is acceptable.

During the course of the matrix effects experiment, the experimental design already exists wherein the first matrix curve is designated as the 'standard' curve and the two replicate curves are acquired as 'analytes'. Use this data to populate the spreadsheets for Verification of linear range.

Sensitivity: The sensitivity of the assay system refers to the ability to reliably produce a signal throughout the entire calibration range, but specifically at the

low-end of the calibration curve (the lower limit of quantitation, LLOQ). In hyphenated mass spec assays, a signal that produces a signal to noise ratio (S/N) of ≥10 is considered to be valid for the LLOQ of an assay system. Further, a S/N ratio of ≥5 is considered to be clear enough for the limit of detection.

Experimental design and acceptance criteria for the determination of sensitivity: Since the LLOQ is the lowest point on the calibration curve, we test the sensitivity of the assay system by injecting 10 replicates of the LLOQ and evaluating the resulting analytical determinations. Standard acceptance criteria of ±20% of nominal concentration apply.

Precision and Accuracy: The precision of a measurement system, related to reproducibility and repeatability, is the degree to which repeated measurements under unchanged conditions show the same results. Simply put, it is determined from how close replicate determinations are to each other, and is used to estimate the amount of random error in a system.

The statistical accuracy of a measurement system defines how close replicate determinations are to the actual target value. Accuracy is a determination of the amount of systematic error in the system.

Experimental design and acceptance criteria for the determination of inter-day and intra-day precision and accuracy:

Inter-day precision and accuracy were determined using six replicates of each of three quality control (QC) sample determinations from across five validation runs. Concentrations of the QC samples ranged across the curve, with the low QC set at approximately 3 times the LLOQ or less, the mid QC near the mid-range of the linear range of the curve, and the high QC set at 90% of the ULOQ.

Percent accuracy was determined for each individual measurement using the equation:

$$\left|\frac{Vd - Vn}{Vn}\right| \times 100\%$$

Where *Vd* is the concentration determined from the calibration curve and *Vn* is the nominal concentration for the QC standard.

Precision was determined for each standard level by first determining the standard deviation of the six replicate standards and then applying the following equation:

$$\frac{SDd}{Vm} * 100\%$$

Where *SDd* is the standard deviation of the six replicates and *Vm* is the mean value of the standard.

In order to be accepted, the precision and accuracy for the replicate determinations must be ≤15% at each level.

Intra-day precision and accuracy were determined using six replicates of each of three quality control (QC low, QC mid, and QC high) sample determinations from a single analytical run performed on separate days. Concentrations of the QC samples ranged across the curve, with the low QC set around 3 times the LLOQ, the mid QC near the mid-range of the linear range of the curve, and the high QC set at 90% of the ULOQ.

Percent accuracy was determined for each individual measurement using the equation:

$$\left|\frac{Vd - Vn}{Vm}\right| \times 100\%$$

Where *Vd* is the concentration determined from the calibration curve and *Vm* is the mean concentration for the QC standard.

Precision was determined for each standard level by first determining the standard deviation of the eighteen replicate standards and then applying the following equation:

$$\frac{SDd}{Vm}$$

Where *SDd* is the standard deviation of the six replicates and *Vm* is the mean value of the standard.

In order to be accepted, the precision and accuracy for the replicate determinations must be ≤15% at each level.

Specificity: Demonstration of the specificity of an assay is an essential part of the validation process. For the purpose of this document, we consider a demonstration of specificity to be a critical component of assay development insomuch as you should not proceed with assay validation if you have not demonstrated that your assay is specific for your analyte of interest.

Demonstration of specificity mandates that you have a unique signal for each analyte and internal standard. The signal for the analyte should be negative in the absence of analyte and positive in the presence of analyte. Further, the signal should be unique for your analyte and internal standard.

When demonstrating specificity for small molecules using LC-MS/MS, the signal that is generated is called a chromatogram and is bases on the MRM signal (parent ion > daughter ion transition) for the target analyte. In some cases, multiple MRM signals can be acquired for a single target molecule and an ion ratio may be calculated based on the area of the peak in the quantitation MRM channel relative to the peak area of the qualifying ion MRM channel.

A unique signal in LC-MS/MS is constituted by a combination of MRM transitions and peak retention time. The parameters that may be used to identify a peak are outlined below.

1. MRM transition of the intended analyte (quantitation trace). The transitions are described as the parent M/Z > daughter M/Z transition. A unique MRM may have a unique parent M/Z, a unique daughter M/Z, or both.

2. Retention time. The retention time of a compound is the amount of time post-injection that a peak takes to elute from a column.
    a. Isobaric compounds have identical MRM traces, and examples are found within pain management, notably codeine/hydrocodone,

morphine/hydromorphone, and Amobarbital/pentobarbital. When you are developing a drug panel that contains these compound pairs (or others), **the isobaric pairs MUST be separated chromatographically** (e.g. have a unique retention time). It is mandatory. Careful attention must be paid during the course of assay development and validation to insure a unique retention time for these, or any other isobaric pairs under development.

3. **Ion ratios.** When more than one MRM is generated for a target analyte, the retention times for both MRMs should be identical and the ion ratio for the pair may be used as an additional point of identification for your target analyte. It is the recommendation of the authors that a minimum of two MRMs are generated for each analyte and that the ion ratio is used for the identification of unknown peaks whenever the science and instrumentation permits.

   Recent changes in regulations require that the signal to noise ration of both the quantification trace and the qualifying ion trace be ≥10. On occasion, an analyte will not have a quantifying ion that passes this criteria while still permitting the quantification trace to remain in a meaningful range. These instances should be documented in the SOP or validation report.

   a. When it is not possible to put multiple qualifying ion MRM traces on all analytes, all effort should be made to utilize qualifying ions on analytes of significant interest. Included among these are analytes for which the presence and quantity of that analyte have significant importance. Examples of this in the pain management setting are heroin and cocaine metabolites (6-monoacetylmorphine and benzoylecgonine respectively), or any drug for which the presence of analyte could result in the dismissal of a patient from the practice (spice cannabinoids, bath salts, etc.).

The most helpful tool that you can make to help ensure that you have a unique signal is a table that is sorted by the M/Z of the parent compound. In the example

below, the morphine/hydromorphone and codeine/hydrocodone pairs are acceptable because the retention times are unique, although the MRMs are similar enough that some bleed may exist between channels. The Amobarbital/Pentobarbital pair is not acceptable because they have identically the same retention time and MRM transitions. Care should be taken to separate these two compounds with chromatography.

Example of a Specificity Table

Analyte name	Retention time	Parent M/Z	Daughter 1 M/Z	Daughter 2 M/Z
Amobarbital	1.43	255.1	182.0	---
Pentobarbital	1.43	255.1	182.0	---
Morphine	1.03	286.2	153.1	165.2
Hydromorphone	1.64	286.2	157.1	185.1
Codeine	2.55	300.2	214.9	165.1
Hydrocodone	2.66	300.2	128.0	171.1

Specificity experiments are performed in separate phases, two of which are done prior to validation, and the third is performed as part of the validation process.

1. Verify that there is no positive signal in the absence of analyte. Inject extracted, blank matrix and verify that no positive signal is seen for any analyte.

2. Verify that your analyte of interest generates a positive signal for only the MRM channel of interest and that all other channels are negative. Inject a high concentration of each analyte individually (5-10 μg/mL is acceptable). Make sure that the only analyte that the only channel that demonstrates a signal consistent with the MRM and retention time of interest is the channel for the target analyte.

3. The third phase involves testing possible interfering substances and is performed as part of the assay validation experiments (see below).

Exogenous interfering substances:

While it is not possible to test all of the prescription drugs that people take, there are several medications that are common over-the counter medications that should be evaluated for potential interference with the test system. Specifically, drugs that are known or suspected of interfering with similar bioanalytical systems should be evaluated to ensure that they do not suppress ionization or cause false-positive results for a given analyte. The following medications are typically evaluated for interference, but this list may vary according to your assay requirements:

Cyrillian part #	Name
O-0334	Over-the-counter mix
	Acetaminophen
	Ibuprofen
	Pseudoephedrine
	Caffeine
	Naproxen
S-019	Salicylic acid
D-013	Dextromethorphan
P-078	Phenylephrine
P-023	Phentermine
D-015	Diphenhydramine

Other pain management medications that are commonly assayed are also assessed for interference. For example, you can include the analyte mixture from an ES positive panel as a spike for the ES negative panels, and vice-versa.

Experimental design and acceptance criteria for concomitant medications and interfering substances: In order to assess the potential for a substance to cause ion suppression or create a false-positive signal, a high concentration of the

possible interfering drug is spiked into a low QC sample (typically 2,000 ng/mL or greater spiked into a 15 – 75 ng/mL low QC). Acceptance criteria for a substance to be deemed as non-interfering is that the quantitated value for the low QC should be within ± 15% of the nominal value. Furthermore, the spiked substance should not cause a false-positive or a false-negative result.

Note: Endogenous interfering substances are addressed in an addendum at the end of this document. *Performing the endogenous interfering substances as part of the initial validation depends on the specimen validity testing that will be performed to monitor these variables. Several of these such as pH, hematuria, turbidity, glycosuria, and proteinuria are likely to be commonly encountered and should be considered as part of the initial validation.*

Partial Volumes and Dilutions: Patient specimens may be diluted under one of two conditions: if there is insufficient sample volume to run the sample according to specification or if the identification of the analyte is passing all parameters and the nominal concentration of the analyte is higher than the upper limit of quantitation.

Experimental design and acceptance criteria for partial volumes and dilutions: A spiked solution was created at a level above the ULOQ, generally at 2X ULOQ. The sample is run at discrete dilutions, typically 1:5, 1:10, 1:20, and 1:50 dilutions. All specimens are diluted with blank matrix as the diluent. In order to pass, concentration determinations for all dilutions should be within ± 15% of the nominal value following correction for the dilution factor.

Carryover: Carryover is the presence of an analyte in a blank injection following a positive injection, resulting in a false-positive sample. The injection needle gets washed in-between samples with a needle wash solution that is intended to remove contamination from the surface of the needle. The efficiency of this process is monitored during validation by assessing carryover in following manner. Samples are injected in the following sequence:

High QC

Wash

Wash

High QC

Wash

Wash

High QC

Wash

Wash

Peak areas are integrated for both the analyte and internal standard. Peak area in the wash solutions should be 0.1 % or less of that found in the High QC standard. In addition the mean of the peak area in the three wash solutions following the high QC replicates should be less than 50% of the LLOQ being used for the assay.

Lab-to-lab correlation study: Lab-to-lab correlation studies are necessary to establish equivalence between methods in different laboratories. This type of study should also be performed every 6 months for non-regulated analytes that are not included in routine proficiency testing programs.

Study Design:

A combination of patient samples and spiked QC materials were prepared and run by at least two labs. The concentration and range of QC materials was selected based on the test menu, assay dynamic ranges, and cutoff values for each participating lab so that all analytes were covered and at least 1 negative and 2 positive QCs were represented for each analyte. QC samples were generated by spiking drug-free human urine (UTAK) with known concentrations of reference materials (Cerilliant). The analytes and their concentrations were not disclosed or discussed with participating laboratories. Study samples were processed as unknown patient specimens and the results from each laboratory were tabulated in Excel. Linear regression analysis was then performed to test how well the results for each analyte correlated between laboratories.

Acceptance Criteria:

Values calculated from patient samples and spiked QC material were plotted against the mean value obtained for participating laboratories. Since quantitative analysis is bound by the lower limit of quantitation (LLOQ or cutoff) and the upper limit of quantitation (ULOQ), values that were above the lowest represented ULOQ for a laboratory under evaluation were designated as the value of that ULOQ. Conversely, values that were below the lowest represented LLOQ for a laboratory were designated as the value of that LLOQ. In cases where only one or two laboratories tested for the represented analyte, calculated values for its quality controls were plotted against their nominal values. To statistically assess how well determined values from each laboratory for a given analyte correlated, the slope and coefficient of determination were calculated by plotting calculated values against the average value for all laboratories. The slope of the regressed line was calculated from the linear regression equation in Excel, where $y = mx + b$ and m is the slope of the line. A slope and correlation coefficient (R^2 value) of 1.0 indicates perfect agreement between compared values.

A slope for a specific analyte within ± 10% of 1.0 ($0.90 \leq m \leq 1.10$) is considered excellent, within ± 20% of 1.0 ($0.80 \leq m \leq 1.20$) is considered good, and within ± 30% of 1.0 ($0.7 \leq m \leq 1.3$) is considered acceptable. An analyte with a slope greater than 30% from 1.0 is considered unacceptable and failing. The rationale for accepting a value within ± 30% variation of 1.0 is that each lab has a 15% acceptable error that when compounded can reach 30%. In addition to the criteria for a failing slope, the coefficient of determination for the line (R^2) must be ≥ 0.85 for that analyte to be considered passing.

Pass / Fail Criteria Used to Score Each Analyte		
Parameter	**Score**	**Pass / Fail?**
$0.90 \leq m \leq 1.10$	Excellent	Pass
$0.80 \leq m \leq 1.20$	Good	Pass
$0.70 \leq m \leq 1.30$	Acceptable	Pass
$0.70 > m > 1.30$	Unacceptable / Failing	Fail
$m = 0$	Failing	Fail
$R^2 \geq 0.85$	Passing	Pass
$R^2 < 0.85$	Failing	Fail

Analytes with failing scores will be subjected to the following trouble-shooting procedures:

1. Review of raw data, processed data, and testing procedures. If all were acceptable, then
2. Repeat testing of the correlation sample. Did this resolve the issue? If not, then
3. Inject a diluted standard containing only the analyte of interest. Did you see a peak? If not, then
4. Cease testing of that analyte until trouble-shooting can be performed and the study repeated.

Stability

Post-preparative stability/ On-instrument Stability: In order to ascertain the post-preparative stability of patient samples for reinjection following an instrument malfunction or process interruption, we monitor the stability of prepared samples over time. An assay validation run is acquired on a Friday (day 0) and then allowed to sit over the weekend and the reinjected the following Monday (day 3), and then again after 7 days. Acceptance criteria are the same for the day 3 run as for the day 0 run.

Freeze-thaw stability: In order to determine if samples are stable for analysis after freeze-thaw cycles, three sets of QC standards (QC low, QC mid, and QC high) were subjected to a series of up to three freeze-thaw cycles. Acceptance criteria are that the determined concentrations for these standards are within ±15% of the nominal value for the QC standard.

Temperature stability: Three levels of QC standards are subjected to storage at room temperature (22 ˚C), refrigerator temperature (4-8 ˚C), and freezer temperature (-20 ˚C) for at least 24 h. Acceptance criteria are that the determined concentrations for these standards are within ±15% of the nominal value for the QC standard.

β-Glucuronidase Optimization: The human body metabolizes many drugs to more water-soluble versions by adding a glucuronide moiety to the drug. This glucuronidation step permits the drug to be readily filtered by the kidneys and eliminated in urine. Due to this metabolic process, the majority of drug that appears in human urine is found as a glucuronide conjugate. As part of the assay development process, a β-glucuronidase deconjugation step is included during sample preparation. In order to evaluate the efficiency of this reaction, glucuronide controls representing the drug classes involved in analysis are included with every run. Acceptance criteria are that the determined concentrations for these standards are within ±15% of the nominal value for the glucuronide control.

Optimization of any enzymatic steps should include

1. Buffer optimization
    a. pH, ionic strength
2. Incubation temperature optimization
3. Duration of incubation
4. Units of enzyme used

Do not forget to factor-in molecular weight changes of standards that are undergoing enzymatic modifications when preparing solutions (such as glucuronide controls) at a target concentration. For example:

Many pain management drugs are subject to conversion to a glucuronidated metabolite. Glucuronidation is the act of adding a glucose moiety to another compound, in this case a foreign substance, usually for the purpose of making it more water soluble. Glucuronidated drug conjugates often remain in circulation better than the parent drugs, and are subjected to renal elimination. Many pain management drugs are subjected to physiological glucuronidation and are eliminated in urine, and so they require conversion back to their parent drug for the purpose of testing for pain management compliance. Glucuronide controls are prepared and run with each analytical batch on the LC-MS/MS. These controls are central to verifying the performance of the β-glucuronidase deconjugation step that is part of the sample preparation process

Glucuronidated controls can be purchased from vendors such as Cerilliant. The concentration in solution refers to the concentration of the glucuronidated compound. If you want to prepare a glucuronide control that will have a specified final concentration, then you must determine the ratio of free drug to the glucuronidated conjugate in order to perform the calculation correctly. Please see the example below for Morphine-3-β-D-glucuronide.

Stock solutions are typically 100 μg/mL

Morphine – 285.34 g/mol

Morphine-3-β-D-glucuronide – 461.47 g/mol

Ratio – $\frac{285.34}{461.47} = 0.61832$

In order to prepare 9 mL of 4,500 ng/mL:

$$X\ mL\ \times \left(100\frac{\mu g}{mL}\right) \times 0.61832 = 9\ mL\ \times \left(4.5\frac{\mu g}{mL}\right)$$

$$X = Add\ 655\ \mu L\ of\ the\ 0.1\frac{mg}{mL} standard\ to\ a\ 9\ mL\ final\ volume\ with\ urine$$

Ratios (conversion factors) and part numbers

0.61832 for morphine-3-β-D-glucuronide: Cerilliant part number M-017
0.63113 for oxymorphone-3-β-D -glucuronide: Cerilliant part number O-030
0.61946 for oxazepam-glucuronide: Cerilliant part number O-023
0.661679 for COOH-THC glucuronide: Cerilliant part number O-038

Acceptance Criteria for All Bioanalytical Runs

i. Standard Curve

In order for a peak to be identified, it must contain all ion transitions and have correct retention time. If more than one MRM is used to identify a peak, then the ion ratio must be correct for the analyte. A minimum of six calibration standards must be used to define a curve. A single point may be excluded from the calibration curve, however, no two consecutive points should be removed from the curve. No values will be reported below the lowest acceptable calibration standard or above the highest acceptable calibration standard. Concentrations below the LLOQ will be resulted as a 'less than' value (e.g. <10 ng/mL) and values greater than the upper limit of quantitation will typically be resulted as a 'greater than' value (e.g. >10,000 ng/mL). Values greater than the upper limit of the calibration curve may also be diluted and re-acquired so that the value is within the assay dynamic range, typically upon request by the ordering physician.

Standard back-calculated values should be within ± 15% of the nominal concentration except at the lower limit of quantitation (LLOQ) where it can be within ± 20% for acceptance.

Standard curve coefficient of determination (r^2) should be a minimum of 0.990, or $r \geq 0.98$ for the curve to be accepted.

A. Relative retention time must be monitored for each analyte. Relative retention time is defined as the difference in retention time between the analyte of interest and its isotopically-labeled internal standard. Monitoring the relative retention time is a new requirement that will help to eliminate false-positives due to peaks that elute very close to the peak of interest, but might be misidentified.

B. The signal to noise ratio should be monitored for all MRM transitions including quantification traces and qualifying ions. The signal to noise ratio should be ≥10 across the dynamic range of the calibration curve.

C. The ion ratio is determined as the ratio between the quantifier ion and a qualifier ion. Ion ratio must be monitored and may be used to determine the identity of a peak that resembles the target analyte in retention time. If the determined ion ratio differs from the target ion ratio (determined from the standards) by >50%, then the unknown peak's identity can be called into question and it is likely that the peak is not the target analyte. Further, attention should be paid to trends with the ion ratio, does if trend lower at higher concentrations, for example. These trends may come into play for analytes with poor qualifier peaks.

ii. Quality Controls

Quality controls should fall within ± 15% of the nominal value to be accepted. In order for a run to be valid, two thirds of the controls must be in range at each QC level, and no two at the same concentration can be out of range.

iii. Unknown Samples

In order for an unknown peak to be identified, it must contain all ion transitions and have the correct retention time and ion ratio (when applicable) for the analyte in question. Samples with results less than the LLOQ will be reported as below limit of quantitation (BLQ). Samples with concentrations above the ULOQ will be reported as '> ULOQ value' . Specimens with concentrations above the ULOQ may be diluted and reanalyzed according to the dilution protocol if requested by the referring physician.

Daily Testing Matrix for Assay Validation

This testing matrix should walk you through all of the experiments required in order to fully validate a bioanalytical method according to current FDA guidelines. These experiments are designed to validate a method that has already been through some degree of assay development. This testing matrix is a suggestion, and may be modified to fit your needs. Before you get to the validation steps, you should have already completed the following during assay development:

1. All target analytes and internal standards should be tuned and MRMs should be determined for each analyte and internal standard.

2. Have your MS method programmed. Note the file names for the final conditions.

3. Determine your chromatographic conditions. The following parameters should be optimized to the analyst's satisfaction prior to commencement of validation runs:
    a. Mobile phase composition
    b. Column type
    c. Column length
    d. Column temperature
    e. Gradient

4. Your inlet (LC) settings should be programmed. Note the file names for the final conditions.

5. Sample preparation should already be worked out.
    a. Determine sample preparation method: solid-phase extraction, liquid-liquid extraction, or dilution.
    b. If sample preparation includes an enzymatic deconjugation step such as beta glucuronidase, then optimize:
        i. pH
        ii. Temperature
        iii. Enzyme concentration

iv. Duration of incubation

c. You should be able to demonstrate through the use of glucuronide controls (or similar) that the enzymatic step has been optimized.

6. You should have already determined the approximate linear range that your assay will cover. Advance work for all target analytes includes:
    a. Preparation of calibration curve standards with a sufficient number of data points (minimum 6) to cover your linear range.
    b. Preparation of appropriate QC standards
        i. QC Low – 3X LLOQ
        ii. QC Mid – Mid-point of curve
        iii. QC High – 90% of ULOQ

7. **Specificity should be demonstrated prior to assay validation.** We consider this to be part of assay development, but it is critical that the specificity of the assay is demonstrated as part of this process. Specificity should have been demonstrated by injecting each analyte and internal standard individually and showing a peak for each individual analyte. Further, you should have made a specificity table sorted by the retention time of each compound to demonstrate that no two analytes exist that have the same retention time and MRM transitions.

8. Prior to commencing assay validation, you will likely have run through sample preparation and detection several times and have fair confidence in the robustness of the analytical method.

It is best to start assay validation experiments on a Monday.

Monday	Matrix Effects/Ion suppression Day 1 Precision and Accuracy Intra-day Precision and Accuracy Linearity / Assay Dynamic range
Tuesday	Matrix lot-to-lot Day 2 Precision and Accuracy
Wednesday	Concomitant medications Day 3 Precision and Accuracy
Thursday	Linearity of Dilution Carryover Day 4 Precision and Accuracy
Friday	Freeze-thaw stability Day 5 Precision and Accuracy
Monday	On-instrument stability Temperature stability Day 6 Precision and Accuracy
Tuesday	7-day post-preparative stability
Wednesday	Lab-to-lab correlation study

Assay Validation Runs contain a minimum of the following*:

1	Wash	24	QC Low 4
2	Sys chk 1	25	QC Low 5
3	Wash	26	QC Low 6
4	Double Blank	27	QC Mid 1
5	Blank	28	QC Mid 2
6	H (Neg QC)	29	QC Mid 3
7	G - Calibration standard (LLOQ)	30	QC Mid 4
8	F - Calibration Standard	31	QC Mid 5
9	E - Calibration Standard	32	QC Mid 6
10	D - Calibration Standard	33	QC High 1
11	C - Calibration Standard	34	QC High 2
12	B - Calibration Standard	35	QC High 3
13	A - Calibration Standard (ULOQ)	36	QC High 4
14	Wash	37	QC High 5
15	LLOQ1	38	QC High 6
16	LLOQ2	39	Wash
17	LLOQ3	40	Morphine-3-β-D-glucuronide
18	LLOQ4	41	Oxymorphone-3-β-D-glucuronide
19	LLOQ5	42	Oxazepam-3-β-D-glucuronide
20	LLOQ6	43	Wash
21	QC Low 1	44	Sys chk 2
22	QC Low 2	45	Wash
23	QC Low 3		

*Select enzymatic reaction controls appropriately

Day 1: Matrix Effects, Linearity, and Ion Suppression

Experimental Design: Matrix effects are signal suppression or signal enhancement that are due to components of the biological matrix that are carried through the sample preparation process. Signal suppression or enhancement due to matrix effects can result in poorly quantitated results.

A standard validation run should be prepared as outlined above, with the following additions.

1. Prepare a calibration curve in water that it exactly the same composition of the calibration curve in matrix.

2. Run at least three replicates of each calibration curve (3-water curves and 3-matrix curves).

Acceptance Criteria For our purposes, we will prepare calibration curves in matrix and in water. Both sets of calibration curves will be subjected to the normal specimen preparation process and they will both be acquired by the acquisition method that is being tested. Plots are created using the nominal value of the standards versus the area counts for each standard level, typically for three replicate calibration curves. If the slopes of the calibration curves created by the water standards deviated by less than ± 15% of the slope of the matrix curves, then no matrix effects were encountered. However, if the slopes of the curves deviated by greater than ± 15%, then matrix effects were deemed to exist.

In order to determine if the internal standard compensated for any matrix effects, internal standard-normalized area ratios (Area of analyte/Area of IS) of the standards prepared in matrix were compared to those prepared in water. Calibration curves are created from the determined concentration of the standards versus the nominal concentration of the standards. If the internal standard-normalized peak area ratios deviated by less than ± 15%, then internal standard compensated for the ion suppression. If the slopes of the curves

deviated by greater than ± 15%, then internal standard did not correct the problem of matrix effects, and the method will fail.

In the case of method failure due to matrix effects, we suggest re-visiting your chromatography and sample preparation techniques to eliminate or minimize matrix effects.

The Matrix Effects and Linearity Batch Queue Should look like this

1	Wash	34	QC High 2	67	C - water curve 3
2	Sys chk 1	35	QC High 3	68	B - water curve 3
3	Wash	36	QC High 4	69	A - water curve 3
4	Double Blank	37	QC High 5	70	Wash
5	Blank	38	QC High 6	71	H - matrix curve 1
6	H (Neg QC)	39	Wash	72	G - matrix curve 1
7	G - Calibration standard (LLOQ)	40	Morphine-3-β-D-glucuronide	73	F - matrix curve 1
8	F - Calibration Standard	41	Oxymorphone-3-β-D-glucuronide	74	E - matrix curve 1
9	E - Calibration Standard	42	Oxazepam-3-β-D-glucuronide	75	D - matrix curve 1
10	D - Calibration Standard	43	Wash	76	C -matrix curve 1
11	C - Calibration Standard	44	H - water curve 1	77	B - matrix curve 1
12	B - Calibration Standard	45	G - water curve 1	78	A - matrix curve 1
13	A - Calibration Standard (ULOQ)	46	F - water curve 1	79	Wash
14	Wash	47	E - water curve 1	80	H -matrix curve 2
15	LLOQ1	48	D - water curve 1	81	G - matrix curve 2
16	LLOQ2	49	C - water curve 1	82	F - matrix curve 2
17	LLOQ3	50	B - water curve 1	83	E - matrix curve 2
18	LLOQ4	51	A - water curve 1	84	D - matrix curve 2
19	LLOQ5	52	Wash	85	C - matrix curve 2
20	LLOQ6	53	H - water curve 2	86	B - matrix curve 2
21	QC Low 1	54	G - water curve 2	87	A - matrix curve 2
22	QC Low 2	55	F - water curve 2	88	Wash
23	QC Low 3	56	E - water curve 2	89	H - matrix curve 3
24	QC Low 4	57	D - water curve 2	90	G - matrix curve 3
25	QC Low 5	58	C - water curve 2	91	F - matrix curve 3
26	QC Low 6	59	B - water curve 2	92	E - matrix curve 3
27	QC Mid 1	60	A - water curve 2	93	D - matrix curve 3
28	QC Mid 2	61	Wash	94	C - matrix curve 3
29	QC Mid 3	62	H - water curve 3	95	B - matrix curve 3
30	QC Mid 4	63	G - water curve 3	96	Wash
31	QC Mid 5	64	F - water curve 3	97	Sys chk 2
32	QC Mid 6	65	E - water curve 3	98	Wash
33	QC High 1	66	D - water curve 3		

Day 2: Matrix lot-to-lot comparison Individual lots of urine matrix differ according to a person's overall health, hydration status, and renal health. Although drug-free human urine is used during validation, a single lot of urine is not enough to demonstrate the ruggedness of the assay system when such variability in the matrix exists. Due to this, and in accordance with current CAP standards, a minimum of 10 lots of human matrix were collected from donors who verify that they are not taking the analytes that are being validated.

Experimental design and acceptance criteria matrix lot-to-lot comparison: Drug-free donor urine samples were blinded as to the donor and were spiked at a low-to-mid-level with each analyte. These samples were prepared and run according to the SOP.

Acceptance Criteria: Acceptance criteria are that the quantitative value for each analyte must be within the precision requirement for the assay, ±15% of the analytical target concentration. Analytes falling outside of this range will not pass validation.

The Matrix Lot-to-Lot Comparison Should be Similar to This

1	Wash	17	QC Low 3	33	Wash 4
2	Syschk 1	18	QC Low 4	34	Matrix 1
3	Wash 2	19	QC Low 5	35	Matrix 2
4	Double blank	20	QC Low 6	36	Matrix 3
5	Blank	21	QC Mid 1	37	Matrix 4
6	Neg QC	22	QC Mid 2	38	Matrix 5
7	Std G	23	QC Mid 3	39	Matrix 6
8	Std F	24	QC Mid 4	40	Matrix 7
9	Std E	25	QC Mid 5	41	Matrix 8
10	Std D	26	QC Mid 6	42	Matrix 9
11	Std C	27	QC High 1	43	Matrix 10
12	Std B	28	QC High 2	44	Wash 5
13	Std A	29	QC High 3	45	Syschk 2
14	Wash 3	30	QC High 4	46	Wash 6
15	QC Low 1	31	QC High 5		
16	QC Low 2	32	QC High 6		

Day 3: Concomitant Medications/ Interfering substances

Experimental: A standard validation run should be prepared as outlined above, with the following additions. The

1. Prepare one additional set of three replicate Low QC samples (three each of the low QC). Spike them at a volume of 10% with Methanol (e.g. if your sample volume is 50 μL, then spike them with 5 μL of methanol; if your sample volume is 200 μL, then spike them with 20 μL of methanol).
    a. Extract these and run as per your normal protocol. Label these as methanol-spiked QCs.

2. Procure the following common medications from Cerilliant. Alternatively, you can use medications of your choice that are appropriate to your patient population. Similarly, lipid, hemoglobin, and bilirubin standards can be prepared for interfering substances in plasma samples to simulate icterus, lipemia, and hemolytic conditions.
    a. Dextromethorphan – D-013 1 mg/mL
    b. Phenylephrine – P-078 1 mg/mL
    c. Diphenhydramine – D-015 1 mg/mL
    d. Salicylic Acid – S-019 1 mg/mL
    e. Phentermine – P-023 1 mg/mL
    f. Over-the counter mix – O-034 100 mg/mL, each component. Contains a mixture of the following drugs:
        i. Acetaminophen
        ii. Caffeine
        iii. Chlorpheniramine
        iv. Ibuprofen
        v. Naproxen
        vi. Pseudoephedrine
    g. Comcomitant medications. When developing multiple drug panels, there are some medications that can be easily accessed and tested. We recommend also preparing spike solutions containing pain management meds that are not included in the current assay

(Perhaps ES- drugs as a spike for ES+) for analysis as possible interfering substances.

3. Dilute each drug or mixture listed in step 2, above to a final concentration of 20 μg/mL in methanol (use a 1:50 dilution for the 1 mg/mL standards, and a 1:5 dilution for the 100 μg/mL standard). You will use these solutions to spike low QC standards at a level of 1:10. Therefore, the final concentration of concomitant medication will be 2 μg/mL in the final preparation.

4. Prepare three replicates of low QC standard spiked with a single interfering substance. Prepare samples and run as per your normal protocol.

Acceptance Criteria: In order to be accepted as 'no interference', the low QC values obtained in the presence of the spike solution should be within ±15% of the values obtained with the methanol spike solutions. If the determined concentrations are outside of this range, then the validation experiment fails and the analyst should note any positive interference or negative interference on the assay validation report.

A mean determined value of ≥ 15% indicates that the interfering substance may cause a false-positive result. In this case, the analyst may need to identify MRM transitions that do not result in the interfering signal, or re-visit the chromatography for the assay so that the positive interference does not result in false-positive results.

A mean determined value of ≤ 15% indicates that the interfering substance may cause a false-negative result, most likely due to ion suppression. This would be an unusual circumstance and would likely be corrected by the isotopically-labeled internal standard. In this case, the analyst may need to re-visit the chromatography for the assay so that the suppressing interference does not result in false-positive results.

Keep the plate from Day 2. Place it in the refrigerator following the acquisition. The samples will be re-acquired on Day 9 – 1 week stability.

The batch sequence for this run is below:

Day 3. The Concomitant Medications Batch Queue Should look like this

1	Wash	23	QC low 3	45	QC low - MeOH 2
2	Sys Check	24	QC low 4	46	QC low - MeOH 3
3	Wash	25	QC low 5	47	Wash
4	Dbl Blank	26	QC low 6	48	QC low - Dextromethorphan 1
5	Blank	27	QC mid 1	49	QC low - Dextromethorphan 2
6	H - Neg QC	28	QC mid 2	50	QC low - Dextromethorphan 3
7	G	29	QC mid 3	51	QC low - Phenylephrine 1
8	F	30	QC mid 4	52	QC low - Phenylephrine 2
9	E	31	QC mid 5	53	QC low - Phenylephrine 3
10	D	32	QC mid 6	54	QC low - Diphenhydramine 1
11	C	33	QC high 1	55	QC low - Diphenhydramine 2
12	B	34	QC high 2	56	QC low - Diphenhydramine 3
13	A	35	QC high 3	57	QC low- Salicylic acid 1
14	Wash	36	QC high 4	58	QC low- Salicylic acid 2
15	LLOQ (Matrix) 1	37	QC high 5	59	QC low- Salicylic acid 3
16	LLOQ (Matrix) 2	38	QC high 6	60	QC low - OTC mix 1
17	LLOQ (Matrix) 3	39	Wash	61	QC low - OTC mix 2
18	LLOQ (Matrix) 4	40	Morphine-3-β-D-glucuronide	62	QC low - OTC mix 3
19	LLOQ (Matrix) 5	41	Oxymorphone-3-β-D-glucuronide	63	Wash
20	LLOQ (Matrix) 6	42	Oxazepam-3-β-D-glucuronide	64	Sys Check
21	QC low 1	43	Wash	65	Wash
22	QC low 2	44	QC low - MeOH 1		

Day 4: Linearity of Dilution and Carryover Assessment

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. Prepare a solution that is two to four times higher as the ULOQ. This solution should be prepared in biological matrix.

2. Prepare dilutions of 1:5, 1:10, 1:20, and 1:50 using blank matrix as the diluent.

3. Prepare a normal validation run, as described above, but then include three replicates of each dilution. Prepare these diluted samples and run as per your normal protocol.

4. Program the dilution factor into your sample queue for all diluted samples. This will permit the analytical software to calculate the undiluted concentration using the calculated concentration times the dilution factor. Dilution factors are as follows:

Dilution prepared	Dilution factor
1:5	5
1:10	10
1:20	20
1:50	50

5. In order to assess carryover, prepare three additional high calibration standards (ULOQ). Inject the standards in the following sequence:
   High Standard (ULOQ)
   Wash
   Wash
   High Standard (ULOQ)
   Wash
   Wash
   High Standard (ULOQ)

Wash
Wash

The batch sequence for Day 3, linearity of dilution and carryover is outlined below.

Acceptance criteria: For this run, the acceptance criteria are the same for the calibration curve and QC samples as for a typical analytical run. Additionally, the back-calculated concentrations of each dilution should fall within ± 15% of the target value.

If the dilutions fail this criteria, then dilutions of that size are not permitted for patient samples and this should be noted in the assay validation report.

Day 4. The Dilution linearity and Carryover Batch Queue Should look like this

1	Wash	23	QC low 3	45	1:5 - 2
2	Sys Check	24	QC low 4	46	1:5 - 3
3	Wash	25	QC low 5	47	1:10 - 1
4	Dbl Blank	26	QC low 6	48	1:10 - 2
5	Blank	27	QC mid 1	49	1:10 - 3
6	H - Neg QC	28	QC mid 2	50	1:20 - 1
7	G	29	QC mid 3	51	1:20 - 2
8	F	30	QC mid 4	52	1:20 - 3
9	E	31	QC mid 5	53	1:50 - 1
10	D	32	QC mid 6	54	1:50 - 2
11	C	33	QC high 1	55	1:50 - 3
12	B	34	QC high 2	56	Wash
13	A	35	QC high 3	57	Std A (ULOQ)
14	Wash	36	QC high 4	58	Wash
15	LLOQ (Matrix) 1	37	QC high 5	59	Std A (ULOQ)
16	LLOQ (Matrix) 2	38	QC high 6	60	Wash
17	LLOQ (Matrix) 3	39	Wash	61	Std A (ULOQ)
18	LLOQ (Matrix) 4	40	Morphine-3-β-D-glucuronide	62	Wash
19	LLOQ (Matrix) 5	41	Oxymorphone-3-β-D-glucuronide	63	Sys Check
20	LLOQ (Matrix) 6	42	Oxazepam-3-β-D-glucuronide	64	Wash
21	QC low 1	43	Wash		
22	QC low 2	44	1:5 - 1		

Day 5: Freeze-Thaw stability

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. Set aside enough QC material (typically 9 aliquots of each QC level) to prepare three replicate samples of each QC.
2. Label one set of three replicate samples as '1', label the second set of three replicate samples as '2', and label the third set of three replicate samples as '3'.
3. Place all of the QCs in the freezer for at least 1 hr, or long enough to freeze.
4. Remove the two sets of three replicates of the QC material labeled '2' and '3', and allow to thaw at room temperature.
5. Place sets '2' and '3' back in the freezer for at least an hour, or long enough to freeze.
6. Remove the set labeled '3' and allow to thaw at room temperature.
7. Place the set labeled '3' back in the freezer for at least an hour, or long enough to freeze.
8. Remove all three sets from the freezer and allow to thaw at room temperature.
9. Mix all samples by vortexing to ensure that the samples have note separated into layers.
10. Prepare and run each aliquot as you would a normal patient sample.

The batch sequence for Day 4, freeze-thaw stability is outlined below.

Acceptance criteria: In order to pass, each QC level must have a determined mean that is within ± 15% of the nominal value. If the experiment fails, then this must be annotated in the assay validation report.

Day 5. The Freeze-Thaw stability Batch Queue Should look like this

1	Wash	26	QC low 6	51	QC High 1-2
2	Sys Check	27	QC mid 1	52	QC High 1-3
3	Wash	28	QC mid 2	53	Wash
4	Dbl Blank	29	QC mid 3	54	QC Low 2-1
5	Blank	30	QC mid 4	55	QC Low 2-2
6	H - Neg QC	31	QC mid 5	56	QC Low 2-3
7	G	32	QC mid 6	57	QC Mid 2-1
8	F	33	QC high 1	58	QC Mid 2-2
9	E	34	QC high 2	59	QC Mid 2-3
10	D	35	QC high 3	60	QC High 2-1
11	C	36	QC high 4	61	QC High 2-2
12	B	37	QC high 5	62	QC High 2-3
13	A	38	QC high 6	63	Wash
14	Wash	39	Wash	64	QC Low 3-1
15	LLOQ 1	40	Morphine-3-β-D-glucuronide	65	QC Low 3-2
16	LLOQ 2	41	Oxymorphone-3-β-D-glucuronide	66	QC Low 3-3
17	LLOQ 3	42	Oxazepam-3-β-D-glucuronide	67	QC Mid 3-1
18	LLOQ 4	43	Wash	68	QC Mid 3-2
19	LLOQ 5	44	QC Low 1-1	69	QC Mid 3-3
20	LLOQ 6	45	QC Low 1-2	70	QC High 3-1
21	QC low 1	46	QC Low 1-3	71	QC High 3-2
22	QC low 2	47	QC Mid 1-1	72	QC High 3-3
23	QC low 3	48	QC Mid 1-2	73	Wash
24	QC low 4	49	QC Mid 1-3	74	Sys Chk
25	QC low 5	50	QC High 1-1	75	Wash

At the beginning of day 5, place triplicate sets of QCs (three each of the low, mid, and high QC standards) at each of the following temperatures:

1. **Triplicate sets in the freezer**
2. **Triplicate sets in the refrigerator**
3. **Triplicate sets at room temperature**

Use these QCs for experiments on day 6.

Day 6: Temperature stability

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. Prepare and run the triplicate sets of QC standards stored at each of three temperatures (room temperature, refrigerator temperature, and freezer temperature) according to your normal protocol. The batch sequence for Day 5, temperature stability is outlined below.

Acceptance criteria: In order to pass, each QC level must have a determined mean that is within ± 15% of the nominal value. If the experiment fails, then this must be annotated in the assay validation report.

Day 6. The Temperature Stability Batch Queue Should look like this

1	Wash	26	QC low 6	51	QC High RT-2
2	Sys Check	27	QC mid 1	52	QC High RT-3
3	Wash	28	QC mid 2	53	Wash
4	Dbl Blank	29	QC mid 3	54	QC Low 4C-1
5	Blank	30	QC mid 4	55	QC Low 4C-2
6	H - Neg QC	31	QC mid 5	56	QC Low 4C-3
7	G	32	QC mid 6	57	QC Mid 4C-1
8	F	33	QC high 1	58	QC Mid 4C-2
9	E	34	QC high 2	59	QC Mid 4C-3
10	D	35	QC high 3	60	QC High 4C-1
11	C	36	QC high 4	61	QC High 4C-2
12	B	37	QC high 5	62	QC High 4C-3
13	A	38	QC high 6	63	Wash
14	Wash	39	Wash	64	QC Low -20C-1
15	LLOQ 1	40	Morphine-3-β-D-glucuronide	65	QC Low -20C-2
16	LLOQ 2	41	Oxymorphone-3-β-D-glucuron	66	QC Low -20C-3
17	LLOQ 3	42	Oxazepam-3-β-D-glucuronide	67	QC Mid -20C-1
18	LLOQ 4	43	Wash	68	QC Mid -20C-2
19	LLOQ 5	44	QC Low RT-1	69	QC Mid -20C-3
20	LLOQ 6	45	QC Low RT-2	70	QC High -20C-1
21	QC low 1	46	QC Low RT-3	71	QC High -20C-2
22	QC low 2	47	QC Mid RT-1	72	QC High -20C-3
23	QC low 3	48	QC Mid RT-2	73	Wash
24	QC low 4	49	QC Mid RT-3	74	Sys chk
25	QC low 5	50	QC High RT-1	75	Wash

Day 6: On-instrument stability. On-instrument stability is designed to simulate the condition in which an instrument error on a Friday afternoon results on the termination of the acquisition sequence and the acquisition is re-started on Monday.

Experimental: Leave the plate from Friday afternoon in the autosampler over the weekend. Monday morning, re-acquire the calibration curve and QC samples.

Acceptance criteria: Acceptance criteria are the same for this run as for a normal analytical run.

Day 7: Week 1 post-preparative stability. Remove the plate from Day 2 from the refrigerator and reacquire the calibration curve and QC samples.

Experimental: Remove the plate from Day 2 from the refrigerator and reacquire the calibration curve, QC standards, and glucuronide controls.

Acceptance criteria: Acceptance criteria are the same for this run as for a normal analytical run.

Day 8: Lab-to-lab correlation study: A minimum of 20 samples comprised of patient samples and spiked QC materials covering the entire test menu should be run by the laboratory validating the new assay and a partner laboratory. We recommend splitting the samples between a minimum of three labs, so if there is disparity between labs, it will be clear which lab had a problem. Further, patient samples will allow you to test the entire process, including the glucuronide deconjugation step, while the spiked QC materials will reveal how accurate and specific the method is. Furthermore, it is extremely difficult to find patient

samples to cover the entire test menu, so spiked materials are good for this purpose.

Acceptance Criteria:

Values calculated from patient samples and spiked QC material were plotted against the mean value obtained for participating laboratories. Since quantitative analysis is bound by the lower limit of quantitation (LLOQ or cutoff) and the upper limit of quantitation (ULOQ), values that were above the lowest represented ULOQ for a laboratory under evaluation were designated as the value of that ULOQ. Conversely, values that were below the lowest represented LLOQ for a laboratory were designated as the value of that LLOQ. In cases where only one or two laboratories tested for the represented analyte, calculated values for its quality controls were plotted against their nominal values. To statistically assess how well determined values from each laboratory for a given analyte correlated, the slope and coefficient of determination were calculated by plotting calculated values against the average value for all laboratories. The slope of the regressed line was calculated from the linear regression equation in Excel, where $y = mx + b$ and m is the slope of the line. A slope and correlation coefficient (R^2 value) of 1.0 indicates perfect agreement between compared values.

A slope for a specific analyte within ± 10% of 1.0 ($0.90 \leq m \leq 1.10$) is considered excellent, within ± 20% of 1.0 ($0.80 \leq m \leq 1.20$) is considered good, and within ± 30% of 1.0 ($0.7 \leq m \leq 1.3$) is considered acceptable. An analyte with a slope greater than 30% from 1.0 is considered unacceptable and failing. The rationale for accepting a value within ± 30% variation of 1.0 is that each lab has a 15% acceptable error that when compounded can reach 30%. In addition to the criteria for a failing slope, the coefficient of determination for the line (R^2) must be ≥ 0.85 for that analyte to be considered passing.

Pass / Fail Criteria Used to Score Each Analyte		
Parameter	**Score**	**Pass / Fail?**
$0.90 \leq m \leq 1.10$	Excellent	Pass
$0.80 \leq m \leq 1.20$	Good	Pass
$0.70 \leq m \leq 1.30$	Acceptable	Pass

$0.70 > m > 1.30$	Unacceptable / Failing	Fail
$m = 0$	Failing	Fail
$R^2 \geq 0.85$	Passing	Pass
$R^2 < 0.85$	Failing	Fail

Analytes with failing scores will be subjected to the following trouble-shooting procedures:

1. Review of raw data, processed data, and testing procedures. If all were acceptable, then
2. Repeat testing of the correlation sample. Did this resolve the issue? If not, then
3. Inject a diluted standard containing only the analyte of interest. Did you see a peak? If not, then
4. Cease testing of that analyte until trouble-shooting can be performed and the study repeated.

Validation Report Contents

This document outlines the appropriate sections that should be present in an LC-MS/MS bioanalytical assay validation.

Once you have completed the initial assay validation, prepare your validation reports. All validation reports should contain a minimum of the following sections:

1. **General Information**
    a. Lab name, test menu, and dynamic ranges
    b. Instrument models and serial numbers
    c. Validation dates, validation scientist information, and lab director signature page.

2. **Instrument settings**
    a. Table of MRM transitions, settings, retention times (MS settings)
    b. Inlet settings (LC)
        i. Mobile phases
        ii. Column type, length, part number, vendor
        iii. Chromatography method (gradient details)
        iv. Column temperature
        v. Autosampler conditions
            1. Loop Volume
            2. Injection volume
            3. Temperature

3. **SOP** outlining how specimens were prepared during validation

4. **Assay Validation SOP** outlining experiments performed for validation

5. **Specificity** – Demonstrate a single peak per single analyte injection for every analyte. Include your specificity table and chromatograms in this section.

6. **Analytical Measurement Range** – Demonstrate a linear response between expected vs measured concentrations on the calibration curve. Usually involves triplicate calibration curves in matrix from matrix effects day of validation data.

7. **Linearity** is confirmed throughout the calibrated range of the curve by running one curve as the 'Standard' curve, and the other two curves as analytes. Determined concentrations should be within ±15% of nominal (target).

8. **Matrix effects** – Look for matrix effects by running water curves vs calibration curves prepared in matrix.
    a. Plot peak areas across the curves vs concentration. If the slopes of the two lines vary by >15%, then matrix effects are present and the assay is limited to the biological matrix that it is validated in.
    b. Prepare a second plot of determined concentration (IS corrected) vs nominal concentration. If the slopes of the two lines are within ±15%, then matrix effects are corrected by utilization of isotopically-labeled internal standard.

9. **Matrix lot-to-lot variability** – Compares different lots of spiked matrix for matrix effects that may be overlooked by preparing all standard materials from a single lot of matrix. CAP requires a minimum of 10 lots of matrix. We recommend challenging the system with matrices exhibiting obvious differences, eg turbidity, flocculation, blood, etc.

10. **Inter-day precision and accuracy** – determined with replicates of QC standards across 5 days of validation.

11. **Intra-day precision and accuracy –** determined with replicates of QC standards within a single run (single validation run, n=6 replicates).

12. **Concomitant medications and interfering substances** – spike low QCs with high concentrations of common interfering substances (pain management meds, OTC meds). Demonstrate that no false-positive or false negative values are obtained by low QC values passing within specifications (±15% of target).

13. **Linearity of dilution -** Create spike standards that are at least 2X the upper limit of quantitation. Demonstrate the ability to dilute these standards and still hit the target concentration within ±15% (typically 1:2, 1:5, 1:10, 1:20, and 1:50 dilutions prepared with blank matrix).

14. **Carryover** – Demonstrate that carryover has been assessed and minimized by injecting the following pattern three times:
    a. High QC
    b. Wash
    c. Wash

    The amount of signal in the washes following a high QC standard should be ≤ (50% of the LLOQ peak area).

15. **Post-preparative stability** – 3-day post-preparative on-instrument stability.

16. **Freeze-thaw stability** – QCs through 3 freeze-thaw cycles.

17. **LLOQ determinations** – Plot replicate injections of the LLOQ across 5 days of validation runs. Values should be within ±20% of nominal.

18. **Sample stability** – Store QC standards (in matrix) under the same conditions as your patient samples. Suggest refrigerated, frozen, and room temperature. Run them daily until a single analyte fails two consecutive runs. The date of the first failing run is the time-limit for storage of samples under those conditions.

19. **Further long-term stability testing** should be performed in order to determine the stability of calibration curve and QC standards under your lab's storage conditions.

Stability Addendum

Assessment of the Two-Week Stability of Patient Samples

If patient samples can be procured that contain all of the analytes in your test menu, then use patient samples. It is often the case that patient samples are not available to cover the entire test menu, so QC materials often suffice for these studies.

Patient samples, spiked QC materials, or a combination of both are stored at room temperature, in the refrigerator (2°-8°C), and in the freezer (≤-10°C). The initial validation typically tests them for up to a week. For this experiment, the samples should be stored for up to 2 weeks and then prepared in triplicate as per your SOP. Acceptance criteria are the same as QC for your analytical run. =

Assessment of light exposure

In the process of urine drug testing, patient samples are collected at a location remote to the lab, typically a medical clinic or collection site, and then prepared for shipment to the receiving laboratory. The laboratory has no control over either the amount of time that the specimens are held, or the conditions under which they are held (typically refrigerated or room temperature).

The following experiment was designed in order to test the stability of analytes tested in this laboratory under the conditions that specimens are likely to encounter at a clinical site. For the sake of this experiment, QC standards prepared in matrix are used to simulate the condition of patient samples when stored under refrigerator or room temperature conditions for up to two weeks.

Since specimens may be exposed to light during this timeframe, a set of light-exposure QC samples may also be assessed at room temperature.

Materials required: QC standards prepared in matrix.

On day 0, place triplicate sets of QCs (three each of the low, mid, and high QC standards) at each of the following temperatures:

1. Triplicate sets in the refrigerator

2. Triplicate sets at room temperature in an opaque container
3. For light exposure, a third set of samples may be kept at room temperature in a container or rack that permits exposure to ambient light, and assayed at the same timepoints as the stability samples.

Day 0: Run QC samples as per your normal protocol (3 replicates each of low, mid, and high QC).

Days 7, 10, and 14: Run QC samples from both storage temperatures, refrigerated and frozen, as per your normal protocol (3 replicates each of low, mid, and high QC). Add the specimens stored in the dark, if desired, for light exposure stability.

Acceptance criteria: At the end of the 14-day study, calculate the mean concentration of each QC standard level for each day of the study. If the concentration of an analyte exceeds -15% difference from day 0, then it will be considered to be degraded. However, if all QC levels are within ±15% of the day 0 values, then they are considered to be stable for 14 days.

The light exposure samples can be compared to the dark, room-temperature exposure samples for an assessment of the effects of light on analyte stability. When the percent difference of the means of these groups of QC standards is compared, a % Difference within ±15% indicates no degradation due to light exposure.

Long-term stability: In order to assess the stability of standards for prolonged periods in storage, they should be periodically assessed. Prepare enough QC standards to perform long-term stability studies. The recommended schedule for testing is as follows:

1-month
2-month
3-month
6-month
9-month

12-month

At each of these timepoints, a fresh calibration curve should be prepared and run as per the normal analytical protocol.

Acceptance criteria: Acceptance criteria are the same for this run as for a normal analytical run. If QCs fail during this time, then samples will be considered stable through the previous passing timepoint.

Shipment Stability

Urine specimens are typically collected at a remote clinical site and then shipped via overnight courier service to the laboratory. The ability of the sample to withstand the rigors of the shipping process should be assessed. During the process of shipping, the samples may experience extremes of temperature, humidity, and light exposure. Furthermore, specimens shipped on a Friday may not be delivered until the following Monday, so care must be taken to assess the quality of the samples at 1 and 3-day timepoints.

QC materials prepared in urine will behave as a surrogate for patient samples.

Elevated temperature: A location should be established that simulates the elevated temperature environment that is likely to be encountered when shipping samples in the summertime. The back of a delivery truck in August is likely to experience temperatures in excess of 120°F (~50°C). In order to assess the stability of specimens subjected to this extreme of temperature, care should be taken to identify a water bath or incubator capable of maintaining the high temperature.

Obtain 6 aliquots of each of three levels of QC sample (6-low QC, mid QC, and high QC). Label one set of 3 aliquots of each level as 24 hours, and the second set of three aliquots as 72 hours. Place both sets of samples in the incubator or water bath. Assay a third set of three each of the low, mid, and high QC samples that have not experienced the temperature extreme on Day 0.

At 24 hour and 72 hour timepoints, remove one set of QC samples from the incubator and perform the assay using your normal protocol.

Once all of the data has been collected, tabulate the results at each level of QC. Determine the mean concentration for each level on each day (Days 0, 1, and 3). If the mean is within ±15% of nominal then no degradation has occurred for that analyte. However if the mean is greater than -15% different from nominal, then analyte degradation has occurred.

Humidity: if an incubator and a water bath are both available, then stability can be performed with specimens under both low and high-humidity conditions. This experiment may not be required, though as urine is aqueous by nature and the sealed containers that contain the specimen do not permit the ambient humidity to contact the patient samples.

Container stability: There are several vendors available for specimen collection devices. Even when the materials that comprise a container are the same, polypropylene plastic, for example, the finishing steps for the plastic or glass can change the affinity of an analyte to bind to the material. Each individual container that a specimen comes in contact with (primary vessel, aliquot tube, etc) should be tested for the ability to recover analyte. Typically, QC material is added to the sample container and allowed to sit for at least as long as a typical sample would sit prior to testing. The QC material recovered from the container or vessel should be prepared according to the SOP and the recovery should be within ±15% of the target value.

Interfering Substances Addendum

Interfering substances are those that occur naturally or may be added to patient samples. In urine these include, but are not limited to, pH, hematuria, turbidity, high nitrite, mucous, proteinuria, bilirubinuria, ketonuria, glutaraldehyde, glycosuria, oxidants (bleach, hydrogen peroxide, peroxidase), pyridinium chlorochromate (sold as urine luck), papain, and vasoconstrictors such as Visine or Clear Eyes (tetrahydrozoline HCL, naphazoline).

The performance of these interfering substances assays should be left to the discretion of the laboratory director as there are implications for specimen validity testing that need to be addressed in the event of a failed assay – one where a substance is deemed to be interfering with one or more analytes in an analytical panel. Acceptance is simple for substances that are deemed to pass, or not affect analysis, then specimen validity tests do not need to include that substance (glucose, for example).

For a 'failed substance', or one that interferes with the panel, all efforts must be made to ensure that the specimens are within the acceptable limits for that substance. For example, if bleach is found to interfere with your assay, then all reasonable measures should be made to determine what concentration of bleach affects analysis and to test specimens as they come into the laboratory for that substance. Specimen rejection criteria should likewise be set to exclude the subset of samples that contain an amount of bleach above the effective cutoff.

General Experimental Design for Interfering Substances

The experimental design for each interfering substance will be similar, but vary only in the preparation of the base material (blank human urine) unless otherwise specified. Blank human urine will be run as a control for interfering substances. Then blank urine will be spiked with varying amounts of the substance being tested for interference. The amount of substance used will vary according to what is appropriate for the substance in question. Once the base matrix has been prepared with the target interferent (modified base), then both blank human urine and the modified base matrix will be spiked with QC material (typically a

dilution of the high QC), prepared according to your SOP, and run in triplicate for each modified base matrix prepared.

Acceptance criteria

The mean value of the spiked human urine will be compared to the mean of the three replicates for the modified base urine. If the values are within your designated acceptance criteria for your assay (typically ±15% or ±20%), then the target interferent is deemed to not affect quantitation at that level. If the mean value for the three replicates of the modified base urine are greater than what is stated in your acceptance criteria, then the substance will be deemed to interfere with your assay at the level (concentration) tested. Any interfering substances should then be tested for in specimen validity testing and written-up in specimen rejection criteria.

Endogenous Interfering Substances

The 'worst case scenarios' for various endogenous substances or conditions with the potential to affect results should be evaluated. The following studies will help prove that your LC-MS/MS method is sufficiently accurate, specific and robust to overcome any variations caused by the presence of diverse endogenous substances in the urine, as well as differences in pH. The following scenarios are intended to reflect "worst case" specimens that might be encountered in the clinical setting:

Hematuria (macroscopic hematuria): ~25 g/L hemoglobin

Bacterial infection: $>10^5$ CFU bacteria/mL

High nitrite (UTI): ~150 μg/mL

High mucus: 1.5 mg/mL

Proteinuria: 1000 mg/dL (dipstick 4+)

Ketonuria: ~1.5 mmol/L

Glycosuria (diabetic/urine glucose dipstick 4+): 2,000 mg/dL

Bilirubinuria (urobilinogen/conjugated bilirubin; e.g., liver cirrhosis): >2 mg/dL/>0.1 mg/dL

Different pH: Test from pH 3-11; 5-8 accepted for screening

Although actual patient samples affected by the above would represent the best source of test material, all of these conditions except bacterial infection can be replicated or mimicked in the laboratory as described below:

Hematuria:

To each 100 mL of "clean" urine, add 20 mL fresh whole blood (no clotting agents or preservatives). Prepare samples for the validation as usual (centrifuge if you would normally centrifuge the samples)

High nitrite:

Nitrite can be ordered commercially as sodium nitrite powder (e.g.: https://www.amazon.com/Sodium-Nitrite-Powder-Ounces-99-6/dp/B00M1OADSW). It can also be ordered directly as nitrite from Sigma as a solution, but at a much higher cost.

If using $NaNO_2$: To each 100 mL of "clean" urine, add 15 mg of $NaNO_2$. If your balance is not sufficiently accurate to measure 15 mg, pool urine samples to obtain 1000 mL and add 1.5 g $NaNO_2$.

High mucus:

Human mucus is not commercially available. However, porcine mucin (as a surrogate) can be purchased from: https://www.sigmaaldrich.com/catalog/product/sigma/m2378?lang=en&region=US

To each 100 mL of urine, add 150 mg of mucin. Vortex well- the mucin will not be fully soluble, just try to get it distributed throughout the sample to the best of your ability (sonication may be attempted for solubilizing the protein). Prepare samples as usual (including centrifuging).

Proteinuria:

Because albumin is the primary protein present in urine, bovine albumin will be used as a surrogate urine protein. This can be purchased as a powder from Sigma (https://www.sigmaaldrich.com/catalog/search?term=albumin&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product)(or as a solution from ThermoFisher: https://www.thermofisher.com/order/catalog/product/15260037?SID=srch-srp-15260037)

To each 100 mL of "clean" urine, add 1 g of albumin (or add 13.33 mL ThermoFisher reagent to 86.67 mL urine). Vortex well. Prepare samples as usual.

Ketonuria:

One of the primary ketones found in urine is beta-hydroxybutyrate. The sodium salt of this ketone can be purchased from Sigma (https://www.sigmaaldrich.com/catalog/search?term=beta-hydroxybutyric+acid&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product).

To each 100 mL of "clean" urine, add 126 mg β-hydroxybutyrate. Vortex well and prepare as usual.

Glycosuria:

D-(+)Glucose can be purchased from a variety of sources, including Sigma (https://www.sigmaaldrich.com/catalog/search?term=glucose&interface=All&N=0&mode=match%20partialmax&lang=en&region=US&focus=product) and Amazon (https://www.amazon.com/HiMedia-GRM6549-500G-Glucose-Monohydrate-L-R/dp/B00DYO7OSK/ref=sr_1_1?ie=UTF8&qid=1539704230&sr=8-1&keywords=D-%28%2B%29glucose)

To each 100 mL of "clean" urine, add 2 g of glucose. Vortex well and prepare as usual.

Bilirubinuria:

Urine may contain high levels of urobilinogen or conjugated bilirubin in certain patient populations. Both products can be purchased from Sigma: https://www.leebio.com/product/1342/urobilinogen-synthetic-reagent-for-urinalysis-651-10

And bilirubin: https://www.sigmaaldrich.com/catalog/product/mm/201102?lang=en&region=US)

Urobilinogen is more commonly found, with a high "normal" concentration of 1.2 mg/dL. Bilirubin is not normally found in urine but may occasionally be present in patients with hepatic injury or cirrhosis.

For urobilinogen: to each 100 mL of "clean" urine, add 2 mg urobilinogen. If the balance is not sufficiently accurate to measure 2 mg, prepare in a larger quantity (e.g. 20 mg/1 L urine) or prepare a stock solution of bilirubin to add to the urine (e.g. add 10 mL of a 20 mg urobilinogen/100 mL stock to 90 mL water)

Conjugated Bilirubin:

1. Prepare a bilirubin solution: Dissolve 10 mg of conjugated bilirubin in 1 L of DI water or clean urine
2. To 90 mL of “clean” urine, add 10 mL of bilirubin stock solution

To reduce the number of samples, you may combine the two types of bilirubin into single samples (e.g. 2 mg or urobilinogen in 90 mL urine, plus 10 mL of the conjugated bilirubin stock). Prepare samples as usual.

Turbidity:

Blank human urine can become turbid for multiple reasons, primarily involving the growth of yeast or bacteria. A pool of human urine can be inoculated with live yeast cultures and incubated at 37°C to encourage growth. McFarland standards or OD may be monitored to quantitate the growth of the organism. Blank urine matrix should be prepared with yeast and growth encouraged by incubation. Samples should be taken at various timepoints, compared to the standards or OD measurements taken at varying timepoints. Label aliquots at different timepoints and place them in the refrigerator in order to suspend bacterial growth at those stages of growth. These modified base matrices may then be spiked and prepared as per your SOP. The comparator matrix will be blank human urine with no yeast or bacterial additive. Acceptance criteria are listed above.

Determining the Impact of pH Extremes:

A pH range from 5 to 8 is considered acceptable for many liquid chemistry assays for drugs of abuse and specimen validity. For the sake of urine drug confirmation, however, we will extend this range to include pH 3-11 for the purpose of finding the limitations of the assay.

Blank human urine may be spiked with acetic acid or ammonium hydroxide to the endpoints of pH 3, 4, 5, 6, 7,8, 9, 10, and 11. Once the base matrix pH has been adjusted, each sample should be spiked with control material to a concentration near the low or mid-level QC. Unaltered blank human urine should be used as a control.

In order to adjust urine to a lower pH: Use acetic or formic acid to adjust base matrix to a lower pH. The acid may be used straight in order to make large changes in urinary pH, or diluted in water and used to make smaller changes in urinary pH. Start with a 10 mL aliquot of blank human urine. Use a pH meter to monitor pH, and begin with a small volume (5-10 μL) of acid. Continue adding acid in 5-10 μL increments (vortexing well between each addition) until you reach your target pH.

In order to adjust urine to a higher pH: Use ammonium hydroxide to adjust your unaltered base matrix to a higher pH. The ammonium hydroxide may be used straight to make large changes in urinary pH, or diluted in water and used to make smaller changes in urinary pH. Start with a 10 mL aliquot of blank human urine. Use a pH meter to monitor pH, and begin with a small volume (5-10 μL) of base. Continue adding base in 5-10 μL increments (vortexing well between each addition) until you reach your target pH.

Prepare three replicates of spiked material at each pH level and the unaltered blank material, and then process according to your SOP.

Acceptance Criteria: The mean value for the three replicates of each sample (unaltered control and pH values from 3-11) will be determined. The mean spike concentration of each pH level will be compared to the unaltered control material and should be within ± 15% to be considered acceptable. If the mean determined value of a pH level exceeds ±15%, then that pH is considered unacceptable and should be written into the acceptance criteria for the assay. Likewise, all acceptable levels of pH should be documented in the assay validation report, SOP, and specimen validity requirements.

Exogenous Adulterants: Urine adulterants come in many forms. Some are consumed internally and some are added to urine after it is collected. Exogenous additive may include, but are not limited to:

Glutaraldehyde

Bleach

Pyridinium Chlorochromate

Papain

Tetrahydrozoline (Visine)

Naphazoline (Clear eyes)

Drano

NaCl

Vinegar

Hydrogen peroxide

Peroxidase

Many of these can be detected using current specimen validity tests, but their effects may vary based on assay systems. Their ability to change outcomes of EMIT tests and some urine drug screens has been well established and samples that fail specimen validity for these are usually rejected for confirmation testing.

If the laboratory director deems it useful, then these substances may be tested as possible adulterants for your LC-MS/MS system.

It is difficult to know exact concentrations that a person may add to their urine sample in order to attempt to pass a drug test, so we suggest preparing matrix spiked with various percents of adulterant from 0% to 20%. Suggested range:

0 % (v/v) - blank human urine

1 % (v/v) - 9.9 mL of blank human urine + 0.1 mL of adulterant

5 % (v/v) - 9.5 mL of blank human urine + 0.5 mL of adulterant

10 % (v/v) - 9.0 mL of blank human urine + 1.0 mL of adulterant

15 % (v/v) - 8.5 mL of blank human urine + 1.5 mL of adulterant

20 % (v/v) -8.0 mL of blank human urine + 2.0 mL of adulterant

This testing paradigm can be applied to any liquid adulterant.

Once blank matrix has been adulterated, each concentration can be spiked to a level in the range of the low or mid QC. The blank control urine and adulterated urine will be run in triplicate, according to your SOP.

Acceptance Criteria: The mean value for the three replicates of each sample (unaltered control and each adulterated concentration) will be determined. The mean spike concentration of each pH level will be compared to the unaltered control material and should be within ± 15% to be considered acceptable. If the mean determined value of adulterant exceeds ±15%, then that concentration of adulterant is considered unacceptable and should be written into the acceptance criteria for the assay. Likewise, all acceptable levels of adulterant should be documented in the assay validation report, SOP, and specimen validity requirements.