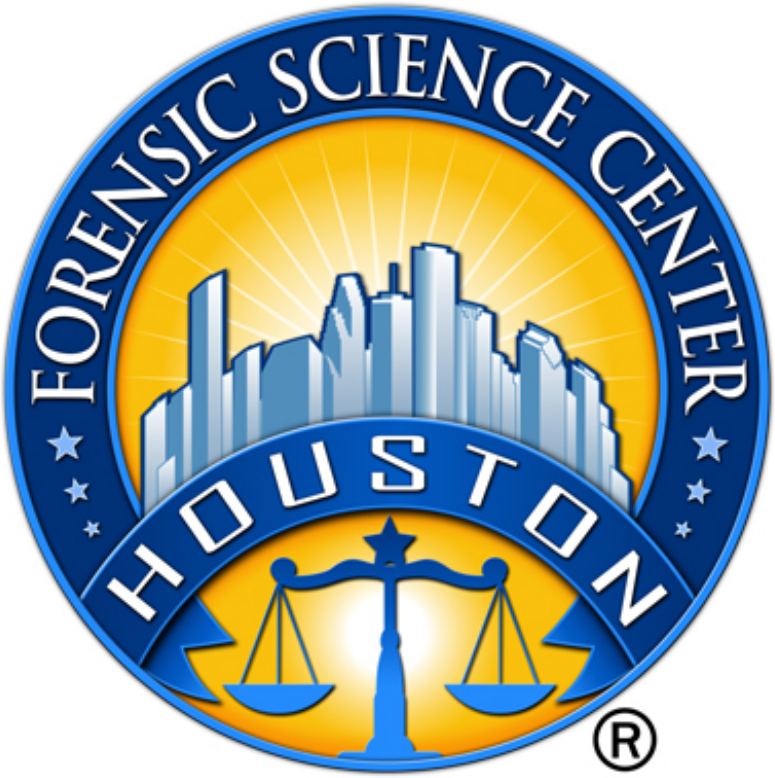


Toxicology Section
Analytical Manual - Standard Operating Procedures (Version 3.2)

Comparative and Analytical Division

Table of Contents

1. Introduction

1.1. This Toxicology Standard Operating Procedures manual ("manual" or "SOP manual") is intended to consolidate the policies and procedures specific to the toxicology section of the Houston Forensic Science Center ("HFSC"). Any conflict between this document and any governing policies established by HFSC, such as the overall quality assurance system, shall be resolved in favor of the HFSC policy. This manual is part of an overall quality assurance system in the Houston Forensic Science Center. Any policies established in the governing quality system or established by the Houston Forensic Science Center will supersede any requirements stated in this manual. However, this document may add additional guidance that supplements what has already been established. This document is an amalgamation of multiple existing policies and procedures specific to the toxicology section. This document supersedes any existing policies and procedures that are not incorporated into the document.

2. Safety

2.1. These procedures must be conducted in accordance with the current HFSC health and safety manual and the current Quality Manual. All biological samples shall be treated with universal precautions. Appropriate personal protective equipment must be worn when performing any type of bench work. Flammable liquids and vapors may cause eye, skin, and respiratory tract irritation. Derivatization reagents are toxic and must be handled in a chemical safety hood or well-ventilated area. Safety Data Sheets (SDS) are available in the laboratory and/or electronically.

3. Evidence Handling

3.1. Purpose

3.1.1. This document outlines the handling procedures of submitted evidence into the Toxicology Section.

3.2. Scope

3.2.1. This procedure is used for the handling and storage of evidence within the Toxicology Section.

3.3. Submission of Evidence

3.3.1. Evidence to be analyzed by the Toxicology Section is received from law enforcement agencies or the judicial system.

3.4. Storage of Evidence

3.4.1. Toxicology evidence is routinely stored in refrigerators within the toxicology section. Freezer storage is an acceptable alternative. All refrigerators and freezers in the toxicology section are monitored using TempAlert, which is further detailed in the Quality Manual, or an equivalent system.

- Acceptable refrigerator temperature range: >0 – 10 °C
- Acceptable freezer temperature range: ≤0 °C

3.4.2. If a refrigerator/freezer stops functioning and exceeds the acceptable temperature range, evidence will be moved to another functioning refrigerator/freezer and transfer documented.

3.4.3. Evidence must be kept separately from drug standards, reagents, and other analytical substances by storage in a separate refrigerator or freezer.

3.5. Receiving Evidence

3.5.1. It is the responsibility of Toxicology personnel to maintain the integrity of evidence at all times while in their custody. All evidence must be protected from loss, cross-transfer, contamination, or deleterious change.

3.6. Upon receipt of evidence into the Toxicology Section:

3.6.1. Client Services and Case Management (CS/CM) Division performs accessioning of toxicology evidence. See CS/CM SOP for details regarding the accessioning procedure and rejection criteria.

3.6.2. If evidence is submitted for both toxicological and biological analyses, the Toxicology Section management will discuss the case with the Biology Section management regarding the workflow.

3.6.3. All evidence transfers must be documented electronically as part of the chain of custody and should include any relevant comments pertaining to evidence processing. If needed, a paper chain of custody will suffice using the HFSC Chain of Custody Form.

3.6.4. Insufficient sample volume will require a court order to proceed with analysis. This applies to instances where (1) evidence consists of one tube of blood containing less than 3 mL or (2) the evidence consists of two tubes of blood, both of which must be opened to complete the analysis. The second instance includes cases with a broken tube; in order for HFSC to analyze these cases, the remaining tube must to be opened and thus a court order is required. HFSC may make exceptions to this procedure (e.g., if analysis of a sample is essential to identify and/or apprehend a suspect not in custody), but any exception must be approved (in writing or by email) by one of the following: HFSC's Chief Executive Office, Chief Operating Officer, General Counsel, or Deputy General Counsel.

3.7. Specimen Condition

3.7.1. If a blood specimen can be aliquoted without the need for homogenization, the specimen is deemed acceptable (normal) for toxicological analysis. The condition of the specimen before analysis will be captured in evidence photographs (refer to 3.8). If a specimen is subject to homogenization prior to analysis, the action will be documented in the case record.

3.7.2. For cases that have multiple color top tubes, the best suited one will be analyzed as follows: grey>lavender>pink>tan>royal blue (if it contains anticoagulant). The following color top tubes require discussion with manager/supervisor and potentially the requester as needed to decide the appropriateness of testing in the event they are the only type provided: gold or red/grey, orange, light green or green/grey, white, red, royal blue (if it contains a clot activator), green, light blue, and replacement top. Yellow top BD brand tubes will not be analyzed.

3.8. Photographs

3.8.1. Evidence must have representative images uploaded into LIMS. Pictures may include:

3.8.1.1. Outer-most evidence container, inner evidence container, blood vial(s)/urine specimen container(s) capturing pertinent information and seal, and any other exhibit(s) including Specimen ID Form if applicable, taken by CS/CM during accessioning; and

3.8.1.2. Tested specimen after analysis to record the initials of analysts having opened the container to conduct analysis.

3.9. Assignments/Tasks

3.9.1. Based on type of offense and type of evidence submitted, the following assignments/tasks will be added to LIMS, unless otherwise requested or specified in the case record. For cases with multiple subjects associated with separate evidence items, an assignment/task will be added for each subject. Four types of reports may be issued: alcohol, negative screening, possible indication of one or more drugs based on immunoassay (see 4.9.2.1 for an example report statement), and confirmation.

3.9.2. DWI or DUID

Blood specimens – alcohol analysis, if <0.10 g/100 mL → Drug Screen, if positive → Confirmation

Urine specimens (if submitted and if blood specimen is not available) – Drug Screen, if positive → Confirmation

3.9.3. Vehicular Homicide or Death Due to Accident

Blood specimens – alcohol analysis → Drug Screen, if positive → Confirmation

Urine specimens (if submitted and if blood specimen is not available) – Drug Screen, if positive → Confirmation

3.9.4. Sexual Assault (Toxicology Kit usually contains both blood and urine specimens)

Blood Specimens only – alcohol analysis → Drug Screen, if positive → Confirmation per request

Blood Specimens – alcohol analysis

Urine Specimen – Drug Screen, if positive → Confirmation per request

3.9.5. Other – Assignment will be made based on client request or consult.

3.10. Preferred Biological Matrix

3.10.1. Blood is the preferred specimen for impairment cases as drug concentrations in blood are more closely associated with performance and behavior than urine. Urine is the preferred specimen for sexual assault cases due to its longer detection window for many drugs. Urine samples are typically not quantified due to variations in volume.

3.11. Outsourced Cases

3.11.1. Evidence to be outsourced to an external laboratory must be processed in the following manner:

3.11.1.1. Verify correct evidence case number.

3.11.1.2. Add a barcode label or manually transfer evidence custody in LIMS.

3.11.1.3. Document outsourcing of evidence through chain of custody in LIMS.

3.11.1.4. Verify a photograph of the evidence has been taken prior to outsourcing.

3.11.1.5. Seal the evidence for shipment.

3.11.2. Evidence that has been outsourced for testing and has been returned must be verified and documented prior to being sealed and returned to the submitting agency. This is accomplished in the following manner:

3.11.2.1. Verify correct evidence case number.

3.11.2.2. Add a barcode label or manually transfer evidence custody in LIMS.

3.11.2.3. Document receiving of evidence through chain of custody in LIMS.

3.11.2.4. Photograph evidence if there is an issue with the condition of the evidence.

3.11.2.5. Repackage with the parent item if applicable.

3.11.2.6. Seal evidence.

3.12. Returning of Evidence

3.12.1. All submitted items will be returned to submitting agency.

3.12.2. Before evidence is sealed, the contents will be checked for proper labeling; one or more pictures would have been taken of the tested specimen after analysis to record the initials of analysts having opened the container to conduct analysis.

3.12.3. Outer evidence containers will be properly sealed and labeled with initials of the individual placing the seal on the item and date the seal was placed before returning evidence to the submitting agency. A part of the initials or date must extend over the edge of the seal onto the container.

4. Technical and Administrative Review

4.1. Purpose

4.1.1. This document outlines the technical and administrative review process for batch analysis of human toxicology specimens.

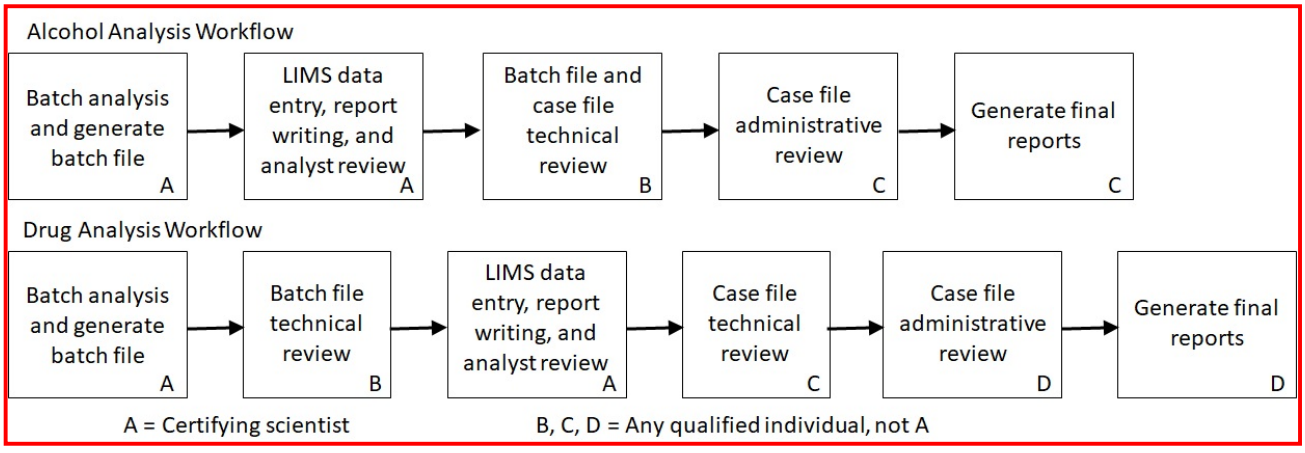
4.2. Scope

4.2.1. This procedure is used to manage the technical and administrative review of toxicology documentation.

4.3. Review Process

4.3.1. Following batch casework analysis, a batch file will be created by the casework analyst conducting the analysis. The casework analyst will then enter case-related information into the Laboratory Information Management System (LIMS). For alcohol analysis and some drug screening and confirmation analyses, this will result in test reports. The certifying scientist is the analyst writing a test report for the case. For alcohol cases, the analyst conducting the alcohol analysis will be the certifying scientist. For toxicology cases where multiple analysts participate in completion of a case, one of the participating analysts will be the certifying scientist.

4.3.2. The batch file and case reports will be initially reviewed by the casework analyst/the certifying scientist and afterwards technically reviewed by another qualified individual using the appropriate Batch Review Checklist. Upon successful completion of the batch and case report technical review (simultaneously for alcohol analysis and separately for drug analysis), each case will then be administratively reviewed by another individual. Instances where more than one drug confirmation analysis is needed, the batch file and case file technical review will be completed at separate times, not necessarily by the same analyst. Below is a schematic of the overall review process:



4.3.3. Any errors caught during technical or administrative review must be addressed before proceeding to the next task in the review process. If an error is caught during administrative review of a case file, the error must be corrected and the case file technically and administratively reviewed once more.

4.3.4. If the batch file is printed, the casework analyst will scan and store the documentation in the proper digital location following successful batch file technical review. If the batch file exists digitally, it will be moved, if not already, to the proper digital location. Printed or digital case-specific documentation will then be moved to each respective case record.

4.3.4.1. If analysis of a certain case sample or analyte fails to meet the acceptance criteria within an acceptable batch, the failed analysis data will be included in the case record.

4.3.4.2. If the entire batch fails, the documentation regarding the failed batch will be included in the batch record.

4.4. Batch File

4.4.1. A batch file will be created, printed or digitally, by the casework analyst.

4.4.2. Batch files must include:

- Batch Review Checklist
- Worklist
- Sequence
- Calibration/Controls
- Data
- Worksheet summarizing the data and detailing other information concerning testing

4.4.3. Batch files may include:

- Method
- Blood and urine batch report
- Any corrective action documentation

4.5. Case File

4.5.1. A case file is generated, printed or digitally, upon submission of evidence to the Houston Forensic Science Center. Each case file must include the following:

4.5.2. Examination Documentation

- Data relevant to the case
- Worksheet summarizing the results and detailing other information concerning testing
- Photos of the evidence

4.5.2.1. **Note**: For each test report generated (e.g., alcohol, negative screening, and/or positive screening/confirmation), the case record will include total number of relevant examination pages associated with the report.

4.5.3. Administrative documentation may include:

- Submission form
- Evidence inventory and description
- Chain of custody
- Correspondence (phone, email, and/or other types of communication)
- Report(s) of analysis
- Discovery orders and subpoenas
- Any incident or corrective action documentation
- Other documentation, *e.g.* outside agency forms

4.6. Batch Review Checklist

4.6.1. Batch review checklists, which are part of the case record, detail the aspects of the batch file requiring inspection for technical accuracy. These have been developed for alcohol (LAB-70), immunoassay screening (LAB-75), and GC-MS screening (LAB-77) and/or confirmation (LAB-73). Equivalent electronic checklists may also be used.

4.7. Case File Review Checklist

4.7.1. Case file review checklists, which are part of the case record, detail the aspects of the case file requiring inspection for technical accuracy for drug screening and/or confirmation. This has been developed for drug screening and confirmation (LAB-74). Equivalent electronic checklists may also be used.

4.8. Administrative Review Checklist

4.8.1. The following items denote what constitutes a case file administrative review:

4.8.1.1. All comments and/or strikethroughs, if any, initialed

4.8.1.2. All pages have correct unique case identifier

4.8.1.3. Added documentation initialed by the individual adding to the case record

4.8.1.4. Evidence Description and Review Form:

4.8.1.4.1. Initials/signature of individual that accessioned and dated

4.8.1.4.2. Outer evidence container and sealed sections completed

4.8.1.4.3. All items received listed, sub-items numbered, and volumes written

4.8.1.5. LIMS:

4.8.1.5.1. Toxicology request added, if needed

4.8.1.5.2. Representative images of evidence uploaded

4.8.1.5.3. Review chain of custody for consistency with documentation, date of analysis, and the evidence pictures

4.8.1.6. Report:

4.8.1.6.1. Review unique case identifier

4.8.1.6.2. Date and name of technical reviewer are listed

4.8.1.6.3. Review appropriate identifiers (i.e., name, DOB, specimen ID, ACN)

4.8.1.6.4. Review results are correct and consistent with examination documentation
4.8.1.6.5. Review all items listed and descriptions are consistent with documentation
4.8.1.6.6. Statement included for each item untested
4.8.1.6.7. Statement included for each item requiring additional testing
4.8.1.6.8. Any comments/discrepancies are clear and consistent with documentation
4.8.1.6.9. Reviewed for clerical errors

4.9. Toxicology Reporting Guidelines

4.9.1. Qualitative vs. Quantitative Results

4.9.1.1. Drugs in urine are reported qualitatively unless specifically noted otherwise in the standard operating procedure or case record. Quantitative drug determinations in urine cannot be interpreted pharmacologically due to differences in total urine volume and elimination rate.

4.9.1.2. Blood is the preferred specimen for quantitative drug determination. Blood drug results may be reported either qualitatively or quantitatively. The laboratory reserves the right to determine whether qualitative or quantitative results are reported as determined by:

- Class of drug
- Case type/offense type
- Other toxicological findings
- Data and/or specimen quality
- Quantity of sample
- Technical abilities of the laboratory

4.9.2. Screening and Confirmatory Tests

4.9.2.1. Laboratory will not report the identity or concentration of a drug based on immunoassay screening results. All immunoassay presumptive positive drug test results are subject to confirmatory analysis using GC-MS or equivalent, provided there is sufficient sample volume. However, the laboratory can report a possible indication of drug(s) (e.g., “this item was screened by ELISA and preliminary results indicate the presence of one or more drugs. If further analysis is needed, please contact HFSC at toxicology@houstonforensicscience.org.”).

4.9.2.2. Preliminary screening tests such as immunoassay have limited scope and utility. They are predominantly used to direct the scope of other analytical testing. Immunoassay results are not reported unless they are negative or provide a possible indication of one or more drugs and no additional tests are performed. In those instances, the report clearly states the scope of testing was limited to immunoassay and lists the drug classes and appropriate cut-offs.

4.9.2.3. Multiple valid results for quantitative assays

4.9.2.3.1. A case sample may need to be analyzed more than once for an assay because one or more of the analytes in the assay do not meet the acceptance criteria. This generates multiple valid results for the other analytes meeting the acceptance criteria after the repeated analyses.

4.9.2.3.2. For such analytes, the average of the valid results will be reported. Each valid result should be within ±20% of the average for GC-MS analysis; and ±5 and ±10% of the average for alcohol analysis if volatile concentrations of reporting analyte(s) are >0.050 g/100 mL and ≤0.050 g/100 mL, respectively.

4.9.2.3.3. For example, a case sample was analyzed for cocaine/metabolite twice because it was positive for cocaine and tentatively benzoylecgonine but benzoylecgonine ion ratios failed in the initial analysis; after the second analysis, you have two valid cocaine results and one valid benzoylecgonine; the average of the two cocaine results will be reported if each cocaine result is within 20% of the average value.

4.9.2.3.4. If the valid results do not acceptably agree with each other as stated 4.9.2.3.2., the concentration of the analyte will not be reported.

5. Preparation and Verification of Drug-Free Matrix

5.1. Purpose

5.1.1. This procedure describes preparation and verification of drug-free matrices.

5.2. Scope

5.2.1. Drug-free matrices are used for the preparation of calibrators and controls for screening and confirmatory toxicology tests.

5.2.2. Drug-free blood containing sodium fluoride preservative and potassium oxalate as anticoagulant can alternatively be purchased from a commercial vendor. Other biological matrices may be prepared as needed.

5.3. Safety/Quality Assurance

5.3.1. This procedure must be conducted in accordance with the current HFSC Health and Safety Manual and the Quality Manual.

5.4. Reagents

- Sodium fluoride (ACS grade or better)
- Potassium oxalate (ACS grade or better)

5.5. Equipment

- Stirrer
- Glass media bottles
- Stir bars
- Top loading balance

5.6. Procedure

5.6.1. Drug-Free Blood:

5.6.1.1. Drug-free blood containing sodium fluoride preservative (1%) and potassium oxalate (0.2%) as anticoagulant is purchased from a commercial vendor. If blood does not contain the preservative and anticoagulant, it can be prepared in-house at the specified concentrations. Store drug-free blood in a refrigerator (expires after 12 months from the preparation date).

5.6.2. Drug-Free Urine:

5.6.2.1. Human urine from drug-free individuals is collected into a collection container and refrigerated.

5.6.2.2. Once approximately 500-2000 mL of urine has been collected it should be pooled.

5.6.2.3. Alternatively, commercial drug-free human urine products can be used once they are demonstrated not to interfere with the analytical assays in service.

5.6.2.4. Store drug-free urine in a refrigerator or a freezer (12-month expiration).

5.6.3. Verification

5.6.3.1. All drug-free matrices are appropriately tested by immunoassay as blank and/or GC-MS screen/basic qualitative confirmation as negative control to ensure they are drug-free prior to being used in casework. Documentation is maintained in a retrievable format.

6. Verification of Relative Concentrations of Working Standard Solutions

6.1. Purpose

6.1.1. This procedure may be performed to compare a new working standard solution with an old working standard solution or to compare the concentration of a calibration working solution to the concentration of a control working solution.

6.2. Scope

6.2.1. This procedure is not routinely required to be performed unless specified in a protocol, method, or SOP.

6.3. Procedure

6.3.1. Working standard solutions are the solutions used to fortify a calibration curve and controls or the solution used to prepare bulk quality control (QC) samples. If multiple working standard solutions are prepared using serial dilution (dilution of one working solution to prepare a second working solution) then this procedure may only need to be performed on the most dilute solution. If the concentration of the most dilute solution is verified to be accurate then it can be assumed that the more concentrated solutions from which it was prepared are also accurate.

6.3.2. Each of the two working standards are to be precisely diluted in triplicate in an appropriate solvent so that they may be analyzed directly by the instrumental technique that will be used for analysis of subject specimens. Each dilution is to be analyzed in duplicate, giving a total of 12 analyses.

6.3.2.1. For example, if the compound of interest is to be analyzed by GC-MS, dilutions will be prepared from each stock solution by diluting each stock solution with reconstitution solvent and then analyzing by GC-MS. Each dilution will be prepared in triplicate and injected in duplicate, giving a total of 12 injections. These dilutions must be prepared so that they are within the linear response range of the GC-MS instrument and the samples must be derivatized if the method requires. A typical procedure would read:

6.3.2.1.1. Prepare triplicate dilutions of each of the working standard solutions by adding 20 µL of the stock standards into conical tubes.

6.3.2.1.2. Add 200 µL reconstitution solvent to each tube and vortex briefly to mix.

6.3.2.1.3. Transfer to autosampler vials and analyze using the normal analytical conditions. Make duplicate injections of each dilution.

6.3.2.1.4. Note: Aliquot volumes may change. However, each method must be evaluated to ensure the final sample has an appropriate concentration. It is important that both working standard solutions are prepared using the same volumes.

6.4. Data Analysis

6.4.1. Using the peak area or peak height response, determine the response factor of the stock solutions by dividing the average response of the duplicate injections for each stock solution by the solution concentration. Calculate the response factor ratio by dividing the response factors of one of the stock solutions by the response factor for the other stock solution. A ratio of 1.0±0.05 gives confidence in the preparation of the stock solutions. Document the verification results on the Excel template located in the section's shared drive.

6.4.2. NOTE: A ratio outside of ±0.05 may be acceptable for a given assay. If the ratio falls outside this range, consult with the section supervisor/manager.

7. Preparation and Verification of Calibration, Control, and Internal Standard Solutions and Reagents

7.1. Purpose

7.1.1. This document outlines the procedure for preparing and verifying calibration, control, and internal standard solutions and reagents.

7.2. Scope

7.2.1. This procedure can be used for the qualitative or quantitative analysis of all toxicology specimens.

7.3. Reagents and Forms

7.3.1. Organic solvents and inorganic reagents (e.g. salts) should be ACS grade or higher. Deionized water should be obtained using a Millipore Direct Q UV3 water system or from an equivalent source. Any internally prepared calibrators or controls may be purchased from an appropriate vendor in lieu of preparation in-house.

7.3.2. All drug standards, working standard solutions, and quality control preparations must be documented on the Reagent and Quality Control (Volatile) Preparation Log (LAB-68), the Working Stock/Standard Preparation Log (LAB-27), or equivalent form and include key information regarding drug standard and chemical names, manufacturers, lot numbers, preparation date, expiration date, by whom the solution was prepared, and the verification information (e.g., batch name, validation). Reference drug standards, controls, and reagents used in the laboratory must be of sufficient quality for their intended use.

7.4. Purchasing, Storage, and Expiration

7.4.1. Vendors should supply a certificate of analysis that contains specific chemical formula, molecular weight, purity, storage conditions, solubility, and a lot number. Information may include an expiration or re-test date.

7.4.2. Solid drug standards and drug standards purchased as liquids in sealed ampoules expire on the date indicated by the manufacturer.

7.4.3. Once a drug standard, or internal standard solution is prepared or diluted, it expires within one year or on the earliest CRM expiration date, unless otherwise specified in the analysis-specific procedures.

7.4.4. If a drug/internal standard is transferred, the expiration date follows the CRM expiration date, unless otherwise specified in the analysis-specific procedures.

7.4.5. Expired drug standards and in-house, multi-component reagents (e.g., 100 mM phosphate buffer and 1% HCl in methanol) should be discarded or clearly labeled not for casework.

7.4.5.1. Expiration dates of the in-house, multi-component reagents are calculated from the date of preparation, regardless of the expiration dates of individual components in the reagents.

7.4.5.2. For troubleshooting purposes only, the section tracks the expiration dates of single-component reagents from the vendors (e.g., derivatizing reagents, chemicals/salts such as sodium fluoride and monobasic sodium phosphate, and solvents such as methanol) if the information is available on the manufacturer's label of the container and/or certificate of analysis (COA) obtained at the time of receipt.

7.5. Use of Calibration, Control, and Internal Standard Solutions and Reagents

7.5.1. Assay calibration must be performed as validated and described in the analytical method.

7.5.2. Control Samples: Types of controls, in order of preference:

7.5.2.1. Commercial controls

7.5.2.2. In-house controls prepared in bulk

7.5.2.3. Controls prepared at the time of analysis using a working standard solution

7.5.3. If CRM is used directly as calibrator or control sample, verification is not required.

7.5.4. Calibration, control, and internal standard solutions used for both blood and urine samples will be verified using the quantitative assays.

7.5.5. Reagents verified during any applicable assay are considered acceptable for all the assays that use the reagents.

7.6. Preparation of Calibration, Control, and Internal Standard Solutions and Reagents

7.6.1. Standards must be made and stored in accordance with the SOP. An equivalent procedure may be used if it is documented on the appropriate preparation log.

7.6.2. The volumes of preparation solutions and the concentrations of CRM for making working standard solutions, internal standard solutions, controls, and reagents can be adjusted as long as the final concentration stays the same. Similarly, sizes of test tubes, flasks, beakers, and other glassware can be different from those specified in SOPs.

7.6.3. In-house prepared controls and/or working standard solutions should be prepared from a different manufacturer than the CRM used to prepare calibration samples.
If a drug standard is not available from a different manufacturer then a different lot from the same manufacturer can be used. If a different lot from the same manufacturer is not available, different ampoules should be used to make separate stock solutions for controls/working standard solutions and calibration samples.

7.6.4. Control stock solutions must be prepared by a different analyst than the stock solutions used for calibration samples.

7.6.5. Information regarding preparation must be documented using the appropriate form or an equivalent form or method, for example:

7.6.5.1. LAB-27: Working Stock/Standard Preparation Log;
7.6.5.2. LAB-68: Reagent and Quality Control (Volatiles) Preparation Log
7.6.6. All solutions/samples must be labeled accordingly. The label must contain at a minimum the solution name and concentration, lot number, initials of the preparer, and expiration date.
7.6.7. Calibration, Control, and IS solutions must be verified to ensure they have been correctly prepared before being used in casework. Upon verification, the verifying analyst shall note the verification batch name and initial the notation on the preparation log.
7.6.8. The concentration of purchased control material (e.g., UTAK control for drug analysis and BQC for alcohol analysis) must be verified prior to being used with casework. Upon verification, the verifying analyst shall note the batch file name (which contains the verification date) and initial the notation. Verification runs must be documented and data kept in a retrievable format in the laboratory.

7.7. Verification of New Lots of Calibration Solutions in Quantitative Assays

7.7.1. Verification batch must include:
7.7.1.1. Current calibrator set
7.7.1.2. New calibrator set
7.7.1.3. Control samples

7.7.2. Evaluation of new calibration solutions
7.7.2.1. Calculate a run as normal, using current calibrator set as “calibrators”
7.7.2.2. Treat the new calibrators and quality controls as unknowns and determine their calculated values
7.7.2.3. If possible, repeat data analysis steps using new calibrator set as “calibrators”
7.7.2.4. Evaluate the data using Calibrator Verification Excel Spreadsheet Template
7.7.3. Acceptance Criteria
7.7.3.1. When the current calibrator lot results and new calibrator lot results are entered into the spreadsheet, a chart will be generated comparing the two sets of data.
7.7.3.2. The slope of best-fit line is acceptable if:
7.7.3.2.1. It is between 0.85 and 1.15, and either
7.7.3.2.2. It is between 0.95 and 1.05, or
7.7.3.2.3. The uncertainty range (95% confidence interval) contains 1.
7.7.3.3. The y-intercept of the best fit line is acceptable if:
7.7.3.3.1. The uncertainty range (95% confidence interval) contains 0.
7.7.3.4. The control values when calculated vs. the new calibrator must be within the percent acceptance used when validating the particular control (typically 20%)
7.7.3.5. If criteria are met, the pass/review fields will read “Pass”.

7.7.3.6. If the criteria are not met, the pass/review fields will read “Review”.

7.7.3.7. Further supplemental information is available to assist in evaluating how the calibrators compare:

7.7.3.7.1. The percent difference between the two calibrator results will be calculated and will be shaded if the new calibrator is more than ±15% different than the current calibrator and will be back-calculated to within ±20% of the target value.

7.7.4. Review/Approval

7.7.4.1. After all appropriate data has been entered to the file, it will be saved and the appropriate supervisor or manager should be notified.

7.7.4.2. If all criteria are met, the supervisor or manager can approve the new calibration lot.

7.7.4.3. If any criteria are not met, review of the new lot of calibrator can only be performed by the supervisor/manager.

7.7.4.4. If upon further review the supervisor/manager decides that the new calibration lot is acceptable, appropriate comments are to be placed in the Comments field specifying why it was accepted. The supervisor/manager can then approve the calibration lot.

7.7.4.5. If upon further review the supervisor/manager decides that the new calibration lot is not acceptable, appropriate comments are to be placed in the Comments field specifying the appropriate steps to be taken. The supervisor/manager can then reject the calibration lot and verify that the review is complete.

7.8. Verification of New Lots of Calibration Solutions in Qualitative Assays

7.8.1. Run the newly prepared calibrators in a minimum of duplicate with the current calibrators.

7.8.1.1. The results of the newly prepared calibrators must be qualitatively and semi-quantitatively acceptable.

7.8.1.1.1. For immunoassay, relative absorbance of the newly prepared calibrator samples should be acceptable: Blank QC > Negative QC > Cut-off Calibrator > Positive QC.

7.8.1.1.2. For GC-MS analysis, relative response of the newly prepared cut-off calibrator should be acceptable: matrix blank/negative control <10% of cut-off calibrator and cut-off calibrator < positive control. The new cut-off calibrators’ semi-quantitative value should be within ±20% of the target value.

7.9. Verification of New Lots of Internal Standard Solutions

7.9.1. Volatiles: Add the amount of internal standard noted in SOP to blank matrix. Run in a minimum of duplicate.

7.9.1.1. Compare the area of the new internal standard to the area of the current internal standard in a blank matrix sample.

7.9.1.2. The average of the new internal standard area counts should match within ±20% of the current internal standard area count. For results outside this range, consult a supervisor or manager.

7.9.2. GC-MS analysis: Prepare two System Suitability samples, one using the current internal standard and the other using the new internal standard.

7.9.2.1. Compare the area of the new internal standard to the area of the current internal standard. The result should match within ±30% of the current internal standard area count.

7.10. Verification of New Lots of In-House Control Solutions

7.10.1. Control Solutions in Quantitative Assays

7.10.1.1. Run the newly prepared control samples in a minimum of duplicate at each concentration along with the current control samples.

7.10.1.1.1. The results of the newly prepared control samples should be within ±20% of the target concentration for drug analysis and within ±10% of the target concentration for alcohol analysis if BAC ≤0.05 g/100 mL and ±5% if BAC >0.05 g/100 mL.

7.10.2. Control Solutions in Qualitative Assays

7.10.2.1. Run the newly prepared control samples in a minimum of duplicate at each concentration along with the current control samples.

7.10.2.1.1. The results of the newly prepared control samples should be qualitatively acceptable (i.e., positive control should be positive).

7.10.2.1.2. For immunoassay, relative absorbance of the newly prepared control samples should be acceptable: Blank QC > Negative QC > Cut-off Calibrator > Positive QC.

7.10.2.1.3. For GC-MS analysis, relative response of the newly prepared control samples should be acceptable: matrix blank/negative control < cut-off calibrator < positive control.

7.11. Establishing Target Concentration and Acceptance Range for Control Solutions in Quantitative Assays

7.11.1. Commercial controls

7.11.1.1. Follow the manufacturer's instructions for material preparation, and then perform four separate analytical runs with three replicates per run or a minimum of 12 replicates over more than one run to establish the mean. Perform these determinations in parallel with the existing controls normally included in casework to verify performance. Acceptable criterion is that the mean value is within ±30% (±15% for alcohol analysis) of the manufacturer's mean if applicable. The controls should have verified control result data from the manufacturer whenever possible to designate the nominal value. This verification is conducted if the lot number for commercial controls changes.

7.11.2. In-house controls, if theoretical target is not used

7.11.2.1. The target is defined to be the average calculated concentration from four separate analytical runs with three replicates per run or a minimum of 12 replicates over more than one run. The verified target must be within ±15% of the nominal value.

7.12. Establishing Performance for Control Solutions for Qualitative Assays

7.12.1. Commercial controls

7.12.1.1. Follow the manufacturer's instructions and perform four separate analytical runs with three replicates per run or a minimum of 12 replicates over more than one run. Perform these determinations in parallel with the existing controls normally included in casework to verify performance. All samples must provide acceptable results.

7.12.2. In-house controls

7.12.2.1. Perform four separate analytical runs with three replicates per run or a minimum of 12 replicates over more than one run. All samples must provide expected results.

7.13. Concurrent Verification of Calibration, Control, and Internal Standard Solutions

7.13.1. Validation data of an analytical method can be used to verify calibration solutions/samples and control solutions/samples or to establish the target value or performance of control samples for the method.

7.13.2. Analyzing in parallel with existing controls or reagents does not apply to newly introduced control samples or reagents. For the newly introduced control samples, performance will be evaluated according to the acceptance criteria of the method. For the newly introduced reagent, it will be considered verified if controls meet the acceptance criteria of the method.

7.13.3. If the current calibration samples, control samples, and/or internal standards are determined to be unacceptable due to analyte instability, contamination, or other reasons, performance of the new set will not be compared against performance of the current set. Rather, performance of the new set will be evaluated according to the acceptance criteria of the method.

7.13.4. Concurrent verification process

7.13.4.1. ELISA: perform three runs, each consisting of Negative QC, Cut-off Calibrator, and Positive QC samples in triplicate.

7.13.4.2. GC-MS screen: perform three runs, each consisting of Negative, Positive QC, and Carryover QC samples in triplicate.

7.13.4.3. Reportable qualitative GC-MS analysis: perform three runs, each consisting of Negative, Cut-off Calibrator, High Calibrator (if applicable), and Positive QC samples in triplicate.

7.13.4.4. GC-MS quantification: perform three runs, each consisting of control samples in triplicate at each concentration and one calibration curve.

7.13.4.5. The results will be evaluated according to the acceptance criteria of the method.

7.14. Verification of Newly Prepared Reagents

7.14.1. Run two negative controls (Blanks for ELISA) using the newly prepared reagent in parallel with controls made using the existing reagent. The 10 M potassium hydroxide and beta-glucuronidase solutions will be verified using two hydrolysis controls and the 1% HCl in methanol solution will be verified using two positive controls.

7.14.2. The reagent will be considered verified if controls meet the acceptance criteria defined by the method.

7.14.3. If the current reagent is not available as reference (e.g., expired), the performance of the new reagent will be evaluated according to the acceptance criteria of the method.

8. In-Process Calibration and Quality Control for Drug Screening/Confirmation Testing

8.1. Purpose

8.1.1. This procedure describes the preparation and implementation of a calibration curve and in-process quality control samples. This procedure is designed to provide a means of detecting potential problems with assay performance and to ensure accurate and reliable test results.

8.2. Scope

8.2.1. These are default procedures for calibrators and quality controls of all validated qualitative and quantitative assays applicable to all analytical SOPs not having specific control protocols.

8.3. Calibration of Quantitative Assays

8.3.1. Calibration protocol must be performed as validated and described in the analytical method.

8.3.2. Unless otherwise specified in the analytical procedure, no fewer than four calibration levels, spanning the linear range of the assay, may be used for a linear and quadratic calibration curve. The four concentrations must span the range of the assay.

8.3.3. For the calibration curve to be accepted, the back-calculated results for each calibrator must calculate to within ±20% of its target value. A variance of ±25% is allowed at the LOQ.

8.3.3.1. For selective ion monitoring (SIM) and multiple reaction monitoring (MRM) analyses, ion ratios for all calibrators must be with in ±20% or two standard deviations relative to the average ion ratio from all calibrators used in the calibration curve, as validated to be appropriate for the ion ratio. The two standard deviation rule applies to the ion ratios that exhibit significant concentration-dependent variability during validation; the ion ratios of the others including internal standards use the 20% rule. Any calibrators that do not meet these requirements must be excluded from the curve.

8.3.4. In some situations, one point may be eliminated from the calibration curve to improve the quality of the curve fit. Elimination of two points should only be made in exceptional circumstances when other evidence supports the use of analytical data from that particular analysis batch. The approval of the technical reviewer is required when two points are discarded.

8.3.5. When a calibrator is excluded, the fact that it was excluded and the reason must be clearly documented with the data for that batch.

8.3.6. The lowest acceptable calibrator for a given batch is the reporting limit, unless otherwise specified in the analytical method.

8.3.7. A negative control should be included after the highest calibrator in each analytical run to monitor for carryover.

8.4. Calibration of Qualitative Assays

8.4.1. A cut-off calibrator must be included in every analytical run.

8.4.2. Immunoassay

8.4.2.1. The cut-off calibrator must have an average %binding greater than the average of the Positive QC and less than the average of the Negative QC.

8.4.3. GC-MS

8.4.3.1. Results of unknown samples are determined to be positive or negative when evaluated against the semi-quantitative cut-off calibrator forced through zero.

8.4.3.2. A high calibrator may be added to account for drugs whose ion ratios are concentration dependent. The high calibrator is not used as a part of the calibration curve, but only used to set acceptable ion ratio limits for concentration dependent drugs. Acceptance criteria for the high calibrator include acceptable ion ratios, positive result, and Gaussian peak shape.

8.4.3.3. For SIM and MRM analyses, ion ratios of the cut-off calibrator and the high calibrator (if applicable) must be averaged to determine the target for all ion ratios. All quality controls and case samples must be within either ±20% or two standard deviations relative to the average ion ratio, as validated to be appropriate for the ion ratio, to meet acceptance criteria.

8.5. Control of Quantitative Assays

8.5.1.1. Each analytical run must contain at least one matrix blank, one negative control sample, and positive control samples, which are extracted at the same time as the calibration curve and case samples; the number of positive control samples must be at least 10% of the number of case samples in the batch.

8.5.1.2. Low control (LQC) concentration should be no more than three times the target LOQ of the assay.

8.5.1.3. Mid control (MQC) concentration should be in the middle of the calibration range.

8.5.1.4. High control (HQC) concentration should be no less than 75% of the upper limit of quantification (ULOQ).

8.5.1.5. An external quality control (i.e., UTAK) should be used when available.

8.5.1.6. Acceptance criteria of quality controls are 1) quantified concentrations ±20% of the target concentration and 2) the retention time ±2% of the average retention time of the calibrators. For SIM and MRM analyses, ion ratios of the all quality controls must be within ±20% or two standard deviations relative to the average ion ratio from all calibrators used in the calibration curve to meet acceptance criteria.

8.5.2. A LQC sample should be injected after the last case sample for each run.

8.5.3. Control samples must be included for every analyte being quantified by the method.
8.5.4. A set of positive controls will bracket all case samples at the beginning and the end of a run (e.g., LQC, HQC, MQC) with a positive control (e.g., MQC, UTAK) bracketing every 10 case samples.
8.5.4.1. Case samples must be bracketed by acceptable positive controls to report quantitative results. Any positive case samples bracketed by a control not meeting the acceptance criteria must be re-analyzed. Negative results may be reported upon documented review of the data by the analyst and technical reviewer.
8.5.4.2. The control sets run at the beginning and the end of the case samples must have a 2/3 passing rate to report quantitative results.
8.5.4.2.1. If both low quality controls fail, the results below the target concentration of the next level quality controls (MQC or UTAK) cannot be reported.
8.5.4.2.2. If both high quality controls fail, the results above the target concentration of the next level quality controls (MQC or UTAK) cannot be reported.

8.6. Control of Qualitative Assays

8.6.1. Each analytical run must contain at least one matrix blank, one negative control sample, and positive control samples, which are extracted at the same time as the cut-off calibrator and case samples; the number of positive control samples must be at least 10% of the number of case samples in the batch.
8.6.2. Immunoassay
8.6.2.1. Positive QC: sample of the control matrix fortified at a concentration no greater than 200% of the cut-off calibrator concentration unless otherwise specified in Section 13.
8.6.2.2. Negative QC: sample of control matrix fortified at a concentration no less than 50% below the cut-off calibrator concentration unless otherwise specified in Section 13.
8.6.3. GC-MS
8.6.3.1. Positive controls: sample of the control matrix fortified at a concentration within two times, excluding carryover control, of the reporting limit for the assay with all drug classes or individual compounds for which the assay is designed to detect.
8.6.3.2. GC-MS Screen: contains a positive control (PQC) that establishes the threshold for sending case samples to confirmatory analysis. The PQC must be at least 10% of the number of case samples in the batch. The carryover control (COQC) is also included once in each batch to determine a threshold for sending case samples for quantitative confirmation; these samples can be sent to an external laboratory for drugs not included in the in-house test panel.
8.6.3.3. Reportable qualitative GC-MS analysis: contains a positive control (PQC) that is no more than 200% of the cut-off calibrator. The PQC must be at least 10% of the number of case samples in the batch.
8.6.3.4. Negative control: sample fortified with internal standard.
8.6.3.5. A run is accepted if system suitability (if applicable) is acceptable, and if all quality

control samples perform as expected. Exceptions will be documented in the batch file. The matrix blank and the negative control must have an analyte response no greater than 10% of the cut-off calibrator, and all case samples must be bracketed by acceptable positive controls to report positive results.

8.6.3.6. Any positive case samples bracketed by a control not meeting the acceptance criteria must be re-analyzed. Negative results may be reported upon documented review of the data by the analyst and technical reviewer.

9. General Guidelines for Instruments and Equipment

9.1. Purpose

9.1.1. Instrumentation and equipment must be regularly maintained to ensure precision and accuracy in the various assays used by the toxicology section.

9.2. Scope

9.2.1. These guidelines are intended to describe proper operation, maintenance, and performance verification procedures for key instrumentation.

9.3. GC-MS

9.3.1. Method of Use

9.3.1.1. Refer to the appropriate operating manual and references for proper handling, use, and troubleshooting.

9.3.2. Tune Verification and Autotune

9.3.2.1. An autotune is used to evaluate the instrument's performance and to check for leaks. An autotune must be performed prior to each analytical run on the instrument, after any maintenance, and may be done at other intervals as deemed necessary by the analyst. During an autotune, the MSD is calibrated by tuning the instrument to ensure the mass-to-charge ratios (m/z) are assigned correctly and the scan ratio is set properly. This procedure also serves as a check for air leaks.

9.3.2.1.1. Each day that an autotune is performed, it should be documented on the GC-MS Maintenance Log (LAB-24) or an equivalent form. A copy of the most recent tune file should be recorded in a retrievable format.

9.3.2.1.2. Following an EI autotune on the mass spectrometer, the tune report should be examined. If the tune does not meet the criteria for the application, then action should be taken to determine why it does not meet said criteria. For example, the system may need a refreshed autotune, a source cleaning, or there may be an air leak.

9.3.2.1.3. If an instrument does not pass the tune verification, no casework will be performed using that instrument until the problem is resolved and the tune verification falls within acceptable specifications.

9.3.2.1.3.1. Tune Specifications

9.3.2.1.3.1.1. The three tuning masses must be within ±0.2 amu of 69.00, 219.00, and 502.00 amu.

9.3.2.1.3.1.2. The peak widths of the three tuning masses must be within ±0.05 amu of 0.60 amu.

9.3.2.1.3.1.3. The ratio of mass 70 to 69 must be within 0.5 – 1.6%.

9.3.2.1.3.1.4. The ratio of mass 220 to 219 must be within 3.2 – 5.4%.

9.3.2.1.3.1.5. The ratio of mass 503 to 502 must be within 7.9 – 12.3%.

9.3.2.1.3.1.6. The ratio of mass 219 to 69 must be >35%.
9.3.2.1.3.1.7. The ratio of mass 502 to 69 must be >3%.
9.3.2.1.3.1.8. The abundance of any peaks less than 69 amu must not be greater than 10% of the base peak abundance. Peaks at 18, 28, and 32 amu are indicative of water, nitrogen, and oxygen, respectively, and may indicate an air leak.

9.3.3. Maintenance

9.3.3.1. Maintenance should be performed following the manufacturer's guidelines or more frequently as needed. Refer to Agilent 5975 Series MSD Operation Manual or equivalent. All maintenance and repairs should be documented on the GC-MS Maintenance Log (LAB-24) or an equivalent form.

9.3.3.1.1. Before each analytical run:

- Perform an autotune
- Verify tank pressure
- Check the wash solvents (Note: The solvent vials may be rinsed and filled or refilled as needed)
- Wash syringe
- Check the rough pump oil level

9.3.3.1.2. Annually:

- Replace the rough pump oil
- Check calibration vial and refill PFTBA as necessary
- Check diffusion pump oil and replace if necessary

9.3.3.1.3. As needed (depending upon instrument and sample throughput):

- Change the septum
- Check and replace the inlet liner
- Check and replace the gold seal
- Clip the column
- Replace/Switch filament(s)
- Replace gas cylinders
- Clean the ion source

9.3.4. Methods

9.3.4.1. Electronic backups of the methods and data files are recommended for each instrument. An electronic copy of the method is located in the Instrument Method Folder or equivalent. Methods are updated regularly following routine instrument maintenance. It is recommended that the analyst initial and date any updated methods excluding changes in the SIM window parameters.

9.3.5. Sample Preparation and Sequence Set-up

9.3.5.1. Samples should be prepared for analysis according to the section's standards or specific SOP.

9.3.5.2. The data file path must clearly identify the location and storage of the data. The convention for the data file storage should include the date and name of the analyst.
9.3.5.3. Retain GC-MS analysis data in the batch file.

9.4. Pipettes

9.4.1. Method of Use
9.4.1.1. Refer to appropriate manuals for proper handling and use.
9.4.2. Calibration
9.4.2.1. Each pipette should be externally calibrated and certified by an approved calibration vendor at least once per calendar year and prior to being placed into service. Calibration performed on a quarterly basis is recommended.
9.4.3. Performance Check and Maintenance
9.4.3.1. Pipette performance checks are performed following maintenance or cleaning.
9.4.3.1.1. External calibration can be performed in lieu of a performance check.
9.4.3.1.2. External calibration of pipettes performed onsite by an approved vendor is not considered maintenance and thus does not require a performance check.
9.4.3.2. Performance check procedure:
9.4.3.2.1. Room temperature deionized water should be pipetted into a weighing vessel on an analytical balance.
9.4.3.2.2. The pipette should be checked at the low end and high end of the relevant range with multiple replicates at each volume unless verified concentrations correspond to critical measurements of the particular pipette. Checking three points with five replicates at each volume is recommended.
9.4.3.2.3. The mass of the water delivered should be recorded on the Pipette Performance Check Form (LAB-41) or an equivalent form.
9.4.3.2.4. The average value of each pipette should fall within the performance limit set by the external vendor's calibration document (±3%) before it may be used for casework.
9.4.3.3. Due to the inability to gravimetrically verify the small volumes (2.0 µL) associated with low volume pipettes that dispense volumes ≤25 µL, these pipettes will not be checked internally. Instead, these shall be checked via an approved calibration vendor before placing back into service.
9.4.3.4. Follow manufacturer's instructions for troubleshooting maintenance if needed.
9.4.3.5. If a pipette fails a performance check or if an analyst has reason to believe that a pipette is not working properly they must:
9.4.3.5.1. Perform a pipette performance check and if the pipette is not in proper working order:
9.4.3.5.1.1. Clearly mark the pipette "OUT OF SERVICE".
9.4.3.5.1.2. Inform the section manager. No laboratory case work will be performed using the pipette until an external calibration is completed.

9.4.3.5.1.3. Repair or send out the pipette for repairs.
9.4.3.5.1.4. Externally calibrate following repair.
9.4.3.5.1.5. Documentation must be maintained in a retrievable format in the laboratory.
9.4.3.5.2. Occasionally, a pipette may be out of service even if no problem has been identified, such as newly purchased pipettes pending performance check or calibration. In the event a pipette is out of service (inactive, in repair, etc.) the pipette shall be marked "OUT OF SERVICE" and the appropriate dates for the period documented using Pipette Performance Check Form (LAB-41) or an equivalent form.
9.4.3.5.3. All pipettes marked as "OUT OF SERVICE" are not subject to routine external calibration unless the pipettes need to be placed back into service.
9.4.3.6. A volumetric or positive displacement pipette is intended for the quantitative transfer of a liquid. On occasion, however, pipettes are used only for qualitative purposes (i.e., transfer steps during derivatizations).
9.4.3.7. Maintenance of Hamilton® Microlab 600 follows as described in Hamilton Maintenance Log (LAB-85) or an equivalent form.
9.4.3.7.1. Maintenance of Hamilton pipettes may include replacing valves, cross tube, syringes, tubing, and batteries as needed. The pipettes will be cleaned annually by Hamilton Company Service Center. Firmware or software versions may also be updated as provided by Hamilton Company.

9.5. pH Meter

9.5.1. Method of Use
9.5.1.1. Refer to the User's Manual for detailed instructions on proper handling and use.
9.5.2. Performance Check and Maintenance
9.5.2.1. A three-point performance check is used for measuring pH of a solution when pH accuracy better than ±0.1 is required. This check is performed prior to each use and documented on pH Meter Performance Check (LAB-48) or an equivalent form.
9.5.2.1.1. Measured pH should be within ±0.022 of the target pH at the reported temperature; if measured temperature falls between the listed temperatures on the pH buffer solution bottle label (e.g., 23 °C), the average of target pH values bracketing the measured temperature (pH 4.00 at 20 °C and pH 4.01 at 25 °C) is used.
9.5.2.2. Weekly maintenance includes refilling electrode and verifying expiration date of the refilling solution, rinsing electrode, verifying electrode storage solution and expiration date, and verifying pH buffer solution expiration dates. It is documented on the pH Meter Performance Check (LAB-48) or an equivalent form.

9.6. Refrigerators

9.6.1. Maintenance

9.6.1.1. Refrigerators and freezers should remain clean and organized at all times. If a spill occurs, appropriate cleaning procedures should be performed.

9.6.2. If a refrigerator or freezer is open for an extended period (e.g., cleaning, inventory), this should be documented on the Temperature Log (LAB-69) or by an equivalent method.

9.6.3. If a refrigerator or freezer stops functioning and exceeds the acceptable temperature range, evidence will be moved to another functioning refrigerator or freezer and the transfer documented.

9.7. NIST Traceable Thermometers

9.7.1. In the event the TempAlert monitoring system is not working, temperature measurements for refrigerators and freezers must be performed using NIST traceable thermometers. Temperature measured with a method other than the TempAlert monitoring system is documented on the Temperature Log (LAB-69) or an equivalent method.

9.7.2. If any thermometer is suspected of not working properly, laboratory management should be notified and a record made. It will then be removed from service and replaced with a working thermometer.

9.8. Balances

9.8.1. Method of Use

9.8.1.1. Refer to the appropriate operating instructions for proper handling and use.

9.8.2. Performance Check and Maintenance

9.8.2.1. Performance check and maintenance should be performed following the manufacturer's guidelines and the schedule described below.

9.8.2.2. Balances

9.8.2.2.1. Balances must be calibrated and certified with a traceable certificate by an external vendor once per year.

9.8.2.2.2. An internal performance check must be conducted weekly using NIST traceable reference weights. This is documented using form Balance Performance Check (LAB-14), or an equivalent method.

9.8.2.2.3. Additional performance checks may be performed as necessary.

9.8.2.2.4. Balances should be checked for accuracy each time the balance is moved and after maintenance is performed.

9.8.2.3. Weights

9.8.2.3.1. Weights used to performance check the balance shall be sent to a vendor for recertification every year.

9.8.2.3.2. Laboratory weights shall be inspected after the annual recertification of the weights by performing an internal performance check. This is documented using form Weights Performance Check (LAB-12), or an equivalent method.

9.8.2.3.3. Laboratory weights should be stored, transported, and handled using pre-cautions to protect them from contamination and deterioration.

9.8.2.4. General

9.8.2.4.1. All records of the performance checks of balances and weight sets, maintenance, and calibration certificates are maintained in a retrievable format.

9.8.2.4.2. If the result from a performance check is outside of the acceptable range, the balance will be immediately taken out of service until maintenance and/or certification are performed by an approved vendor. Laboratory management should be notified and the problem documented on LAB-12, LAB-14, or an equivalent method.

9.8.2.4.3. Since the tolerances of electronic balances vary, instrument specifications must be checked to determine the appropriate criteria for satisfactory performance. The following general specifications may be used.

Balance Class	Weights	Acceptable Range
Analytical	0.002 g	0.0015-0.0025 g
	1 g	0.9500-1.0500 g
	5 g	4.9500-5.0500 g
Top Loading	1 g	0.95-1.05 g
	5 g	4.95-5.05 g
	100 g	99.50-100.50 g

9.9. Heating Block

9.9.1. Method of Use

9.9.1.1. Refer to the appropriate operating instruction manual for proper handling and use.

9.9.2. Performance Check and Maintenance

The thermometer in the heat block will be checked using a NIST traceable thermometer once a month. The temperature on the thermometer in the heat block must read within ±5 °C of the NIST traceable thermometer and the performance is documented in Heat Block Thermometer Performance Check (LAB-84).

9.10. Millipore Water Purification System

9.10.1. Method of Use

9.10.1.1. Refer to the appropriate operating instruction manual for proper handling and use.

9.10.2. Performance Check and Maintenance

9.10.2.1. Ensure the resistivity of the Millipore water is above 18 megohm.

9.10.2.2. Maintenance includes SmartPak replacement, final filter replacement, vent filter replacement, flow calibration, tank level calibration, UV lamp replacement and UV timer reset, screen filter cleaning, sanitization of the system, and sanitization of the tank, performed as described in Millipore Maintenance Log (LAB-83) and the User Manual.

9.11. Tecan System

9.11.1. Method of Use

9.11.1.1. Refer to the appropriate operating instructions for proper handling, use, and troubleshooting.

9.11.2. Reagents and Materials

9.11.2.1. Deionized water.

9.11.2.2. 1 M HCl (hydrochloric acid): Add 84.6 mL concentrated hydrochloric acid (HCl) to a 1-liter volumetric flask containing deionized water. Bring to volume with deionized water. Store at room temperature (12-month expiration).

9.11.2.3. 1 M NaOH (sodium hydroxide): Dissolve 40.0 g sodium hydroxide pellets in 1 L deionized water. Store at room temperature (12-month expiration).

9.11.3. Maintenance

9.11.3.1. Tecan Freedom EVO 75

9.11.3.1.1. Follow the routine preventative maintenance procedure below as needed or before running case work. Documentation of the maintenance performed is on the Tecan Maintenance Log (LAB-92) or an equivalent method.

9.11.3.1.2. Daily Maintenance Tasks

9.11.3.1.2.1. Fill system fluid containers with deionized water.

9.11.3.1.2.2. Thoroughly prime (flush) the system with deionized water from the system fluid container.

9.11.3.1.2.3. Check the green Teflon coating of the stainless steel pipette tip for any damage.

9.11.3.1.2.4. Check the syringes for leaks, bubbles or internal contamination.

9.11.3.1.2.5. Check around the valve for signs of moisture.

9.11.3.1.2.6. Check for air bubbles or contamination in the pipetting tubing.

9.11.3.1.2.7. Empty all waste containers.

9.11.3.1.3. Monthly Maintenance Tasks

9.11.3.1.3.1. Remove racks and waste reservoir from the instrument surface. Carefully clean the work surface using a disinfectant wipe and a KimWipe.

9.11.3.1.3.2. Clean the waste reservoir using cotton tip applicators and wire brush.

9.11.3.1.3.3. Clean the Teflon sample tip by gently wiping it with a lint-free tissue and isopropanol, and then deionized water.

9.11.3.1.3.4. Clean the system liquid container with a mild soap. Make sure to thoroughly rise out container before filling with deionized water.

9.11.3.1.3.5. Perform an Acid/Base Wash

9.11.3.1.3.5.1. Follow system maintenance prompts to perform an Acid/Base wash using 1N HCl and 1N NaOH. An acid-base wash must be completed after preventative maintenance has been performed.

9.11.3.2. Tecan HydroFlex Plate Washer

9.11.3.2.1. Documentation of the maintenance performed is on the Tecan Maintenance Log (LAB-92) or an equivalent method.

9.11.3.2.2. Monthly or as needed, use appropriate wires to remove any buildup in washer tips. A wire brush may be used if buildup is evident around base of tips.

9.11.3.2.3. Monthly or as needed, perform Rinse Night using the plate washer menu to flush the liquid system and to prevent needle blockages.

9.11.3.2.3.1. During the rinse night procedure, the needles are soaked in deionized water in the prime tray for at least 12 hours.

9.12. Headspace GC-FID

9.12.1. Method of Use

9.12.1.1. Refer to the appropriate operating manual and references for proper handling, use, and troubleshooting.

9.12.2. Maintenance

9.12.2.1. Verification is performed after maintenance or changes in data acquisition. The verification run must meet acceptance criteria outlined in this procedure. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification runs must be documented in the maintenance log or equivalent form and data kept in a retrievable format in the laboratory.

9.12.2.2. If maintenance, repair, or changes in data acquisition significantly affects retention times of analytes (e.g., installing a new GC column), the mean retention time of calibrators included in the verification will be used to establish the new retention time of each analyte.

9.12.2.3. A major verification, which includes three analyses of calibrators and at least three replicates of MQC1, BQC2, EQC and LMQC, is performed following any changes in data acquisition.

9.12.2.4. A minor verification, which includes an analysis of calibrators and at least three replicates of MQC1, BQC2, EQC, and LMQC, is performed following any preventative maintenance and/or major repairs.

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9.13. Centrifuge

9.13.1. Method of Use

9.13.1.1. Refer to the appropriate instruction manual for proper handling, use, and troubleshooting.

9.14. NIST Traceable Volumetric Flasks

9.14.1. Class A volumetric flasks will be used for the preparation of calibrators and will be dedicated for this purpose. The flasks will be replaced every ten years.

9.15. Literature References

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9.15.27. Tecan HydroFlex Plate Washer Operating Manual, Document Part No: 30026397, 2008-02
9.15.28. Tecan Sunrise Plate Reader Operating Manual, Document Part No.: 30041769, 2008-11
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10. Operation and Maintenance of Zymark Turbo Vap LV

10.1. Purpose

10.1.1. During toxicological extraction, samples require evaporation in order to concentrate the isolated material. The Zymark Turbo Vap® LV is a microprocessor-controlled evaporator that provides simultaneous and automated concentration of multiple samples, unattended operation, convenience, and speed. The Turbo Vap will allow high capacity evaporation at a maximum of 50 samples processed simultaneously.

10.2. Scope

10.2.1. The Turbo Vap will be used routinely during extractions in order to concentrate extracts or as needed. Analysts should be knowledgeable regarding the use, precautions, and maintenance of this evaporator.

10.3. Equipment

- Zymark Turbo Vap LV Workstation and accessories
- Compatible test tube rack
- Inert gas supply (nitrogen)
- Distilled or deionized water
- Timer
- Siphon tube
- Spectrum Clear Bath

10.4. Precautions

10.4.1. The workstation must be placed in an appropriate location with available gas and electrical sources, as well as adequate ventilation. The workstation may either be placed inside a fume hood or the exhaust duct supplied with the unit must be utilized. This must go to a suitable ventilation system vented outside the laboratory.

10.4.1.1. **Note:** Exhaust gases may be hazardous. Consult the Safety Data Sheets for all solvents used.

10.4.2. The workstation must be used on a flat, level, stable surface.

10.4.3. The workstation must never be used with hydrogen or other flammable gases which may explode or catch on fire.

10.4.4. DO NOT move the unit when the bath is full of water. It is a burn hazard.

10.4.5. DO NOT operate the Turbo Vap LV without water in the bath to avoid the risk of fire or burn injuries.

10.4.6. To avoid injury, DO NOT EXCEED 100 psi maximum inlet pressure.

10.5. Procedure

10.5.1. Turn on the unit and gas supply.

10.5.2. Check the water bath and fill to the appropriate level with distilled or deionized water. The water bath level should be as high as the initial level of solvent in sample tubes unless micro-centrifuge racks and tubes are utilized.

10.5.3. Check the gas supply and pressure.

10.5.4. Set the water bath temperature as specified in the respective procedure. The bath will be at the correct temperature when the TEMP light stops blinking.

10.5.5. Set the gas pressure and time setting in accordance with the SOP.

10.5.6. Turn on the rows containing sample tubes by pressing the corresponding tube stations pushbuttons. There are 5 rows of 10 stations available for use. Partial rows may have unused gas nozzles. These should be plugged with supplied stoppers for optimum gas flow and sample protection against moisture.

10.5.6.1. **Note:** Leaving empty rows unplugged while in use can cause condensation on the unit lid, which may in turn contaminate the sample.

10.5.7. Load sample tubes into the evaporator by opening the cover and placing the sample racks into the water bath.

10.5.8. Close the cover and press START. To stop an evaporation run, simply press STOP. To pause an evaporation run, press the START/PAUSE pushbutton. To shut off the evaporation in any row of test tubes, press the corresponding TUBE STATIONS pushbutton.

10.5.9. When the cycle is complete, the gas automatically shuts off and the evaporator buzzer will sound every 30 seconds. Lift lid and leave open as soon as possible. Remove tubes and blot with absorbent material to remove moisture if desired.

10.5.9.1. **Note:** Highly volatile samples can be lost if they are allowed to remain in the unit.

10.5.10. When use of the Turbo Vap is complete for the day, turn the unit power and gas supply off. Lift the cover and keep it open.

10.6. Maintenance

10.6.1. Routine maintenance of the Turbo Vap LV eliminates the need for frequent cleaning due to cloudy or bacteria infested water.

10.6.1.1. **Note:** Cleaning the water bath may cause exposure to bacterial or viral hazards. Use good laboratory operating procedures when dealing with liquids.

10.6.2. Turn the evaporator's AC power OFF and unplug the power cord.

10.6.3. Open the cover and remove the rack.

10.6.4. Siphon the water out of the bath.

10.6.5. Use an appropriate cleaner if desired, to wipe any residue from the bath walls. Rinse the bath and re-siphon the liquid. Also clean the rack itself.

10.6.6. Pour approximately 1 L distilled or deionized water into bath. Add several drops of Clear Bath as needed.

10.6.7. Add more water until liquid level is at standard operating height.

10.6.8. Refer to user manual for other maintenance issues including but not limited to fuse replacement, leak checks, and troubleshooting.

10.7. Literature and Supporting Documentation

10.7.1. Zymark Corporation. TurboVap LV Evaporator Workstation: Operator's Manual. P/N 44248, Rev. 11.

10.7.2. Biotage. TurboVap: User's Manual CD. P/N C128079/02, ISO Rev. A.

10.7.3. Biotage. TurboVao LV: User's Manual. P/N C128098 Rev. 02, 2010.

11. Operation and Maintenance of CEREX Pressure Processors

11.1. Purpose

11.1.1. During solid phase extraction (SPE), samples require processing and drying which may be carried out using positive pressure. The CEREX® Pressure Processor is equipped with 48 individual restricted gas ports to allow for positive pressure processing of solid phase extraction columns. It is also equipped with on/off switches, in order to preserve gas and save time by not having to plug ports not in use.

11.2. Scope

11.2.1. Each of two positive pressure manifolds, CEREX System 48 and CEREX System 48-II, will be used routinely during solid phase extraction in the toxicology section of the laboratory. Analysts should be knowledgeable regarding use and maintenance of these processors.

11.3. Equipment

- CEREX System 48 ("System 48") or CEREX System 48-II ("CEREX 48")
- Collection tube rack, 16x100 mm
- SPE Rack, 6CC, for 48 Place Processor
- Waste bin(s) and waste bin rack
- Nitrogen and Air compressed gas cylinder/supply

11.4. Precautions

11.4.1. The SPE Processor does not include a filter on the source gas input. A clean, oil-free gas source is essential to prevent sample contamination.

11.4.2. Compressed nitrogen is the recommended pressure source. If necessary, the unit can operate using high purity filtered air.

11.4.2.1. **Note:** The positive pressure processor must be used in a fume hood in order to prevent inhalation of aspirated biological fluids and organic solvents.

11.5. Procedure

11.5.1. Once samples are ready for extraction, place SPE columns into SPE rack.

11.5.2. Place a waste bin in the waste bin rack and set the SPE rack on top. The black marker on the rack leg should be in the back right corner for correct alignment of racks, column openings, and gas flow.

11.5.3. Turn on gas flow to the unit.

11.5.4. To compress the manifold, place the assembled collection and column racks on the slide platform. Slide rack to the back of the unit using the small handle on the front of

the slide platform. The platform will stop when it reaches the stop located at the back of the unit, under the manifold.

11.5.5. To begin applying pressure to the rack assembly:

11.5.5.1. System 48: Simultaneously lift the black switches on each side of the unit. This will move the rack assembly up and against the manifold.

11.5.5.2. CEREX 48: Simultaneously push the blue buttons on each side of the front of the unit until the manifold moves down onto the rack assembly.

11.5.5.2.1. **Note:** Releasing the blue buttons too soon will cause the depression mechanism to automatically open and return to the manifold to its original position.

11.5.6. Using the 4 toggle switches on the top of the manifold, turn on the rows that will be needed. (If rows are not needed, they may be turned off, if desired, to save nitrogen.)

11.5.7. To adjust the flow, use the "SPE flow rate" knob located on the front of the unit. Three different settings are available to use during extraction.

11.5.7.1. 'Off' – No gas flows to the manifold. This setting should be used when compression and decompression of the manifold are taking place.

11.5.7.2. 'Adjust Flow' – The gas is delivered through the ADJ FLOW regulator and then through the rotameter which is controlled by the needle valve located at its base. This flow is optimal when precise, slow flow is required for the columns. This is the most gentle, limiting flow setting.

11.5.7.3. 'Max Flow' – The gas delivery system provides rapid gas flow to the manifold which can be controlled by adjusting the regulator located below the 'Max Flow' pressure gauge on the front of the unit. This pressure range can be used to maximize flow through the columns and during column drying.

11.5.7.3.1. **Note:** The 'max flow' regulator is a 'locking' knob. It must be adjusted by pulling out the knob. Once the desired pressure is set, it can be 'locked in' by pushing in on the knob.

11.5.8. Decompression of the manifold is done by the following procedure

11.5.8.1. System 48: Simultaneously push down the black switches on each side of the unit. This will move the rack assembly down and away from the column assembly.

11.5.8.2. CEREX 48: Simultaneously push the blue buttons on each side of the front of the unit until the manifold moves up, away from the rack assembly.

11.6. Maintenance

11.6.1. Both System 48 and CEREX 48 are constructed of anodized aluminum, stainless steel, and solvent resistant plastics. However, the following items should be observed during the operation:

11.6.2. Solvent spillage or overflow should be cleaned immediately in order to prevent instrument damage.

11.6.3. The column seal is silicone rubber and should be cleaned with methanol as needed.

11.7. Literature

11.7.1. SPEware Coporation. Cerex 48 Pressure Processor: Operating Instructions, Rev. A.

12. Drug Screen by Enzyme-Linked Immunosorbent Assay (ELISA)

12.1. Purpose

12.1.1. Preliminary screening of blood or urine samples for certain drugs or classes of drugs may be performed using Enzyme-Linked Immunosorbent Assay (ELISA). ELISA relies on drug-specific antibodies which are attached to polystyrene wells on a 96 well microtiter plate. The unknown sample is fortified to the plate with a drug-enzyme conjugate (horseradish peroxidase). Any free drug in the sample competes with the conjugate for antibody binding sites on the surface of the well. After the well is washed, a chromogenic substrate is added and a color is produced by catalysis by horseradish peroxidase. The enzymatic reaction is stopped by using dilute hydrochloric acid and then the absorbance is measured at 450 and 620 nm. The intensity of the color is inversely proportional to the concentration of drug in the sample.

12.2. Scope

12.2.1. This protocol describes usage of ELISA as a preliminary, qualitative screen for certain drugs or classes of drugs.

12.3. Reagents and Kits

12.3.1. Antibody coated polystyrene microtiter plates. These are purchased as commercial kits (Immunalysis Corporation).

- Amphetamine Direct ELISA Kit
- Barbiturates Direct ELISA Kit
- Benzodiazepines Direct ELISA Kit
- Cocaine Metabolite (Benzoylecgonine Specific) Direct ELISA Kit
- Cannabinoids (THCA/CTHC) Direct ELISA Kit
- Carisoprodol Direct ELISA Kit
- Methadone Direct ELISA Kit
- Methamphetamine Direct ELISA Kit
- Opiates Direct ELISA Kit
- PCP Direct ELISA Kit
- Oxycodone Direct ELISA Kit
- Oxycodone/Oxymorphone Direct ELISA Kit
- Zolpidem Direct ELISA Kit

12.3.2. Enzyme Conjugate: Horseradish peroxidase labeled drug and diluted in a protein matrix with protein stabilizers.

12.3.3. Substrate Reagent: Each bottle contains 3, 3', 5, 5'- tetramethylbenzidine (TMB) and urea peroxide in buffer.
12.3.4. Stop Reagent: Each bottle contains 1 N hydrochloric acid (HCl).
12.3.5. Phosphate Buffer Saline (PBS), pH 7.0: 150 mM saline in 100 mM phosphate buffer.
12.3.5.1. PBS may be prepared in-house:
12.3.5.1.1. Add 14.0 g of dibasic sodium phosphate to a 1000 mL volumetric flask and bring to volume with deionized water while stirring.
12.3.5.1.2. In a 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring.
12.3.5.1.3. Mix the two solutions together until a pH of 7.0±0.05 is obtained.
12.3.5.1.4. Add sufficient sodium chloride to bring the concentration to 150 mM.
12.3.5.1.4.1. For 1.5 L phosphate buffer, add 13.2 g sodium chloride.
12.3.5.1.5. Store refrigerated (expiration 6 months).
12.3.6. Note: Allow all reagents to come to room temperature before use.

12.4. Equipment and Materials

12.4.1. Tecan Freedom EVO 75
12.4.2. Tecan HydroFlex Plate Washer
12.4.3. Tecan Sunrise Plate Reader
12.4.4. Vortex mixer
12.4.5. Air displacement pipettes
12.4.6. Repeater pipette

12.5. Stock Standards and Solutions

12.5.1. Blank: Unfortified Blood (Synthetic)
12.5.1.1. Source: Immunalysis Corp., UTAK Laboratories, or equivalent
12.5.1.1.1. For Blank: unfortified blood (synthetic) store in freezer until thawed. Thawed synthetic blood is stored refrigerated.
12.5.1.1.2. Before using a new lot of calibrators/controls in casework, run a blank control for all analytes to ensure there is no contamination. The absorbance of the blank control must be above 1.000 for all analytes.
12.5.2. Blank: Unfortified Urine
12.5.2.1. Before using a new lot of calibrators/controls in casework, run a blank control for all analytes to ensure there is no contamination. The absorbance of the blank control must be above 1.000 for all analytes.
12.5.3. ELISA Stock standards for blood and urine: Concentrations of certified reference materials different from the charts below may be used to obtain the same final concentration.

12.5.4. Mixed Standards for Blood Analysis

12.5.4.1. Blood Cut-off Calibrator

12.5.4.1.1. Blood Cut-off Calibrator Stock

12.5.4.1.1.1. Prepare by spiking the following amounts of the CRMs into a 10 mL class A volumetric flask and QS with methanol.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
Secobarbital	1	250	25,000
(-)-11-nor-9-carboxy-THC	0.1	250	2,500
Methadone	1	125	12,500
Morphine	1	100	10,000
d-Amphetamine	1	100	10,000
d-Methamphetamine	1	100	10,000
Oxazepam	1	100	10,000
Benzoylecgonine	1	100	10,000
Phencyclidine	1	50	5,000
Zolpidem	1	50	5,000

*Cerilliant or equivalent

12.5.4.1.2. Blood Cut-off Calibrator Sub-stock

12.5.4.1.2.1. Transfer 1 mL of the Blood Cut-off Calibrator Stock to a 5 mL class A volumetric flask. Add 250 µL of Carisoprodol 1 mg/mL CRM and QS with PBS.

12.5.4.1.3. Blood Cut-off Calibrator

12.5.4.1.3.1. Add 250 µL of Blood Cut-off Calibrator Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified blood (synthetic), and thoroughly mix.

12.5.4.1.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Blood Cut-off Calibrator	Final Concentration (ng/mL)
d-Amphetamine	20
Secobarbital	50
Oxazepam	20
Benzoylecgonine	20
(-)-11-nor-9-carboxy-THC	5
Carisoprodol	500
Methadone	25
d-Methamphetamine	20
Morphine	20
Phencyclidine	10
Zolpidem	10

12.5.4.2. Blood Controls

12.5.4.2.1. Blood Negative Control Stock

12.5.4.2.1.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with methanol.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
(-)-11-nor-9-carboxy-THC	0.1	250	2,500
Methadone	1	125	12,500
Morphine	1	100	10,000
d-Amphetamine	1	100	10,000
d-Methamphetamine	1	100	10,000
Oxazepam	1	100	10,000
Benzoylecgonine	1	100	10,000
Secobarbital	1	100	10,000
Phencyclidine	1	50	5,000
Zolpidem	1	25	2,500

*Lipomed or equivalent

12.5.4.2.2. Blood Negative Control Sub-Stock

12.5.4.2.2.1. Transfer 1 mL of the Blood Negative Control Stock to a 10 mL class A volumetric flask. Add 100 µL of Carisoprodol 1 mg/mL CRM and QS with PBS.

12.5.4.2.3. Blood Negative Control

12.5.4.2.3.1. Add 250 µL of Blood Negative Control Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified blood (synthetic), and thoroughly mix.

12.5.4.2.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Blood Negative Control	Final Concentration (ng/mL)
d-Amphetamine	10
Secobarbital	10
Oxazepam	10
Benzoylecgonine	10
(-)-11-nor-9-carboxy-THC	2.5
Carisoprodol	100
Methadone	12.5
d-Methamphetamine	10
Morphine	10
Phencyclidine	5
Zolpidem	2.5

12.5.4.2.4. Blood Positive Control Stock

12.5.4.2.4.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with PBS.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
Carisoprodol	1	1,000	100,000
(-)-11-nor-9-carboxy-THC	0.1	200	2,000
Secobarbital	1	100	10,000
Methadone	1	50	5,000
Morphine	1	40	4,000
d-Amphetamine	1	40	4,000
d-Methamphetamine	1	40	4,000
Oxazepam	1	40	4,000
Benzoylecgonine	1	40	4,000
Phencyclidine	1	20	2,000
Zolpidem	1	20	2,000

*Lipomed or equivalent

12.5.4.2.5. Blood Positive Control

12.5.4.2.5.1. Add 500 µL of Blood Positive Control Stock to a 50 mL class A volumetric flask, QS with synthetic blood, and thoroughly mix.

12.5.4.2.5.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Blood Positive Control	Final Concentration (ng/mL)
d-Amphetamine	40
Secobarbital	100
Oxazepam	40
Benzoylecgonine	40
(-)-11-nor-9-carboxy-THC	20
Carisoprodol	1,000
Methadone	50
d-Methamphetamine	40
Morphine	40
Phencyclidine	20
Zolpidem	20

12.5.5. Mixed Standards for Urine Analysis

12.5.5.1. Urine Cut-off Calibrator

12.5.5.1.1. Urine Cut-off Calibrator Stock

12.5.5.1.1.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with methanol.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
Secobarbital	1	300	30,000
Methadone	1	300	30,000
Morphine	1	200	20,000
d-Amphetamine	1	200	20,000
d-Methamphetamine	1	200	20,000
(-)-11-nor-9-carboxy-THC	0.1	200	2,000
Benzoylecgonine	1	150	15,000
Oxazepam	1	100	10,000
PCP	1	25	2,500

*Cerilliant or equivalent

12.5.5.1.2. Urine Cut-off Calibrator Sub-Stock

12.5.5.1.2.1.1. Transfer 1 mL of the Urine Cut-off Calibrator Stock to a 10 mL class A volumetric flask. Add 50 µL of Carisoprodol 1 mg/mL CRM and QS with PBS, and thoroughly mix.

12.5.5.1.3. Urine Cut-off Calibrator

12.5.5.1.3.1. Add 2.5 mL of Urine Cut-off Calibrator Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

12.5.5.1.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Urine Cut-off Calibrator	Final Concentration (ng/mL)
d-Amphetamine	200
Secobarbital	300
Oxazepam	100
Benzoylecgonine	150
(-)-11-nor-9-carboxy-THC	20
Carisoprodol	500
Methadone	300
d-Methamphetamine	200
Morphine	200
Phencyclidine	25

12.5.5.2. Urine Controls

12.5.5.2.1. Urine Negative Control Stock

12.5.5.2.1.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with methanol.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
Methadone	1	150	15,000
Secobarbital	1	150	15,000
Morphine	1	100	10,000
d-Amphetamine	1	100	10,000
d-Methamphetamine	1	100	10,000
(-)-11-nor-9-carboxy-THC	0.1	100	1,000
Benzoylecgonine	1	75	7,500
Oxazepam	1	50	5,000
Phencyclidine	1	12.5	1,250

*Lipomed or equivalent

12.5.5.2.2. Urine Negative Control Sub-Stock

12.5.5.2.2.1. Transfer 1 mL of the Urine Negative Control Stock to a 10 mL class A volumetric flask. Add 25 µL of Carisoprodol 1 mg/mL CRM and QS with PBS, and thoroughly mix.

12.5.5.2.3. Urine Negative Control

12.5.5.2.3.1. Add 2.5 mL of Urine Negative Control Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

12.5.5.2.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Urine Negative Control	Final Concentration (ng/mL)
d-Amphetamine	100
Secobarbital	150
Oxazepam	50
Benzoylecgonine	75
11-nor-9-carboxy-THC	10
Carisoprodol	250
Methadone	150
d-Methamphetamine	100
Morphine	100
Phencyclidine	12.5

12.5.5.2.4. Urine Positive Control Stock

12.5.5.2.4.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with PBS.

Certified Reference Material*	Drug Standard Concentration (mg/mL)	Amount into 10 mL of Methanol (µL)	Final Concentration (ng/mL)
Carisoprodol	1	100	10,000
Secobarbital	1	90	9,000
Methadone	1	60	6,000
(-)-11-nor-9-carboxy-THC	0.1	60	600
Morphine	1	40	4,000
d-Amphetamine	1	40	4,000
d-Methamphetamine	1	40	4,000
Benzoylecgonine	1	30	3,000
Oxazepam	1	30	3,000
PCP	1	5	500

*Lipomed or equivalent

12.5.5.2.5. Urine Positive Control

12.5.5.2.5.1. Add 5 mL of Urine Positive Control Stock to a 50 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

12.5.5.2.5.2. Aliquot out into appropriately labeled tubes and store in the freezer.

Urine Positive Control	Final Concentration (ng/mL)
d-Amphetamine	400
Secobarbital	900
Oxazepam	300
Benzoylecgonine	300
(-)-11-nor-9-carboxy-THC	60
Carisoprodol	1,000
Methadone	600
d-Methamphetamine	400
Morphine	400
Phencyclidine	50

12.5.6. Oxycodone Standards for Blood and Urine Analysis
12.5.6.1. Oxycodone Cut-off Calibrators
12.5.6.1.1. Oxycodone Cut-off Calibrator Stock - 5 µg/mL
12.5.6.1.1.1. Prepare by spiking 50 µL of the Oxycodone 1 mg/mL CRM in methanol into 10 mL class A volumetric flask and QS with PBS.
12.5.6.1.2. Oxycodone Blood Cut-off Calibrator - 10 ng/mL
12.5.6.1.2.1. Transfer 50 µL of the Oxycodone Cut-off Calibrator Stock to an appropriately labeled 25 mL class A volumetric flask and QS with unfortified blood (synthetic) and thoroughly mix.
12.5.6.1.2.2. Aliquot out into appropriately labeled tubes and store in the freezer.
12.5.6.1.3. Oxycodone Urine Cut-off Calibrator - 100 ng/mL
12.5.6.1.3.1. Transfer 500 µL of the Oxycodone Cut-off Calibrator Stock to an appropriately labeled 25 mL class A volumetric flask and QS with unfortified urine and thoroughly mix.
12.5.6.2. Oxycodone Controls
12.5.6.2.1. Oxycodone Control Stock - 5 µg/mL
12.5.6.2.1.1. Prepare by spiking 50 µL of the Oxycodone 1 mg/mL CRM in methanol into 10 mL class A volumetric flask and QS with PBS.
12.5.6.2.2. Oxycodone Blood Negative Control - 5 ng/mL
12.5.6.2.2.1. Transfer 25 µL of the Oxycodone Control Stock to an appropriately labeled 25 mL class A volumetric flask and QS with unfortified blood (synthetic) and thoroughly mix.
12.5.6.2.2.2. Aliquot out into appropriately labeled tubes and store in the freezer.
12.5.6.2.3. Oxycodone Blood Positive Control - 20 ng/mL
12.5.6.2.3.1. Transfer 200 µL of the Oxycodone Control Stock to an appropriately labeled 50 mL class A volumetric flask and QS with unfortified blood (synthetic) and thoroughly mix.
12.5.6.2.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.
12.5.6.2.4. Oxycodone Urine Negative Control - 50 ng/mL
12.5.6.2.4.1. Transfer 250 µL of the Oxycodone Control Stock to an appropriately labeled 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.
12.5.6.2.4.2. Aliquot out into appropriately labeled tubes and store in the freezer.
12.5.6.2.5. Oxycodone Urine Positive Control - 200 ng/mL
12.5.6.2.5.1. Transfer 2 mL of the Oxycodone Control Stock to an appropriately labeled 50 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.
12.5.6.2.5.2. Aliquot out into appropriately labeled tubes and store in the freezer.

12.6. Procedure

12.6.1. Allow all biological specimens and reagents to come to room temperature before starting the procedure.

12.6.2. Label 12x75 mm test tubes accordingly for the following calibrators and quality controls:

12.6.2.1. Blank QC (in duplicate) – drug free matrix.

12.6.2.2. Cut-off Calibrator (in duplicate) – fortified with target drugs at the specified threshold.

12.6.2.3. Negative QC (in duplicate) – fortified with target drugs at half the concentration found in the calibrator with the exception of zolpidem (blood), barbiturates (blood), and carisoprodol (blood).

12.6.2.4. Positive QC (in duplicate) – fortified with target drugs at two times the concentration found in the calibrator with the exception of barbiturates (urine), benzodiazepines (urine), and cannabinoids (blood and urine).

12.6.3. Pipet 100 µL of calibrators, quality controls, and case samples into the appropriately labeled 12x75 mm test tube.

12.6.3.1. Mix all calibrators, quality controls and case samples well prior to sampling by gentle priming of the pipette tip.

12.6.3.2. Aliquoting occurs one case sample at a time.

12.6.3.3. Every 10 case samples must be bracketed by a Positive QC.

12.6.4. For blood, pipet 900 µL of PBS into each test tube. For urine, pipet 1900 µL of PBS into each test tube. Vortex.

12.6.5. The order of samples loaded into the sample racks must be verified against the sequence list by another analyst prior to analysis. This must be documented by initialing and dating the sequence list.

12.6.5.1. The instrument will pipet the following sample volumes for each assay, followed by 100 µL of the appropriate conjugate:

Assay	Volume Blood (µL)	Volume Urine (µL)
Amphetamines	10	10
Barbiturates	20	10
Benzodiazepines	30	40
Benzoylecgonine	75	10
Cannabinoids	50	50
Carisoprodol	10	10
Methadone	25	10
Methamphetamine	25	10
Opiates	10	10
Phencyclidine	10	10
Zolpidem	100	N/A
Oxycodone	10	40

12.6.5.2. After conjugate addition and approximately 1 hour incubation, each plate is washed with deionized water. The plate washer will wash each well six times, two strips at a time.

12.6.5.3. Tecan will pipet 100 µL of the appropriate TMB substrate into each sample well.

12.6.5.4. After TMB incubation, the instrument will pipet 100 µL of the stop solution into each sample well.

12.6.5.5. Each plate is read using the Tecan Sunrise plate reader. The UV spectrophotometer will measure the absorbance at two wavelengths, 450 nm and 620 nm.

12.6.5.6. Print appropriate ELISA data.

12.7. Sequence Table

12.7.1. Every ELISA batch must contain matrix blanks, negative controls, cut-off calibrators, and positive controls of applicable matrix.

ELISA sequence example:
Blank-1
Blank-2
Negative-1
Negative-2
Calibrator-1
Calibrator-2
Positive-1
Positive-2
Samples 1-10
Positive-3
Samples 11-20
Positive-4
Sample 21-30
Positive-5

12.7.2. Microplate Sequence

	1	2	3	4	5	6
A	Blank	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Positive
B	Blank	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE
C	Negative	SAMPLE	Positive	SAMPLE	SAMPLE	SAMPLE
D	Negative	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE
E	Calibrator	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE
F	Calibrator	SAMPLE	SAMPLE	Positive	SAMPLE	SAMPLE
G	Positive	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE
H	Positive	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Positive

12.8. Interpretation of Results

12.8.1. All samples with B/B_0 (%binding) readings BELOW the cut-off calibrator (mean of duplicate analysis) are presumptive positive.

12.8.1.1. %Binding is calculated numerically as follows:

Cut-off Calibrator and Controls: $\%Binding = (Avg.\ Abs._{calibrator}/Avg.\ Abs._{blank\ control}) \times 100$

Case Samples: $\%Binding = (Abs._{case\ sample}/Avg.\ Abs._{blank\ control}) \times 100$

Example:

Cut-off calibrators produce an average absorbance reading of 0.66 (Avg. $Abs._{calibrator}$= 0.66); the average absorbance for the blank controls is 2.0 (Avg. $Abs._{blank\ control}$ = 2.0). The average %Binding is 0.66/2.0) x 100 = 33%.

12.8.2. Results are qualitative in nature. Quantitative results are not possible due to the non-linear binding characteristics of the assay and antibody specificity.

12.9. Acceptance Criteria

12.9.1. During technical review of the ELISA batch file, the following acceptance criteria apply to all assays:

12.9.2. %CV for the Blank QCs (n = 2) must be <20%

12.9.3. The average absorbance and %binding values should be as follows: Blank QC > Negative QC > Cut-off Calibrator > Positive QC

12.9.4. The average absorbance of the Blank QC must be greater than 1.000 for all assays.

12.9.5. Assays not meeting these criteria must be repeated.

12.9.6. Positive case samples must be immediately bracketed by acceptable positive controls to report the results. Any positive case samples bracketed by a control not meeting the acceptance criteria must be re-analyzed. Negative results may be reported upon documented review of the data by the analyst and technical reviewer; the negative case samples with %binding 1-1.5 times the Cut-off Calibrator must be re-analyzed.

12.9.6.1. A Positive QC is considered acceptable if %binding of the Positive QC is less than the average %binding of the Cut-off Calibrators and the average %binding of the Negative QCs.

12.10. Literature and Supporting Documents

12.10.1. Logan, B.K. Recommendations for Toxicological Investigation of Drug Impaired Driving and Motor Vehicle Fatalities. Journal of Analytical Toxicology, 2013.

12.10.2. Smith, M. Immunoassay in Principles of Forensic Toxicology, 4th Edition. Levine B (Ed). AACC Press, Washington DC, 2013, pp. 149-169.

12.10.3. Kerrigan, S and Phillips Jr., W.H. Comparison of ELISAs for Opiates, Methamphetamine, Cocaine Metabolite, Benzodiazepines, Phencyclidine and Cannabinoids in Whole Blood and Urine, Clin Chem: 47, 540-547 (2001).

12.10.4. Tecan Operating Manual Freedom EVO 75 BG/N: 30023958.02.

12.10.5. Tecan HydroFlex Plate Washer Operating Manual, Document Part No: 30026397, 2008-02

12.10.6. Tecan Sunrise Plate Reader Operating Manual, Document Part No.: 30041769, 2008-11

12.10.7. Immunalysis ELISA Kit Inserts, Pomona, CA.

13. Preparation of Reagents for Drug Screening/Confirmation Analyses

13.1. Purpose

13.1.1. This procedure outlines the preparation of reagents used in multiple drug screening and/or confirmation analyses.

13.2. Scope

13.2.1. This procedure applies to drug screening/confirmation analyses that use the reagents in 13.3.

13.3. Reagents and Forms

13.3.1. Organic solvents and inorganic reagents (e.g., salts, acids) should be ACS grade or higher. Deionized water should be obtained using a Millipore Direct Q UV3 water system or from an equivalent source. Preparations of reagents are documented on the Reagent and Quality Control (Volatiles) Preparation Log (LAB-68) or an equivalent form, and include key information regarding chemical names, manufacturers, lot number, preparation date, expiration date, by whom the solution was prepared, and the verification batch name.

- Glacial acetic acid
- Hydrochloric acid
- Ammonium hydroxide
- Dibasic sodium phosphate
- Monobasic sodium phosphate
- Methylene chloride
- Isopropanol
- Methanol
- Ethyl acetate
- Beta-glucuronidase enzyme
- Deionized (DI) water

13.3.2. **1 M Acetic acid**

13.3.2.1. Add 57.2 mL of concentrated glacial acetic acid to a 1 L volumetric flask half-filled with deionized water. Bring to volume with deionized water and thoroughly mix.

13.3.2.2. Store: Room temperature

13.3.2.3. Expiration: 1 year

13.3.3. **100 mM Phosphate buffer, pH 6.0**

13.3.3.1. Weigh approximately 7.0 g of dibasic sodium phosphate and add to a 500 mL volumetric flask. Bring to volume with deionized water while stirring. Weigh approximately 12.0 g of monobasic sodium phosphate and add to a 1000 mL volumetric flask. Bring to volume with deionized water while stirring. Adjust the pH of the monobasic sodium phosphate by adding the dibasic sodium phosphate until the pH reaches 6.0±0.05.

13.3.3.2. Store: Room temperature

13.3.3.3. Expiration: 6 months

13.3.4. **2% Ammonium hydroxide (concentrated) in 80:20 methylene chloride/isopropanol – Elution solvent**

13.3.4.1. In a 100 mL graduated cylinder add 80 mL of methylene chloride and 20 mL of isopropanol. Cap and mix thoroughly. Remove 2 mL of 80:20 methylene chloride/isopropanol and add 2 mL of ammonium hydroxide. Cap and thoroughly mix. Prepare fresh daily.

13.3.4.2. Store: Room temperature

13.3.4.3. Expiration: N/A

13.3.5. **1% Hydrochloric acid in methanol – Acidic methanol**

13.3.5.1. Add 1 mL of concentrated hydrochloric acid to a 100 mL volumetric flask and bring to volume with methanol.

13.3.5.2. Store: Room temperature

13.3.5.3. Expiration: 3 months

14. Evaluation of Results from Gas Chromatography-Mass Spectrometry

14.1. Purpose

14.1.1. The GC-MS is composed of two major building blocks: the gas chromatograph (GC) and the mass spectrometer (MS). The GC utilizes a capillary column. The dimensions (length, diameter, film thickness) and the stationary phase properties of the column facilitate the separation of complex mixtures. Different compounds in a sample and their varied relative affinity for the stationary phase of the column will promote separation of these compounds as the sample travels through the length of the column. The compounds are retained by the column and then elute from the column at different times (retention time). The mass spectrometer is the detector portion of the instrument. As the compounds elute off the column and enter the MS, they are ionized, fragmented, separated and detected based upon their mass-to-charge ratio (m/z). The results obtained from GC-MS analysis must be evaluated to determine the acceptability of the results. This applies to both quantitative and qualitative results.

14.2. Scope

14.2.1. This policy applies to all GC-MS analyses unless otherwise stated in the drug-specific SOP.

14.3. Qualitative Analysis

14.3.1. All mass spectral libraries and/or reference spectra must be approved by the technical reviewer.

14.3.2. When library search is used to identify a substance, case samples will be evaluated for library match %, retention time difference, peak shape (if applicable), and whether there is a sufficient number of mass spectral fragments before comparing the case (unknown) spectrum to the reference spectrum.

14.3.3. The following acceptance criteria may be used as guidelines, *but not as absolutes*, to identify a substance through its mass spectrum:

14.3.3.1. Usually the base peak will be the same and isotope clusters will contain the same major ions and relative abundances.

14.3.3.2. When compared to a reference spectrum, the spectra must contain strong similarities.

14.3.3.3. Any differences between the reference spectrum and the unknown spectrum must be carefully evaluated for acceptability.

14.3.4. The following criteria must be met for qualitative identification of a compound:

14.3.4.1. Retention times (RT) and/or relative retention times (RRT) must be within ±2% of the target for SIM analyses.

14.3.4.2. Case mass spectrum must be of comparable quality to a reference spectrum in mass assignment and intensity.

14.3.4.3. Any extraneous ions of significant intensity that are not found in the reference spectrum must be demonstrated as being from background or known interference by using an extracted ion chromatogram.

14.3.5. Qualitative identification is determined using characteristic retention time and mass spectral characteristics. Deuterated internal standards in each sample are used to identify the correct retention time of the drug. For SIM, all three characteristic ions must be present. Ion intensity and ratios should be taken into consideration. Ion ratios should be within ±20% or two standard deviations of the average ratio from all calibrators used in the calibration response curve, as validated to be appropriate for the ion ratio, to meet acceptance criterion.

14.4. Quantitative Analysis

14.4.1. Qualitative identification described in sections 14.3.1 – 14.3.4 must be met.

14.4.2. Concentration of a drug is determined using linear regression analysis. At least 4 calibrators and a blank blood sample must be used for each quantitation. R^2 values of at least 0.99 should be attained following linear regression analysis.

14.4.2.1. If at least 6 calibrators are included in the run, two may be excluded if the response factor is not within ±20% of the remaining calibrators or the ion ratios are unacceptable. The response factor is defined as follows: (Peak Area of the Internal Standard/Peak Area of the Calibrator) x Calibrator Concentration.

14.4.2.2. If the response factor is within range, it is permissible to exclude the point from the calibration provided that the independent control bracketing reporting analytes is still within acceptable limits and the reviewer is in agreement. The calculated concentration of a calibrator must be within ±20% of the expected value, with the exception of the LOQ, which must be within ±25% of the expected value.

14.4.2.3. When determining the acceptability of a calibrator, the concentration range is taken into account. Acceptable ion ratios must be met as described in “In-Process Calibration and Quality Control for Drug Screening/Confirmation Testing,” section 8.3.3.1 in order for a calibrator to be included in the calibration response curve. If a calibrator needs to be excluded from the curve, the action should be described and justified in the batch file.

14.4.3. Quantitative values above the highest calibrator must be reported as greater than the appropriate calibrator concentration if no dilution has performed. Quantitative values below the lowest calibrator will be reported as none detected. Dilution of a specimen may be necessary for samples that contain an elevated concentration of drug. If a sample volume used is different from the volume stated in the method SOP (e.g.,

dilution, insufficient sample volume, interference), the sample volume must be documented in the case record.

14.4.4. For quantitative reporting, the calculated value of the in-house or external control should be within ±20% of the target concentration.

14.5. Reinjection Guidelines

14.5.1. Reinjections can be made up to 24 hours after the completion of the initial injection of the particular sample, unless otherwise stated in specific SOP.

14.5.2. Case samples: Reinjections must be run with negative control and bracketed by a high quality control at the beginning and a low quality control at the end (positive controls for qualitative assays). The quality controls must meet the acceptance criteria in order for the reinjections to be reported.

14.5.2.1. If reinjections can be inserted into the sequence before the batch has completed, they should be included before the washes along with an acceptable low quality control at the end. The quality controls must meet the acceptance criteria in order for the reinjections to be reported.

14.5.3. Calibrators or control samples: the samples with known analyte concentrations can be re-injected if the samples have initially misinjected (i.e., analyte as well as internal standard data are not collected).

14.6. Carryover Guidelines

14.6.1. If a case sample is greater than the ULOQ, it may be necessary to dilute the sample as stated in section 14.4.3.

14.6.2. The sample immediately following the case sample greater than the ULOQ needs to be evaluated for the potential of carryover. This is done by re-injecting the case sample greater than the ULOQ followed by a negative control. The Laboratory will deem no carryover has occurred and results for the sample following the case sample greater than the ULOQ may be reported if 1) the negative control remains negative and 2) the response of the re-injected case sample with high concentration has not been decreased by more than 50% compared to its response of the initial injection.

14.6.2.1. If the outcome fails to meet the carryover criteria, the case sample that followed the case sample with a drug concentration greater than the ULOQ will be re-extracted and re-analyzed for that particular drug.

15. Drug Screen by Gas Chromatography-Mass Spectrometry

15.1. Purpose

15.1.1. A full-scan analysis is performed for the screening of basic, acidic, and neutral drugs using solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) in electron ionization (EI) mode. Mepivacaine and pentobarbital-d5 are used as internal standards.

15.2. Scope

15.2.1. This procedure describes an initial screening analysis for commonly encountered basic, acid, and neutral drugs of interest in biological specimens, including blood and urine.

15.3. Reagents and Solvents

15.3.1. 1 M Acetic acid
15.3.2. 100 mM Phosphate buffer, pH 6.0
15.3.3. Elution solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol
15.3.4. Acidic methanol (1% HCl in methanol)
15.3.5. Methanol
15.3.6. Ethyl acetate
15.3.7. Hexane
15.3.8. Deionized (DI) water
15.3.9. Blank blood (preserved with potassium oxalate and sodium fluoride)
15.3.10. Blank urine

15.4. Equipment and Materials

15.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
15.4.2. Repeater pipette
15.4.3. Cerex® Clin II, 6mL columns, 50 mg
15.4.4. Positive pressure SPE manifold
15.4.5. Analytical/Top-loading balance
15.4.6. pH meter
15.4.7. Evaporator
15.4.8. Vortex mixer
15.4.9. Centrifuge

15.5. Instrumentation

15.5.1. Parameters

15.5.1.1. Capillary Column: 15 m DB-5MS UI Agilent J&W Column (or equivalent), 0.25 mm id X 0.25 µm film thickness. The flow rate is 6.6 mL/min for column 1 and 3.4 mL/min for column 2, with an injection volume of 1 µL in splitless mode. NOTE: This is a retention time locked method to pentobarbital-d5 at 2.75 minutes so the pressure and flow are subject to change.

15.5.1.2. GC-MS: Agilent 7890B-5977B MSD

Initial Temperature:	100 °C hold for 0.25 minutes 40 °C/min to 325 °C hold for 1.1 minutes
Total Run Time:	6.97 minutes
Injector Temperature:	280 °C
Interface Temperature:	310 °C
MS Quads:	180 °C
MS Source:	300 °C

15.5.1.3. Full-Scan acquisition: FAST-BAN.M

15.5.2. Performance Check

15.5.2.1. Wash solvents for the autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 2 pre and 8 post injection rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

15.5.2.2. Ensure that the tune verification and system suitability injection have been completed, all acceptance criteria met, and maintenance log filled out.

15.6. Standards and Solutions

15.6.1. Preparation of the Positive Control: a methanolic solution of commonly encountered drugs. Refer to the Working Stock/Standard Preparation Log (LAB-27) for the composition and concentration of the current drug mixture (for blood and urine).

15.6.2. Preparation of the Carryover Control: a methanolic solution of commonly encountered drugs. Refer to the Working Stock/Standard Preparation Log (LAB-27) for composition and concentration of the current drug mixture (for blood and urine).

Analyte	Positive Control		Carryover Control	
	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)
Fluoxetine	32	800	160	4000
Butalbital	10	250	100	2500
Phenytoin	10	250	50	1250
Phenobarbital	10	250	20	500
Secobarbital	10	250	100	2500
Nortriptyline	8	200	80	2000
Trazodone	8	200	40	1000
Methadone	4	100	40	1000
Tramadol	4	100	40	1000
Doxylamine	4	100	20	500
Venlafaxine	4	100	20	500
Diphenhydramine	4	100	10	250
Ketamine	2	50	10	250
Amitriptyline	2	50	20	500
Cyclobenzaprine	2	50	20	500
Imipramine	2	50	20	500
Meperidine	2	50	20	500
Propoxyphene	2	50	20	500
Sertraline	2	50	20	500
Zolpidem	1	25	10	250

The above drug panel and concentrations are subject to change depending on the capability of the instrument.

15.6.3. GC-MS Screen Working Standards for Internal Standards (for blood and urine).

15.6.3.1. This analysis requires preparation of three solutions. Each solution contains mepivacaine at 1 mg/mL, mepivacaine at 10 µg/mL, or pentobarbital-d5 at 10 µg/mL.

15.6.3.1.1. Preparation of the 1 mg/mL Basic Internal Standard Stock Solution: add 10 mg of mepivacaine to a 10 mL volumetric flask and bring to volume with methanol.

15.6.3.1.2. Preparation of the 10 µg/mL Basic Internal Standard: add 100 µL of the 1 mg/mL Basic Internal Standard Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

15.6.3.1.3. Preparation of the 10 µg/mL Acid-Neutral (AN) Internal Standard: add 100 µL of 1 mg/mL CRM of pentobarbital-d5 to a 10 mL volumetric flask and bring to volume with methanol.

15.7. Fortification Guide

Volume of Matrix (mL)	Target Concentration	Drug Standard Concentration	Volume Added (µL)
2	Mixed	Positive QC	50
2	Mixed	Carryover QC	50

15.8. Extraction Procedure

15.8.1. Allow all biological specimens to come to room temperature before starting the procedure.
15.8.2. Label all round bottom screw cap tubes accordingly.
15.8.3. Add corresponding drug standard to controls. Subsequently dry down at approximately 50 °C under nitrogen at 20 psi for 1 minute.
15.8.4. Pipet 2 mL of drug-free blood/urine for matrix blank, negative control, positive controls, and case samples into the appropriate labeled tubes.
15.8.5. Using a repeater pipette, add 50 µL of both internal standards to each sample to obtain the final concentration of 250 ng/mL. Vortex.
15.8.6. Add 4 mL of 100 mM sodium phosphate buffer, pH 6. Vortex.
15.8.7. Centrifuge tubes at approximately 3082 rcf for 10 minutes.
15.8.8. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
15.8.8.1. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
15.8.8.2. Add 1 mL of deionized water and aspirate.
15.8.8.3. Add 1 mL of 1M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.
15.8.8.4. Add 1 mL of hexane and aspirate.
15.8.8.5. Place conical tubes into the vacuum manifold.
15.8.8.6. Elute acidic and neutral drugs with 1 mL ethyl acetate.
15.8.8.7. Remove conical tubes and replace with waste reservoir.
15.8.8.8. Add 1 mL methanol and aspirate. Dry column under full pressure for 5 minutes.
15.8.8.9. Replace the conical tubes containing the acidic/neutral fraction into the vacuum manifold.
15.8.8.10. Elute basic drugs by adding 1 mL of elution solvent, prepared fresh daily.
15.8.9. Add 30 µL of acidic methanol to all elution tubes.
15.8.10. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi for 15 minutes.
15.8.11. Reconstitute in 30 µL of ethyl acetate using the repeater pipet, vortex, and transfer to appropriately labeled autosampler vials with inserts and cap tightly.
15.8.12. Inject 1 µL onto the GC-MS using FAST-BAN.M.

15.9. Sequence Table

15.9.1. Every screen batch must contain a matrix blank, carryover control, and 10% positive controls of applicable matrix.

BAN sequence example (blood and urine):
Matrix Blank (Blood)
Carryover Control (Blood)
Negative Control (Blood)
Positive Control (Blood)
10 Blood Case Samples
Positive Control (Blood)
10 Blood Case Samples
Positive Control (Blood)
Matrix Blank (Urine)
Carryover Control (Urine)
Negative Control (Urine)
Positive Control (Urine)
10 Urine Case Samples
Positive Control (Urine)

15.10. Data Analysis

15.10.1. Interpretation of results using Deconvolution Reporting Software (DRS)

15.10.1.1. The following criteria will be used to consider a positive finding without doing additional library searches.

15.10.1.2. The match factor should be within the range of 60 to 100.

15.10.1.3. Retention time has already been factored into the deconvolution match factor. Components with good spectral matches but a retention time not matching the databases are automatically penalized according to difference in expected and actual retention times. There may be occasions where retention time shifts occur due to the presence of overloading constituents. These instances may require alternate means of peak identification (i.e., screener or library searching).

15.10.1.4. Additional confirmatory data can be generated within the AMDIS software to display ion overlays and the ability to search unidentified deconvolution spectra using the NIST library.

15.10.1.5. When determining the presence or absence of a drug, the following table should be used as a guideline.

Determining Factors	Positive	None Detected
Spectra	Most of the characteristic ions are present, the most abundant ones, at the correct ratio.	Many of the characteristic ions are missing, including the ones with a significant relative abundance and/or with the correct ion ratio.
DRS Match	60-100%	Less than 60%

15.10.2. Screen Data Analysis

15.10.2.1. General review of the Batch (to be done at the end of the run)

15.10.2.1.1. Examine the MS chromatogram of the matrix blank for significant peaks that are not considered normal. If unusual peaks are found, identify on the MS. If unusual findings are observed, review data with supervisor/manager for decision on acceptability.

15.10.2.1.2. Examine the entire run to assure that samples are in proper order and the analysis was performed successfully (e.g., no baseline injections, no baseline problems, etc.).

15.10.2.1.3. Spectral match will not be evaluated for rejection or acceptance for drugs that have not been validated.

15.10.2.2. Batch Review of Controls

15.10.2.2.1. For screen analysis, the compounds contained in these controls will be identified using DRS.

15.10.2.2.2. All drugs in positive controls must be detected by DRS to be acceptable.

15.10.2.2.3. All blanks must be negative for reporting analyte(s); if a blank is positive for reporting analyte(s), it must be evaluated for carry-over to ensure the results of case samples are not compromised.

15.10.2.3. Case Sample Review

15.10.2.3.1. Case samples should be reviewed in order they were injected to allow for monitoring of performance over the course of the run.

15.10.2.3.2. Case samples must be bracketed by acceptable positive controls.

15.10.2.3.3. Internal standards in all case samples must be detected by DRS.

15.10.2.3.4. Case samples with a DRS match greater than 60 will be sent to confirmation.

15.10.2.3.5. If DRS identifies a compound not presently in the current drug mixtures at a significant concentration, the case sample will be evaluated to see if outsourcing is necessary for confirmation.

15.10.2.3.6. If a case sample has a drug response greater than the carryover control it will be evaluated by toxicology management to see if outsourcing is necessary for quantitative confirmation.

15.11. Literature and Supporting Documents

15.11.1. Chen, Xiao-Hua, et al. Isolation of Acidic, Neutral, and Basic Drugs from Whole Blood Using a Single Mixed-Mode Solid-Phase Extraction Column. Journal of Analytical Toxicology. 1992;16:351-355.

15.11.2. Method File: FAST-BAN.M

16. Basic, Acidic, and Neutral Drug Qualitative Confirmation by Gas Chromatography-Mass Spectrometry

16.1. Purpose

16.1.1. A full-scan analysis is performed for the qualitative confirmation of basic, acidic, and neutral drugs using solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) in electron ionization (EI) mode. Mepivacaine and pentobarbital-d5 are used as internal standards.

16.2. Scope

16.2.1. This procedure functions as an initial screen as well as a qualitative confirmation for commonly encountered drugs of interest in blood and urine specimens.

16.2.2. This procedure may be suitable for the qualitative confirmation of benzodiazepines, barbiturates, anticonvulsants, antihistamines, tricyclic antidepressants, non-tricyclic antidepressants (e.g., selective serotonin reuptake inhibitors (SSRIs) and related compounds), muscle relaxants, narcotic analgesics, dissociative anesthetics, and other abused drugs. Other targeted procedures are available for quantitative analysis of several basic, acidic, and neutral drugs.

16.3. Reagents and Solvents

16.3.1. 1 M Acetic acid

16.3.2. 100 mM Phosphate buffer, pH 6.0

16.3.3. Elution solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol

16.3.4. Acidic methanol (1% HCl in methanol)

16.3.5. Methanol

16.3.6. Ethyl acetate

16.3.7. Hexane

16.3.8. Deionized (DI) water

16.3.9. Blank blood (preserved with potassium oxalate and sodium fluoride)

16.3.10. Blank urine

16.4. Equipment and Materials

16.4.1.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)

16.4.1.2. Repeater pipette

16.4.1.3. Cerex® Clin II, 6mL columns, 50 mg

16.4.1.4. Positive pressure SPE manifold

16.4.1.5. Analytical/Top-loading balance

16.4.1.6. pH meter

16.4.1.7. Evaporator

16.4.1.8. Vortex mixer
16.4.1.9. Centrifuge

16.5. Instrumentation

16.5.1. Parameters

16.5.1.1. Capillary Column: 30 m DB-5MS Agilent J&W Column (or equivalent), 0.25 mm id X 0.25 µm film thickness. The flow rate is 1.38 mL/min with an injection volume of 2 µL in pulsed split mode (4:1). NOTE: This is a retention time locked method to mepivacaine at 13.1 minutes so the pressure and flow are subject to change.

16.5.1.2. GC-MS: Agilent 7890A-5975C MSD

Initial Temperature:	110 °C hold for 0.5 minutes 20 °C/min to 180 °C for 2.5 minutes 5 °C/min to 230 °C for 0.1 minutes 25 °C/min to 320 °C for 4.5 minutes
Total Run Time:	24.7 minutes
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

16.5.1.3. Full-Scan acquisition: QConfirm.M

16.5.2. Performance Check

16.5.2.1. Wash solvents for the autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post injection rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

16.5.2.2. Ensure that the tune verification and system suitability have been completed, all acceptance criteria are met, and maintenance log is filled out.

16.6. Standards and Solutions

16.6.1. Basic Qualitative Confirm Working Standards (for blood and urine)

16.6.1.1. This analysis requires preparation of four solutions. Refer to the Working Stock/Standard Preparation Log (LAB-27) for the compositions and concentrations of the current basic cut-off calibrator and positive control. The internal standard solutions contain mepivacaine at 1 mg/mL and 10 µg/mL.

16.6.1.1.1. Preparation of the Basic Cut-off Calibrator: a methanolic solution of commonly encountered basic drugs. Refer to the Working Stock/Standard Preparation Log (LAB-27) for the composition and concentration of the current basic drug mixture.

16.6.1.1.2. Preparation of the Basic Positive Control: a methanolic solution of commonly encountered basic drugs. Refer to the Working Stock/Standard Preparation Log (LAB-27) for composition and concentration of the current basic drug mixture.

Analyte	Cut-off Calibrator		Positive Control	
	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)
Phenytoin	20	500	20	1000
Fluoxetine	4	100	4	200
Meperidine	4	100	4	200
Nortriptyline	4	100	4	200
Tramadol	4	100	4	200
Trazodone	4	100	4	200
Amitriptyline	2	50	2	100
Diphenhydramine	2	50	2	100
Doxylamine	2	50	2	100
Ketamine	2	50	2	100
Imipramine	2	50	2	100
Methadone	2	50	2	100
Propoxyphene	2	50	2	100
Sertraline	2	50	2	100
Venlafaxine	2	50	2	100
Cyclobenzaprine	1	25	1	50
Zolpidem	1	25	1	50

The above drug panel and concentrations are subject to change depending on the capability of the instrument.

16.6.1.1.3. Preparation of the 1 mg/mL Basic Internal Standard Stock Solution: add 10 mg of mepivacaine to a 10 mL volumetric flask and bring to volume with methanol.

16.6.1.1.4. Preparation of the 10 µg/mL Basic Internal Standard: add 100 µL of the 1 mg/mL Basic Internal Standard Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

16.6.2. Acidic-Neutral (AN) Qualitative Confirm Working Standards (for blood and urine)

16.6.2.1. This analysis requires preparation of three solutions. The two solutions contain butalbital, carisoprodol, meprobamate, phenobarbital, and secobarbital at 10 µg/mL. The internal standard solution contains pentobarbital-d5 at 10 µg/mL.

16.6.2.1.1. Preparation of the 10 µg/mL Acidic-Neutral (AN) Cut-off Calibrator: add 100 µL of each 1 mg/mL CRM of butalbital, carisoprodol, meprobamate, phenobarbital, and secobarbital to a 10 mL volumetric flask and bring it to volume with methanol.

16.6.2.1.2. Preparation of the 10 µg/mL Acidic-Neutral (AN) Positive Control: add 100 µL of each 1 mg/mL CRM of butalbital, carisoprodol, meprobamate, phenobarbital, and secobarbital to a 10 mL volumetric flask and bring it to volume with methanol.

16.6.2.1.3. Preparation of the 10 µg/mL Acidic-Neutral (AN) Internal Standard: add 100 µL of 1 mg/mL CRM of pentobarbital-d5 to a 10 mL volumetric flask and bring to volume with methanol.

16.7. Fortification Guide

16.7.1. Basic Qualitative Confirmation

Volume of Matrix (mL)	Target Concentration	Drug Standard Concentration	Volume Added (µL)
2	Mixed	Cut-off Calibrator	50
2	Mixed	Positive QC	100

16.7.2. Acidic-Neutral Qualitative Confirmation

Volume of Matrix (µL)	Target Concentration (ng/mL)	Drug Standard Concentration	Volume Added (µL)
500	500	Cut-off Calibrator	25
500	1000	Positive QC	50

16.8. Extraction Procedure

16.8.1. Basic Extraction

16.8.1.1. Allow all biological specimens to come to room temperature before starting the procedure.

16.8.1.2. Label all round bottom screw cap tubes accordingly.

16.8.1.3. Add corresponding drug standard to cut-off calibrator and positive controls. Subsequently dry down at approximately 50 °C under nitrogen at 20 psi for 1 minute.

16.8.1.4. Pipet 2 mL of drug-free blood/urine for matrix blank, negative control, positive controls, and case samples into the appropriate labeled tubes.

16.8.1.5. Using a repeater pipette, add 100 µL of internal standard (mepivacaine) to each sample to obtain the final concentration of 500 ng/mL. Vortex.

16.8.1.6. Add 4 mL of 100 mM sodium phosphate buffer, pH 6. Vortex.

16.8.1.7. Centrifuge tubes at approximately 3082 rcf for 10 minutes.

16.8.1.8. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.

16.8.1.8.1. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
16.8.1.8.2. Add 1 mL of deionized water and aspirate.
16.8.1.8.3. Add 1 mL of 1M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.
16.8.1.8.4. Add 1 mL of methanol and aspirate. Dry columns under full pressure for 5 minutes.
16.8.1.8.5. Elute basic drugs by adding 1 mL of elution solvent, prepared fresh daily.
16.8.1.9. Add 30 µL of acidic methanol to all elution tubes.
16.8.1.10. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi for 7-9 minutes.
16.8.1.11. Reconstitute in 30 µL of ethyl acetate using a repeater pipette, vortex, and transfer to appropriately labeled autosampler vials with inserts and cap tightly.
16.8.1.12. Inject 2 µL onto the GC-MS using QConfirm.M method. Data analysis method is Basicdrugs.M.

16.8.2. Acidic-Neutral Extraction

16.8.2.1. Allow all biological specimens to come to room temperature before starting the procedure.
16.8.2.2. Label all round bottom screw cap tubes accordingly.
16.8.2.3. Add corresponding drug standard to cut-off calibrator and positive controls. Subsequently dry down at approximately 50 °C under nitrogen at 20 psi for 1 minute.
16.8.2.4. Pipet 500 µL of drug-free blood/urine for matrix blank/negative control/positive controls and case samples into the appropriate labeled tubes.
16.8.2.5. Using a repeater pipette, add 50 µL of internal standard (pentobarbital-d5) to each sample to obtain the final concentration of 1000 ng/mL. Vortex.
16.8.2.6. Add 4 mL of 100 mM sodium phosphate buffer, pH 6. Vortex
16.8.2.7. Centrifuge tubes at approximately 3082 rcf for 10 minutes.
16.8.2.8. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
16.8.2.8.1. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
16.8.2.8.2. Add 1mL of deionized water and aspirate.
16.8.2.8.3. Add 1mL of 1M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.
16.8.2.8.4. Add 1mL of hexane and aspirate. Dry columns at 20 psi pressure for 2 minutes.
16.8.2.8.5. Elute acidic and neutral drugs by adding 1 mL of ethyl acetate.
16.8.2.9. Add 30 µL of acidic methanol to all elution tubes.
16.8.2.10. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi for 5-7 minutes.

16.8.2.11. Reconstitute in 30 µL of ethyl acetate using a repeater pipette, vortex, and transfer to appropriately labeled autosampler vials with insert and cap tightly.

16.8.2.12. Inject 2 µL onto the GC-MS using QConfirm.M. Data analysis method is Acidicdrugs.M.

16.9. Sequence Table

16.9.1. Every batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls of applicable matrix.

QConfirm sequence example (blood and urine):

Matrix Blank (Blood)
Cut-off Calibrator (Blood)
Negative Control (Blood)
Positive Control (Blood)
10 Blood Case Samples
Positive Control (Blood)
10 Blood Case Samples
Positive Control (Blood)
Matrix Blank (Urine)
Cut-off Calibrator (Urine)
Negative Control (Urine)
Positive Control (Urine)
10 Urine Case Samples
Positive Control (Urine)

16.10. Data Analysis

16.10.1. Interpretation of results using the Deconvolution Reporting Software (DRS)

16.10.1.1. The following criteria will be used to consider a positive finding without doing additional library searches.

16.10.1.1.1. The match factor should be within the range of 60 to 100. Typically, any component having a match factor greater than 80 may be considered positive, depending on the abundance. Components with a match factor less than 80 but greater than 60 may also be considered positive but require careful scrutiny of the data to ensure that retention times and peak shape are acceptable and unique ions are present.

16.10.1.1.2. Retention time has already been factored into the deconvolution match factor. Components with good spectral matches but a retention time not matching the databases are automatically penalized according to difference in expected and actual retention times. There may be occasions where retention time shifts occur

due to the presence of overloading constituents. These instances may require alternate means of peak identification (i.e., screener or library searching).

16.10.1.1.3. Additional confirmatory data can be generated within the AMDIS software to display ion overlays and the ability to search unidentified deconvolution spectra using the NIST library.

16.10.1.1.4. When determining the presence or absence of a drug, the following table should be used as a guideline.

16.10.1.2. Data Analysis Methods: Basicdrugs.M and Acidicdrugs.M.

Determining Factors	Positive	None Detected
Response	Unambiguous/Solid. Approximately more than 3 times signal to noise ratio.	Less than approximately 3 times the signal to noise ratio.
Peak Shape	Defined Peak Shape	No symmetrical peak shape or almost down to the baseline
Spectra	Most of the characteristic ions are present; the most abundant ones, at the correct ratio.	Many of the characteristic ions are missing, including the ones with a significant relative abundance and/or with the correct ion ratio.
DRS Match	60-100%	Less than 60%
ChemStation Quantification	Must be greater than or equal to the concentration of the cut-off and acceptable positive control must bracketed the case sample.	The concentration is less than the cut-off and the sample is bracketed by acceptable positive controls.

16.10.2. Qualitative Confirmation Data Analysis

16.10.2.1. QC Review

16.10.2.2. General Review of the Batch (to be done at the end of the run)

16.10.2.2.1. Examine the MS chromatogram of the matrix blank for significant peaks that are not considered normal. If unusual peaks are found, identify on the MS. If unusual findings are observed, review data with supervisor/manager for decision on acceptability.

16.10.2.2.2. Review chromatography of positive controls throughout the batch by the MS for unusual peak shape, tailing, fronting, splitting, or interferences. Questionable performance must be reviewed with the supervisor/manager for acceptability.

16.10.2.2.3. Examine the entire run to assure that samples are in proper order and the analysis was performed successfully (e.g., no baseline injections, no baseline problems, etc.).

16.10.2.2.4. Evaluate run recovery by comparing IS recovery of case samples to IS recovery of the cut-off calibrator and controls.

16.10.2.3. Batch Review of Controls

16.10.2.3.1. For qualitative-only analysis, the compounds contained in these controls will be identified using DRS and ChemStation.

16.10.2.3.2. All drugs in positive controls must be present and above the cut-off concentration.

16.10.2.3.3. All blanks must be negative for reporting analyte(s); if a blank is positive for reporting analyte(s), it must be evaluated for carry-over to ensure the results of case samples are not compromised.

16.10.2.4. Case Sample Review

16.10.2.4.1. Case samples should be reviewed in order they were injected to allow for monitoring of performance over the course of the run.

16.10.2.4.2. Internal standard response must be above 50% of the average of the cut-off calibrator and all controls and no higher than 200% to report qualitative, confirmed results.

16.10.2.4.3. Case samples must be bracketed by acceptable positive controls.

16.10.2.5. Batch outcomes not described in 16.10.2.4 will be evaluated on a case by case basis and acceptability will be determined by toxicology management.

16.10.3. Reinjection Guidelines:

16.10.4. Reinjections can be made up to 48 hours after the completion of the initial injection of the particular sample.

16.11. Literature and Supporting Documents

16.11.1. Chen, Xiao-Hua, et al. Isolation of Acidic, Neutral, and Basic Drugs from Whole Blood Using a Single Mixed-Mode Solid-Phase Extraction Column. Journal of Analytical Toxicology. 1992;16:351-355.

16.11.2. Method File: QConfirm.M

17. Amphetamines Confirmation by Gas Chromatography-Mass Spectrometry

17.1. Purpose

17.1.1. A targeted analysis is performed for confirmatory analysis of amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA) by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Drugs are isolated from the matrix using a basic extraction. Primary and secondary amines are derivatized with PFPA to form perfluoroalkyl derivatives. Deuterated internal standard and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

17.2. Scope

17.2.1. This procedure describes a confirmatory analysis of amphetamines in biological specimens including, but not limited to blood. Urine confirmations are reported only qualitatively.

17.3. Reagents and Solvents

17.3.1. 0.1 M Acetic acid: add approximately 5.7 mL of concentrated glacial acetic acid to a 1 L volumetric flask half-filled with deionized water. Bring to volume with deionized water and thoroughly mix. Store at room temperature. Expiration: 1 year

17.3.2. 100 mM Phosphate buffer, pH 6.0

17.3.3. Elution Solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol. Prepare fresh daily.

17.3.4. Methanol

17.3.5. Ethyl acetate

17.3.6. Deionized (DI) water

17.3.7. Acidic methanol (1% HCl in methanol)

17.3.8. PFPA (pentafluoropropionic anhydride)/PFAA (pentafluoropropionic acid anhydride)

17.3.9. Utak Drugs of Abuse Level 1

17.3.10. Blank blood (preserved with potassium oxalate and sodium fluoride)

17.3.11. Blank urine

17.4. Equipment and Materials

17.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)

17.4.2. Repeater pipette

17.4.3. UCT Clean Screen® DAU, 6 mL columns, 200 mg

17.4.4. Positive pressure SPE manifold

17.4.5. Analytical/Top-loading balance

17.4.6. pH meter
17.4.7. Evaporator
17.4.8. Vortex mixer
17.4.9. Centrifuge
17.4.10. Heating block

17.5. Instrumentation

17.5.1. Parameters

17.5.1.1. Capillary Column: 30 m HP-1MS Agilent J&W GC Column (or equivalent), 0.25 mm id X 0.25 µm film thickness. The flow rate is 1.07 mL/min with an injection volume of 1 µL in split mode (12:1).

17.5.1.2. GC-MS: Agilent GC 7890A-5975C MSD

Initial Temperature:	100 °C hold for 1.8 min 20 °C/min to 200 °C hold for 4 min 50 °C/min to 300 °C
Total Run Time:	12.8 min
Injector Temperature:	190 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

17.5.1.3. SIM acquisition: AMPS.M/AMPS_U.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
+/-Amphetamine-d11	194.0	128.0, 98.0	4.69	1/x
+/-Amphetamine	190.0	118.0, 91.0	4.73	
+/-Methamphetamine-d14	211.0	163.0, 128.0	5.45	1/x
+/-Methamphetamine	204.0	160.0, 118.1	5.50	
+/-MDMA-d5	208.0	344.0	7.55	$1/x^2$
+/-MDMA	204.0	339.0, 162.0	7.58	
+/-MDEA-d6	224.0	165.0, 194.0	7.88	$1/x^2$
+/-MDEA	218.0	190.0, 162.0	7.90	
*Retention Time (RT) varies with column length.				

17.5.2. Performance Check

17.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash

solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

17.5.3. Ensure that the tune verification and system suitability have been completed, all acceptance criteria met, and maintenance log filled out.

17.6. Standards and Solutions

17.6.1. Amphetamines Working Standards for Calibrators (for blood and urine)

17.6.1.1. This analysis requires preparation of two solutions. The solutions contain (±)-amphetamine, (±)-methamphetamine, (±)-MDMA, (±)-MDEA at 5 µg/mL and 1 µg/mL.

17.6.1.1.1. Preparation of the 5 µg/mL Amphetamines Calibrator: add 50 µL of 1 mg/mL CRM of (±)-amphetamine, (±)-methamphetamine, (±)-MDMA, (±)-MDEA to a 10 mL volumetric flask and bring to volume with methanol.

17.6.1.1.2. Preparation of the 1 µg/mL Amphetamines Calibrator: add 25 µL of 1 mg/mL CRM of (±)-amphetamine, (±)-methamphetamine, (±)-MDMA, (±)-MDEA to a 25 mL volumetric flask and bring to volume with methanol.

17.6.2. Amphetamines Working Standards for Quality Controls (for blood and urine)

17.6.2.1. This analysis requires preparation of two solutions. The solutions contain (±)-amphetamine, (±)-methamphetamine, (±)-MDMA, (±)-MDEA at 10 µg/mL and 1 µg/mL.

17.6.2.1.1. Preparation of the 10 µg/mL Amphetamines Control: add 100 µL of 1 mg/mL CRM of (±)-amphetamine, (±)-methamphetamine, (±)-MDMA, (±)-MDEA to a 10 mL volumetric flask and bring to volume with methanol.

17.6.2.1.2. Preparation of the 1 µg/mL Amphetamines Control: add 1 mL of the 10 µg/mL Amphetamines Control to a 10 mL volumetric flask and bring it to volume with methanol.

17.6.3. Amphetamines Internal Standard (for blood and urine)

17.6.3.1. This solution contains (±)-amphetamine-d11, (±)-methamphetamine-d14, (±)-MDMA-d5, (±) MDEA-d6 at a concentration of 5 µg/mL.

17.6.3.1.1. Preparation of the 5 µg/mL Amphetamines Internal Standard: add 50 µL of 1 mg/mL CRM of (±)-amphetamine-d11, (±)-methamphetamine-d14, (±)-MDMA-d5, (±) MDEA-d6 to a 10 mL volumetric flask and bring to volume with methanol.

17.7. Fortification Guide

17.7.1. Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	20	1	20	80
1	30	1	30	70
1	50	1	50	50
1	100	1	100	--
1	150	5	30	70
1	250	5	50	50
1	500	5	100	--

17.7.2. Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	40	1	40	60
1	300	10	30	70

17.7.3. Urine Calibrator and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	20	1 (Calibrator)	20	80
1	40	1 (QC)	40	60

17.8. Extraction Procedure

17.8.1. Allow all biological specimens to come to room temperature before starting the procedure.

17.8.2. Label all round bottom screw cap tubes accordingly.

17.8.3. Pipet 1 mL of drug-free blood/urine for matrix blank, negative control, calibrators, positive controls, and case samples into the appropriate labeled tubes.

17.8.4. Add corresponding drug standards to calibrator and positive controls, followed by the appropriate amount of methanol. Vortex.

17.8.5. Pipet 1 mL of case samples into appropriate labeled tubes followed by 100 µL of methanol. Vortex.

17.8.6. Using a repeater pipette, add 20 µL of internal standard to each sample to obtain the final concentration of 100 ng/mL. Vortex.

17.8.7. Add 2 mL of 100 mM phosphate buffer, pH 6.0 to each tube. Vortex.

17.8.8. Centrifuge tubes at approximately 1510 rcf for 10 minutes.
17.8.9. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
17.8.9.1. Add 3 mL methanol and aspirate.
17.8.9.2. Add 3 mL deionized water and aspirate.
17.8.9.3. Add 1 mL of 100mM phosphate buffer, pH 6.0 and aspirate.
17.8.9.4. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
17.8.9.5. Add 3 mL of deionized water and aspirate.
17.8.9.6. Add 1 mL of 0.1 M acetic acid and aspirate.
17.8.9.7. Add 3 mL of methanol and aspirate. Dry columns under full pressure for 5 minutes.
17.8.9.8. Elute amphetamines by adding 3 mL of elution solvent, prepared fresh daily.
17.8.10. Evaporate eluates at approximately 40 °C under nitrogen at 20 psi for 3 minutes.
17.8.11. Add 50 µL of acidic methanol to all tubes.
17.8.12. Evaporate eluates to dryness at approximately 40 °C under nitrogen at 20 psi. NOTE: avoid over drying.
17.8.13. Reconstitute in 50 µL of PFPA and 50 µL of ethyl acetate to each tube. Cap and vortex.
17.8.14. Incubate for 15 minutes at 70 °C. Allow extracts to cool to room temperature.
17.8.15. Evaporate derivatized extracts to dryness at approximately 40 °C under nitrogen at 20 psi. NOTE: avoid over drying.
17.8.16. Reconstitute derivatized extracts in 100 µL of ethyl acetate.
17.8.17. Transfer derivatized extracts to appropriately labeled autosampler vials with inserts and cap tightly.
17.8.18. Inject 1 µL onto the GC-MS using the AMPS.M/AMPS_U.M method.

17.9. Sequence Table

17.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, in-house quality controls, and an external Utak quality control (if available). Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC, or Utak).
17.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.

AMPS sequence example:

Blood	Urine
Matrix blank	Matrix blank
20 ng/mL Calibrator	Cut-off Calibrator
30 ng/mL Calibrator	Negative Control
50 ng/mL Calibrator	Positive Control
100 ng/mL Calibrator	10 Case Samples
150 ng/mL Calibrator	Positive Control
250 ng/mL Calibrator	10 Case Samples
500 ng/mL Calibrator	Positive Control
Negative Control	10 Case Samples
LQC	Positive Control
HQC	
Utak	
10 Case Samples	
Utak	
10 Case Samples	
Utak	
10 Case Samples	
Utak	
HQC	
LQC	

17.10. Data Analysis

17.10.1. Blood

17.10.1.1. The calibration curve for amphetamines ranges from 20-500 ng/mL.

17.10.1.2. The low quality control (LQC) for amphetamines is 40 ng/mL.

17.10.1.3. The high quality control (HQC) for amphetamines is 300 ng/mL.

17.10.2. Urine

17.10.2.1. The cut-off calibrator for amphetamines is 20 ng/mL forced through zero.

17.10.2.2. The positive control (PQC) for amphetamines is 40 ng/mL.

17.11. Lieterature and Supporting Documents

17.11.1. Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 11th ed. Seal Beach, CA: Biomedical Publications, 2017.

17.11.2. Levine, Barry, ed. “Amphetamines/Sympathomimetic Amines.” Principles of Forensic Toxicology, 4th ed. Washington, DC: AACC Press, 2013, 353-370.

17.11.3. “Sympathomimetic Amines in Blood, Plasma/Serum, Urine, and Tissue for GC or GC-MS Confirmations Using: 200 mg of UCT Clean Screen® Extraction Column.” United Chemical Technologies. 2009; 12-14.
17.11.4. Method File: AMPS.M and AMPS_U.M

18. Benzodiazepines Confirmation by Gas Chromatography-Mass Spectrometry

18.1. Purpose

18.1.1. A targeted analysis is performed for confirmatory analysis of nordiazepam, oxazepam, lorazepam, temazepam, α-hydroxyalprazolam, diazepam, and alprazolam using liquid-liquid extraction (LLE) and gas chromatography-mass spectrometry (GC-MS). Hydroxyl groups of oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam and the secondary amine on nordiazepam are derivatized with BSFTA with 1% TMCS to form trimethylsilyl derivatives. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode. Benzodiazepines are extracted and analyzed by two different methods. Alprazolam and diazepam are analyzed by the Alp.M method and all other benzodiazepines are analyzed by the Benzos.M method.

18.2. Scope

18.2.1. This procedure describes a confirmatory analysis of benzodiazepines in biological specimens, including blood. Urine confirmations are reported only qualitatively.

18.3. Reagents and Solvents

18.3.1. 0.5 M Sodium phosphate buffer, pH 8.0: weigh approximately 35.5 g of dibasic sodium phosphate and add to a 500 mL volumetric flask. Bring to volume with deionized water while stirring. In a 100 mL volumetric flask, weigh and add approximately 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring. Adjust the pH of the dibasic sodium phosphate by adding the monobasic sodium phosphate dropwise until the pH reaches 8.0±0.05. Store at room temperature. Expiration: 6 months

18.3.2. Hexane saturated with acetonitrile (HSA): combine approximately 400 mL of hexane and 100 mL of acetonitrile, thoroughly mix and allow the two layers to separate. Store at room temperature. Expiration: 1 year

18.3.3. 100 mM Sodium phosphate buffer, pH 6.8: weigh approximately 7.0 g of dibasic sodium phosphate and add to a 500 mL volumetric flask. Bring to volume with deionized water while stirring. In a second 500 mL flask, weigh and add approximately 6.0 g of monobasic sodium phosphate. Bring to volume with deionized water while stirring. Adjust the pH of the monobasic sodium phosphate by adding the dibasic sodium phosphate drop-wise until the pH reaches 6.8±0.05. Store at room temperature. Expiration: 1 year

18.3.4. Butyl acetate

18.3.5. Acetonitrile

18.3.6. Hexane

18.3.7. Methanol

18.3.8. Deionized (DI) water
18.3.9. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)
18.3.10. Utak Benzodiazepines Plus 100 Control
18.3.11. Blank blood (preserved with potassium oxalate and sodium fluoride)
18.3.12. Blank urine

18.4. Equipment and Materials

18.4.1.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
18.4.1.2. Repeater pipette
18.4.1.3. Pasteur pipettes
18.4.1.4. Analytical/Top-loading balance
18.4.1.5. pH meter
18.4.1.6. Evaporator
18.4.1.7. Vortex mixer
18.4.1.8. Centrifuge
18.4.1.9. Heating block

18.5. Instrumentation

18.5.1. Parameters
18.5.1.1. Capillary Column: 15 m DB-5MS-UI Agilent J&W GC Column (or equivalent), 0.250 mm id X 0.25 µm film thickness. The flow rate is 5.5 mL/min on column 1 and 2.9 mL/min column 2, with an injection volume of 1 µL for Benzos.M/Benzo_U.M (2 µL for Alp.M/Alp_U.M) in pulsed splitless mode.
18.5.1.2. GC-MS: Agilent GC 7890B-5977B MSD

Initial Temperature:	150 °C hold for 0.6 minutes 40 °C/min to 230 °C 10 °C/min to 310 °C
Total Run Time:	10.6 minutes
Injector Temperature:	250 °C
Interface Temperature:	310 °C
MS Quads:	180 °C
MS Source:	300 °C

18.5.1.3. SIM acquisition: Alp.M/Alp_U.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
Alprazolam-d5	284.0	313.0, 209.0	6.82	1/x
Alprazolam	279.0	204.0, 308.0	6.83	
Diazepam-d5	289.0	226.0, 261.0	4.10	1/x
Diazepam	283.0	221.0, 256.0	4.11	
*Retention Time (RT) varies with column length.				

18.5.1.4. SIM acquisition: Benzos.M/Benzos_U.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
Nordiazepam-d5	347.0	332.0, 232.0	3.40	1/x
Nordiazepam	341.0	327.0, 342.0	3.41	
Oxazepam-d5	435.0	420.0, 406.0	3.75	1/x
Oxazepam	429.0	415.0, 313.0	3.76	
Lorazepam-d4	433.0	453.0, 351.0	4.20	1/x
Lorazepam	429.0	449.0, 431.0	4.21	
Temazepam-d5	348.0	261.0, 288.0	4.81	1/x
Temazepam	343.0	345.0, 256.0	4.82	
α-Hydroxyalprazolam-d5	401.0	388.0, 386.0	7.19	1/x
α-Hydroxyalprazolam	396.0	383.0, 381.0	7.20	
*Retention Time (RT) varies with column length.				

18.5.2. Performance Check

18.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 2 pre and 8 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

18.5.2.2. Ensure that the tune verification and system suitability injection have been completed, all acceptance criteria are met, and maintenance log filled out.

18.6. Standards and Solutions

18.6.1. Benzodiazepines Working Standards for Calibrators (for blood and urine)

18.6.1.1. This analysis requires preparation of two solutions. The solutions contain nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam at 5 µg/mL and 1 µg/mL.

18.6.1.1.1. Preparation of the 5 µg/mL Benzodiazepines Calibrator: add 50 µL of each 1 mg/mL CRM of nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.1.1.2. Preparation of the 1 µg/ mL Benzodiazepines Calibrator: add 25 µL of each 1 mg/mL CRM of nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam to a 25 mL volumetric flask and bring to volume with methanol.

18.6.2. Benzodiazepines Working Standards for Quality Controls (for blood and urine)

18.6.2.1. This analysis requires preparation of four solutions; three solutions contain concentrations of nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam at 10 µg/mL, 6 µg/mL, and 1 µg/mL. The fourth solution contains oxazepam-glucuronide at 1 µg/mL.

18.6.2.1.1. Preparation of the 10 µg/mL Benzodiazepines Control Stock Solution: add 100 µL of each 1 mg/mL CRM of nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.2.1.2. Preparation of the 6 µg/mL Benzodiazepines Control: add 60 µL of each 1 mg/mL CRM of nordiazepam, oxazepam, lorazepam, temazepam, and α-hydroxyalprazolam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.2.1.3. Preparation of the 1 µg/mL Benzodiazepines Control: add 1 mL of the 10 µg/mL Benzodiazepines Control to a 10 mL volumetric flask and bring to volume with methanol.

18.6.3. Benzodiazepines Internal Standard

18.6.3.1. This solution contains nordiazepam-d5, oxazepam-d5, lorazepam-d4, temazepam-d5, and α-hydroxyalprazolam-d5 at 5 µg/mL.

18.6.3.1.1. Preparation of the 5 µg/mL Benzodiazepines Internal Standard: add 50 µL of each 1 mg/mL CRM of nordiazepam-d5, oxazepam-d5, lorazepam-d4, temazepam-d5, and α-hydroxyalprazolam-d5 to a 10 mL volumetric flask and bring to volume with methanol.

18.6.4. Alprazolam-Diazepam (ALP) Working Standards for Calibrators (for blood and urine)

18.6.4.1. This analysis requires preparation of two solutions. The solutions contain alprazolam and diazepam at 5 µg/mL and 1 µg/mL.

18.6.4.1.1. Preparation of the 5 µg/mL ALP Calibrator: add 50 µL of each 1 mg/mL CRM of alprazolam and diazepam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.4.1.2. Preparation of the 1 µg/mL ALP Calibrator: add 25 µL of each 1 mg/mL CRM of alprazolam and diazepam to a 25 mL volumetric flask and bring to volume with methanol.

18.6.5. Alprazolam-Diazepam (ALP) Working Standards for Quality Controls (for blood and urine)

18.6.5.1. This analysis requires preparation of three solutions. The solutions contain alprazolam and diazepam at 10 µg/mL, 6 µg/mL, and 1 µg/mL.

18.6.5.1.1. Preparation of the 10 µg/mL ALP Control Stock Solution: add 100 µL of each 1 mg/mL CRM of alprazolam and diazepam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.5.1.2. Preparation of the 6 µg/mL ALP Control: add 60 µL of each 1 mg/mL CRM of alprazolam and diazepam to a 10 mL volumetric flask and bring to volume with methanol.

18.6.5.1.3. Preparation of the 1 µg/mL ALP Control: add 1 mL of the 10 µg/mL ALP Control to a 10 mL volumetric flask and bring to volume with methanol.

18.6.6. Alprazolam-Diazepam (ALP) Internal Standard

18.6.6.1. This solution contains alprazolam-d5 and diazepam-d5 at 5 µg/mL.

18.6.6.1.1. Preparation of the 5 µg/mL ALP Internal Standard: add 50 µL of each 1 mg/mL CRM of alprazolam-d5 and diazepam-d5 to a 10 mL volumetric flask and bring it to volume with methanol.

18.7. Fortification Guide

18.7.1. Benzodiazepines Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	15	1	15	85
1	30	1	30	70
1	50	1	50	50
1	100	1	100	--
1	150	5	30	70
1	200	5	40	60
1	250	5	50	50

18.7.2. Benzodiazepines Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	25	1	25	75
1	75	1	75	25
1	180	6	30	70

18.7.3. Benzodiazepines Urine Calibrator and Positive Controls

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	50	1 (Calibrator)	50	50
1	100	1 (QC)	100	--

18.7.4. ALP Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	20	1	20	80
1	30	1	30	70
1	50	1	50	50
1	100	1	100	--
1	150	5	30	70
1	200	5	40	60
1	250	5	50	50

18.7.5. ALP Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	25	1	25	75
1	180	6	30	70

18.7.6. ALP Urine Calibrator and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	50	1 (Calibrator)	50	50
1	100	1 (QC)	100	--

18.8. Extraction Procedure

18.8.1. Allow all biological specimens to come to room temperature before starting the procedure.
18.8.2. Label all round bottom screw cap tubes accordingly.
18.8.3. Pipet 1 mL of drug-free blood for matrix blank, negative control, calibrators, and positive controls into the appropriate labeled tubes.
18.8.4. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
18.8.5. Pipet 1 mL of case samples into appropriate labeled tubes followed by 100 µL of methanol. Vortex.
18.8.6. Using a repeater pipette, add 20 µL of internal standard to each sample to obtain the final concentration of 100 ng/mL. Vortex.
18.8.7. Add 1 mL of 0.5 M sodium phosphate buffer, pH 8.0 to each tube. Vortex.
18.8.8. Add 3 mL of butyl acetate to each tube. Cap tightly and pulse vortex for approximately 30 seconds.
18.8.9. Centrifuge tubes at approximately 1510 rcf for 10 minutes to achieve separation.
18.8.10. Transfer the organic (upper) layer into appropriately labeled conical tube.
18.8.11. Evaporate to dryness at approximately 75 °C under nitrogen at 20 psi.
18.8.12. Add 500 µL of hexane saturated with acetonitrile to each tube. Vortex.
18.8.13. Add 40 µL (50 µL for ALP) of acetonitrile to each tube. Vortex.
18.8.14. Centrifuge tubes at approximately 1510 rcf for 3 minutes to achieve separation.
18.8.15. Aspirate the upper layer to waste.
18.8.16. Benzodiazepines Procedure
18.8.16.1. Add 35 µL of BSTFA with 1% TMCS. Cap tightly and vortex.
18.8.16.2. Incubate for 15 minutes at 70 °C. Allow extracts to cool to room temperature.
18.8.16.3. Transfer derivatized extracts to appropriately labeled autosampler vials with inserts and cap tightly.
18.8.16.4. Inject 1 µL onto the GC-MS using the Benzos.M/Benzos_U.M method.
18.8.17. ALP Procedure
18.8.17.1. Transfer the lower (organic) layer to appropriately labeled autosampler vials with inserts and cap tightly.
18.8.17.2. Inject 2 µL onto the GC-MS using the Alp.M/Alp_U.M method.

18.9. Sequence Table

18.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, in-house quality controls, and an external Utak quality control (if available). Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC, or Utak).
18.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, hydrolysis control (Benzos only), and 10% positive controls that bracket case samples.

Benzos sequence example:

Blood	Urine
Matrix blank	Matrix blank
Calibrator 15 ng/mL	Cut-off Calibrator
Calibrator 30 ng/mL	Negative Control
Calibrator 50 ng/mL	Positive Control
Calibrator 100 ng/mL	10 case samples
Calibrator 150 ng/mL	Positive Control
Calibrator 200 ng/mL	10 case samples
Calibrator 250 ng/mL	Positive Control
Negative Control	
LQC	
HQC	
MQC	
10 case samples	
Utak Benzodiazepines Plus 100	
10 case samples	
HQC	
MQC	
LQC	

ALP sequence example:

Blood	Urine
Matrix blank	Matrix blank
Calibrator 20 ng/mL	Cut-off Calibrator
Calibrator 30 ng/mL	Negative Control
Calibrator 50 ng/mL	Positive Control
Calibrator 100 ng/mL	10 case samples
Calibrator 150 ng/mL	Positive Control
Calibrator 200 ng/mL	10 case samples
Calibrator 250 ng/mL	Positive Control
Negative Control	
LQC	
HQC	
Utak Benzodiazepines Plus 100	
10 case samples	
Utak Benzodiazepines Plus 100	
10 case samples	
Utak Benzodiazepines Plus 100	
HQC	
LQC	

18.10. Data Analysis

18.10.1. Blood

18.10.1.1. The calibration curve for α-hydroxyalprazolam, nordiazepam, oxazepam, and temazepam ranges from 15-250 ng/mL, lorazepam from 30-250 ng/mL, and alprazolam and diazepam from 20-250 ng/mL.

18.10.1.2. The low quality control (LQC) for benzodiazepines and ALP is 25 ng/mL. NOTE: Lorazepam is not included in the LQC because its linear range is 30-250 ng/mL.

18.10.1.3. The mid quality control (MQC) for benzodiazepines is 75 ng/mL. NOTE: The MQC is considered the LQC for lorazepam because the linear range is 30-250 ng/mL.

18.10.1.4. The high quality control (HQC) for benzodiazepines and ALP is 180 ng/mL.

18.10.2. Urine

18.10.2.1. The cut-off calibrator for benzodiazepines and ALP is 50 ng/mL forced through zero.

18.10.2.2. The positive control (PQC) for benzodiazepines and ALP is 100 ng/mL.

18.10.3. General

18.10.3.1. Ion ratio (308.0) uses ±2 standard deviations for its acceptance criteria, based on the validation data.

18.11. Literature and Supporting Documents

18.11.1. Cairns, Eric R., et al. Quantitative Analysis of Alprazolam and Triazolam in Hemolysed Whole Blood and Liver Digest by GC/MS/NICI with Deuterated Internal Standards. Journal of Analytical Toxicology. 1994;18:1-6.

18.11.2. Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 11th ed. Seal Beach, CA: Biomedical Publications, 2017. 77-80.

18.11.3. Levine, Barry, ed. "Benzodiazepines." Principles of Forensic Toxicology, 4th ed. Washington, DC: AACC, 2013. 237-252.

18.11.4. Tiscione, Nicholas B., et al. Quantitation of Benzodiazepines in Whole Blood by Electron Impact-Gas Chromatography-Mass Spectrometry. Journal of Analytical Toxicology. 2008;32:644-652.

18.11.5. Method File: Alp.M/Alp_U.M and Benzos.M/Benzos_U.M

19. Cannabinoids Confirmation by Gas Chromatography-Mass Spectrometry

19.1. Purpose

19.1.1. A targeted analysis is performed for confirmatory analysis of Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THC-COOH) by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Carboxyl and hydroxyl groups are derivatized with BSFTA containing 1% TMCS to form trimethylsilyl derivatives. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

19.2. Scope

19.2.1. This procedure describes a confirmatory analysis of THC and target metabolites in biological specimens including blood. Urine confirmations are reported only qualitatively.

19.3. Reagents and Solvents

19.3.1. 10 M Potassium hydroxide: weigh approximately 56.1 g of potassium hydroxide pellets and add to a 100 mL volumetric flask containing approximately 50 mL of deionized water. Stir. Let the solution completely dissolve and cool before bringing to volume with deionized water. Store at room temperature. Expiration: 1 year

19.3.2. Wash solvent: 1% ammonium hydroxide (concentrated) in 85:15 deionized water/acetonitrile. In a 100 mL graduated cylinder, add 85 mL of deionized water and 15 mL of acetonitrile. Cap and mix thoroughly. Remove 1 mL of 85:15 deionized water/acetonitrile mixture and add 1 mL of ammonium hydroxide. Cap and thoroughly mix. Prepare fresh daily.

19.3.3. Elution solvent: 3% glacial acetic acid (concentrated) in 90:10 hexane/ethyl acetate. In a 100 mL graduated cylinder add 90 mL of hexane and 10 mL of ethyl acetate. Cap and mix thoroughly. Remove 3 mL of 90:10 hexane/ethyl acetate mixture and add 3 mL of glacial acetic acid. Cap and thoroughly mix. Prepare fresh daily.

19.3.4. Ethyl acetate

19.3.5. Deionized (DI) water

19.3.6. BSTFA containing 1% TMCS

19.3.7. Blank blood (preserved with potassium oxalate and sodium fluoride)

19.3.8. Blank urine

19.4. Equipment and Materials

19.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)

19.4.2. Repeater pipette

19.4.3. Cerex® THC, 6mL columns, 65 mg

19.4.4. Positive pressure SPE manifold

19.4.5. Analytical/Top-loading balance
19.4.6. Evaporator
19.4.7. Vortex mixer
19.4.8. Centrifuge
19.4.9. Heating block

19.5. Instrumentation

19.5.1. Parameters

19.5.1.1. Capillary Column: 30 m HP-1MS Agilent J&W GC Column or equivalent (DB-5MS for urine), 0.25 mm id X 0.25 µm film thickness. The flow rate is 0.95 mL/min with an injection volume of 2 µL in pulsed splitless mode.

19.5.1.2. GC-MS: Agilent GC 7890A-5975C MSD

Initial Temperature:	160 °C hold for 1 minute 25 °C/min to 230 °C hold for 6 minutes 30 °C /min to 260 °C hold for 4 minutes 10 °C/min to 270 °C hold for 0.5 minutes 30 °C /min to 300 °C hold for 5 minutes
Total Run Time:	22.3 minutes
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

19.5.1.3. SIM acquisition: THC.M/THC_U.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
THC-d3	389.3	374.3, 306.1	10.21	1/x
THC	386.3	371.3, 303.1	10.24	
11-OH-THC-d3	374.0	477.0, 462.0	13.17	1/x
11-OH-THC	371.0	474.0, 459.0	13.21	
THC-COOH-d3	476.0	374.0, 491.0	15.16	1/x
THC-COOH	473.0	371.0, 488.0	15.20	
*Retention Time (RT) displayed are from HP-1MS column. RT varies with column length.				

19.5.2. Performance Check

19.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

19.5.2.2. Ensure that the tune verification and system suitability have been completed, all acceptance criteria are met, and maintenance log filled out.

19.6. Standards and Solutions

19.6.1. Cannabinoids Working Standards for Calibrators (for blood and urine)

19.6.1.1. This analysis requires preparation of two solutions. The solutions contain THC, THC-COOH, and 11-OH-THC at 1 µg/mL and 0.1 µg/mL.

19.6.1.1.1. Preparation of the 1 µg/mL Cannabinoids Calibrator: add 25 µL of each 1 mg/mL CRM of THC, THC-COOH, and 11-OH-THC to a 25 mL volumetric flask and bring to volume with methanol.

19.6.1.1.2. Preparation of the 0.1 µg/mL Cannabinoids Calibrator: add 1 mL of the 1 µg/mL Cannabinoids Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2. Cannabinoids Working Standards for Quality Controls (for blood and urine)

19.6.2.1. This analysis requires preparation of five solutions. The three solutions contain THC, THC-COOH, and 11-OH-THC at 10 µg/mL, 1 µg/mL, and 0.1 µg/mL. The other two solutions contain THC-COOH-glucuronide at 1 µg/mL and 0.1 µg/mL.

19.6.2.1.1. Preparation of the 10 µg/mL Cannabinoids Control Stock Solution: add 100 µL of each 1 mg/mL CRM of THC, THC-COOH, and 11-OH-THC to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2.1.2. Preparation of the 1 µg/mL Cannabinoids Control: add 1 mL of the 10 µg/mL Cannabinoids Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2.1.3. Preparation of the 0.1 µg/mL Cannabinoids Control: add 1 mL of the 1 µg/mL Cannabinoids Control to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2.1.4. Preparation of the 1 µg/mL Cannabinoids Hydrolysis Control Stock Solution: Add 100 µL of the 100 µg/mL CRM of THC-COOH-glucuronide to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2.1.5. Preparation of the 0.1 µg/mL Cannabinoids Hydrolysis Control: Add 1 mL of the 1 µg/mL Cannabinoids Hydrolysis Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

19.6.3. Cannabinoids Internal Standard Solutions (for blood and urine)

19.6.3.1. This analysis requires preparation of two solutions. The solutions contain concentrations of THC-d3, THC-COOH-d3, and 11-OH-THC-d3 at a 10 µg/mL and 2 µg/mL.

19.6.3.1.1. Preparation of the 10 µg/mL Internal Standard Stock Solution: add 100 µL of each 1 mg/mL CRM of THC-d3, THC-COOH-d3, and 11-OH-THC-d3 to a 10 mL volumetric flask and bring to volume with methanol.

19.6.3.1.2. Preparation of the 2 µg/mL Internal Standard: add 2 mL of the 10 µg/mL Internal Standard Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

19.7. Fortification Guide

19.7.1. Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	4	0.1	40	60
1	10	0.1	100	--
1	25	1	25	75
1	50	1	50	50
1	75	1	75	25
1	100	1	100	--

19.7.2. Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	8	0.1	80	20
1	40	1	40	60
1	80	1	80	20

19.7.3. Urine Calibrators and Positive Controls

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	4	0.1 (Calibrator)	40	60
1	8	0.1 (QC)	80	20
1	8	0.1 (Hydrolysis QC)	80	20
1	80	1 (Calibrator)	80	20

19.8. Extraction Procedure

19.8.1. Blood Extraction

19.8.1.1. Allow all biological specimens to come to room temperature before starting the procedure.

19.8.1.2. Label all round bottom screw cap tubes accordingly.

19.8.1.3. Pipet 1 mL of drug-free blood for matrix blank, negative control, calibrators, and positive controls into appropriate labeled tubes.

19.8.1.4. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
19.8.1.5. Pipet 1 mL of case samples into appropriate labeled tubes followed by 100 µL of methanol. Vortex.
19.8.1.6. Using a repeater pipette, add 25 µL of internal standard to each sample to obtain the final concentration of 50 ng/mL. Vortex.
19.8.1.7. Add 2 mL of cold acetonitrile. Vortex immediately.
19.8.1.8. Centrifuge tubes at approximately 3082 rcf for 15 minutes to achieve separation.
19.8.1.9. Transfer the organic (upper) layer into an appropriately labeled round bottom glass tube.
19.8.1.10. Add 2 mL of deionized water. Vortex.
19.8.1.11. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
19.8.1.11.1. Pour sample into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
19.8.1.11.2. Wash SPE columns by adding 1 mL of deionized water: acetonitrile (85:15) with 1% ammonium hydroxide. Prepare fresh daily.
19.8.1.11.3. Dry columns under full pressure for 5 minutes.
19.8.1.11.4. Elute THC and 11-OH-THC by adding 2 mL ethyl acetate. Dry columns under full pressure for 5 minutes.
19.8.1.11.5. Elute THC-COOH by adding 2 mL of hexane: ethyl acetate (90:10) with 3% glacial acetic acid. Prepare fresh daily.
19.8.1.12. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi.
19.8.1.13. Reconstitute in 30 µL of BSTFA with 1% TMCS and 20 µL of ethyl acetate to each tube. Cap and vortex.
19.8.1.14. Incubate for 15 minutes at 70 °C. Allow extracts to cool to room temperature.
19.8.1.15. Transfer derivatized extracts to appropriately labeled autosampler vials with inserts and cap tightly.
19.8.1.16. Inject 2 µL onto the GC-MS using THC.M method.

19.8.2. Urine Extraction
19.8.2.1. Pipet 1 mL of drug-free urine for matrix blank, negative control, calibrators, and positive controls into appropriate labeled tubes.
19.8.2.2. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
19.8.2.3. Pipet 1 mL of case samples into appropriate labeled tubes followed by 100 µL of methanol. Vortex.
19.8.2.4. Add 50 µL of 10 M potassium hydroxide to all tubes. Vortex.

19.8.2.5. Incubate for 15 minutes at 60 °C. Allow tubes to cool to room temperature.
19.8.2.6. Using a repeater pipette, add 25 µL of internal standard to each sample to obtain the final concentration of 50 ng/mL. Vortex.
19.8.2.7. Centrifuge tubes at approximately at 3082 rcf for 10 minutes if the samples are cloudy or contain particulates.
19.8.2.8. Follow the blood extraction procedure from 19.8.1.9-19.8.1.15.
19.8.2.9. Inject 2 µL onto the GC-MS using the THC_U.M method.

19.9. Sequence Table

19.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, and in-house quality controls. Case samples must be bracketed by 10% positive controls (LQC, MQC, or HQC).
19.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, high calibrator, hydrolysis control, and 10% positive controls that bracket case samples.

THC sequence example:

Blood	**Urine**
Matrix blank	Matrix blank
Calibrator 4 ng/mL	Cut-off Calibrator
Calibrator 10 ng/mL	High Calibrator
Calibrator 25 ng/mL	Negative Control
Calibrator 50 ng/mL	Hydrolysis Control
Calibrator 75 ng/mL	Positive Control
Calibrator 100 ng/mL	10 case samples
Negative Control	Positive Control
LQC	10 case samples
HQC	Positive Control
MQC	10 case samples
10 case samples	Positive Control
MQC	
10 case samples	
MQC	
10 case samples	
MQC	
HQC	
LQC	

19.10. Data Analysis

19.10.1. Blood

19.10.1.1. The calibration curve for cannabinoids ranges from 4-100 ng/mL.

19.10.1.1.1. The low quality control (LQC) for cannabinoids is 8 ng/mL.

19.10.1.1.2. The mid quality control (MQC) for cannabinoids is 40 ng/mL.

19.10.1.1.3. The high quality control (HQC) for cannabinoids is 80 ng/mL.

19.10.2. Urine

19.10.2.1. The cut-off calibrator for cannabinoids is 4 ng/mL forced through zero.

19.10.2.2. The high calibrator for cannabinoids is 80 ng/mL. NOTE: this calibrator is not used as part of the calibration curve, but only to set the average for ion ratios.

19.10.2.3. The positive control (PQC) for cannabinoids is 8 ng/mL.

19.11. Literature and Supporting Documents

19.11.1. Abraham, T., et al. Simultaneous GC–EI-MS Determination of Δ^9-Tetrahydrocannabinol, 11-Hydroxy-Δ^9-Tetrahydrocannabinol, and 11-nor-9-Carboxy-Δ^9-Tetrahydrocannabinol in Human Urine Following Tandem Enzyme-Alkaline Hydrolysis. Journal of Analytical Toxicology. 2007; Vol. 31: 477-485.

19.11.2. Baselt, Randall C. “Tetrahydrocannabinol.” Disposition of Toxic Drugs and Chemicals in Man,11th ed. Seal Beach, CA: Biomedical Publications, 2017. 2063-2068.

19.11.3. Levine, Barry, ed. “Cannabis.” Principles of Forensic Toxicology, 4th ed. Washington, DC: AACC, 2013. 269-303.

19.11.4. Method File: THC.M. and THC_U.M.

20. Cocaine Confirmation by Gas Chromatography-Mass Spectrometry

20.1. Purpose

20.1.1. A targeted analysis is performed for confirmatory analysis of benzoylecgonine (BE), cocaine, and cocaethylene (CE) by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). The hydroxyl group on benzoylecgonine is derivatized with PFPA and HFIP to form perfluoroalkyl derivative. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

20.2. Scope

20.2.1. This procedure describes a confirmatory analysis of cocaine and target metabolites in biological specimens including blood. Urine confirmations are reported only qualitatively.

20.3. Reagents and Solvents

20.3.1. 1 M Acetic acid
20.3.2. 100 mM Phosphate buffer, pH 6.0
20.3.3. Elution Solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol. Prepare fresh daily.
20.3.4. Hexane
20.3.5. Methanol
20.3.6. Ethyl acetate
20.3.7. Deionized (DI) water
20.3.8. PFPA (pentafluoropropionic anhydride)/PFAA (pentafluoropropionic acid anhydride)
20.3.9. HFIP (1,1,1,3,3,3-hexafluoro-2-propanol)
20.3.10. Utak Drug of Abuse Level 1
20.3.11. Blank blood (preserved with potassium oxalate and sodium fluoride)
20.3.12. Blank urine

20.4. Equipment and Materials

20.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
20.4.2. Repeater pipette
20.4.3. UCT Clean Screen® DAU, 6 mL columns, 200 mg
20.4.4. Positive pressure SPE manifold
20.4.5. Analytical/Top-loading balance
20.4.6. Evaporator
20.4.7. Vortex mixer
20.4.8. Centrifuge
20.4.9. Heating block

20.5. Instrumentation

20.5.1. Parameters

20.5.1.1. Capillary Column: 30 m DB-5MS Agilent J&W GC Column (or equivalent), 0.25 mm X 0.25 µm film thickness. The flow rate is 1.2 mL/min with an injection volume of 1 µL in splitless mode.

20.5.1.2. GC-MS: Agilent GC 7890A-5975C MSD

Initial Temperature:	130 °C hold for 1 minute 17 °C/min to 280 °C hold for 3 minutes
Total Run Time:	12.82 minutes
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

20.5.1.3. SIM acquisition: Cocaine.M/Cocaine_U.M.

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
Benzoylecgonine-d3	442.0	321.0, 275.0	7.68	1/x
Benzoylecgonine	439.0	318.0, 272.0	7.69	
Cocaine-d3	185.0	306.0, 85.0	9.02	1/x
Cocaine	182.0	303.0, 198.0	9.03	
Cocaethylene-d3	320.0	215.0, 199.0	9.31	1/x
Cocaethylene	317.0	212.0, 196.0	9.32	
*Retention Time (RT) varies with column length.				

20.5.2. Performance Check

20.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

20.5.2.2. Ensure that the tune verification and system suitability have been completed, all acceptance criteria met, and maintenance log filled out.

20.6. Standards and Solutions

20.6.1. Cocaine Working Standards for Calibrators (for blood and urine)

20.6.1.1. This analysis requires preparation of three solutions. The solutions contain cocaine, benzoylecgonine, and cocaethylene at 10 µg/mL, 1 µg/mL, and 0.1 µg/mL.

20.6.1.1.1. Preparation of the 10 µg/mL Cocaine Calibrator: add 100 µL of each 1 mg/mL CRM of cocaine, benzoylecgonine, and cocaethylene to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.1.1.2. Preparation of the 1 µg/mL Cocaine Calibrator: add 25 µL of each 1 mg/mL CRM of cocaine, benzoylecgonine, and cocaethylene to a 25 mL volumetric flask and bring to volume with acetonitrile.

20.6.1.1.3. Preparation of the 0.1 µg/mL Cocaine Calibrator: add 1 mL of the 1 µg/mL Cocaine Calibrator to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.2. Cocaine Working Standards for Quality Controls (for blood and urine)

20.6.2.1. This analysis requires preparation of three solutions. The two solutions contain cocaine, benzoylecgonine, and cocaethylene at 10 µg/mL and 1 µg/mL. The third solution contains cocaine and cocaethylene at 5 µg/mL and benzoylecgonine at 10 µg/mL.

20.6.2.1.1. Preparation of the 10 µg/mL Cocaine Control Stock Solution: add 100 µL of each 1 mg/mL CRM of cocaine, benzoylecgonine, and cocaethylene to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.2.1.2. Preparation of the 1 µg/mL Cocaine Control: add 1 mL of the 10 µg/mL Cocaine Control to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.2.1.3. Preparation of the 10/5 µg/mL Cocaine Control: add 50 µL of each 1 mg/mL CRM of cocaine and cocaethylene, and 100 µL of benzoylecgonine to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.3. Cocaine Internal Standard

20.6.3.1. This solution contains cocaine-d3, benzoylecgonine-d3, and cocaethylene-d3 at 5 µg/mL.

20.6.3.1.1. Preparation of the 5 µg/mL Internal Standard: add 50 µL of each 1 mg/mL CRM of cocaine-d3, benzoylecgonine-d3, and cocaethylene-d3 to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.7. Fortification Guide

20.7.1. Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	10	0.1	100
1	25	1	25
1	50	1	50
1	100	1	100
1	250	10	25
1	500	10	50
1	1000	10	100

20.7.2. Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	20	1	20
1	800/400	10/5	80

20.7.3. Urine Calibrator and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	10	0.1 (Calibrator)	100
1	20	1 (QC)	20

20.8. Extraction Procedure

20.8.1. Allow all biological specimens to come to room temperature before starting the procedure.

20.8.2. Label all round bottom screw cap tubes accordingly.

20.8.3. Add corresponding drug standards to calibrators and positive controls. Subsequently dry down at approximately 50 °C under nitrogen at 20 psi for 1 minute.

20.8.4. Pipet 1 mL of drug-free blood/urine for matrix blank, negative, calibrators, positive controls, and case samples into the appropriate labeled tubes. Vortex.

20.8.5. Using a repeater pipette, add 20 µL of internal standard to each sample to obtain the final concentration of 100 ng/mL. Vortex.

20.8.6. Add 2 mL of deionized water to all tubes. Vortex and let stand for approximately 10 minutes.

20.8.7. Centrifuge tubes at approximately 1734 rcf for 15 minutes. NOTE: urine samples do not need to be centrifuged unless they are cloudy.

20.8.8. Decant supernatant into appropriately round bottom screw cap tubes.
20.8.9. Add 2 mL of 100 mM phosphate buffer, pH 6.0 to all tubes. Vortex.
20.8.10. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
20.8.10.1. Add 2 mL hexane and aspirate.
20.8.10.2. Add 2 mL of methanol and aspirate.
20.8.10.3. Add 2 mL of deionized water and aspirate.
20.8.10.4. Add 1 mL of 100 mM phosphate buffer, pH 6.0 and aspirate.
20.8.10.5. Pour samples into appropriated SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
20.8.10.6. Add 4 mL of deionized water and aspirate.
20.8.10.7. Add 1 mL of 1 M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.
20.8.10.8. Add 2 mL of methanol and aspirate.
20.8.10.9. Add 1 mL of hexane and aspirate. Dry columns under full pressure for 5 minutes.
20.8.10.10. Elute drugs by adding 1.5 mL of elution solvent, prepared fresh daily.
20.8.11. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi.
20.8.12. Reconstitute in 50 µL of PFPA and 50µL of HFIP to each tube. Cap and vortex.
20.8.13. Incubate for 15 minutes at 70 °C. Allow extracts to cool to room temperature.
20.8.14. Evaporate derivatized extracts to dryness at approximately 50 °C under nitrogen at 20 psi.
20.8.15. Reconstitute in 50 µL of ethyl acetate. Vortex.
20.8.16. Transfer derivatized extracts to appropriately labeled autosampler vials with inserts and cap tightly.
20.8.17. Inject 1 µL onto the GC-MS using the Cocaine.M/Cocaine_U.M method.

20.9. Sequence Table

20.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, and in-house quality controls. Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC, or Utak).
20.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.

Cocaine sequence example:

Blood	**Urine**
Matrix blank	Matrix blank
Calibrator 10 ng/mL	Cut-off Calibrator

Calibrator 25 ng/mL	Negative Control
Calibrator 50 ng/mL	Positive Control
Calibrator 100 ng/mL	10 case samples
Calibrator 250 ng/mL	Positive Control
Calibrator 500 ng/mL	10 case samples
Calibrator 1000 ng/mL	Positive Control
Negative Control	10 case samples
LQC	Positive Control
HQC	
Utak	
10 case samples	
Utak	
10 case samples	
Utak	
10 case samples	
Utak	
HQC	
LQC	

20.10. Data Analysis

20.10.1. Blood

20.10.1.1. The calibration curve for cocaine and cocaethylene ranges from 10-500 ng/mL, whereas the calibration curve for benzoylecgonine is 10-1000 ng/mL.

20.10.1.2. The low quality control (LQC) for cocaine, cocaethylene, and benzoylecgonine is 20 ng/mL.

20.10.1.3. The high quality control (HQC) for cocaine and cocaethylene is 400 ng/mL, whereas the HQC for benzoylecgonine is 800 ng/mL.

20.10.2. Urine

20.10.2.1. The cut-off calibrator for cocaine, cocaethylene, and benzoylecgonine is 10 ng/mL forced through zero.

20.10.2.2. The positive control (PQC) for cocaine, cocaethylene, and benzoylecgonine is 20 ng/mL.

20.10.3. Reinjection Guidelines:

20.10.3.1. Reinjections can be made up to 48 hours after the completion of the initial injection of the particular sample.

20.11. Literature and Supporting Documents

20.11.1. Crouch, Dennis J., et al. Analysis of Cocaine and its Metabolites from Biological Specimens using Solid-Phase Extraction and Positive Ion Chemical Ionization Mass Spectrometry. Journal of Analytical Toxicology. 1995;19:352-358.

20.11.2. United Chemical Technologies, Inc. Cocaine and Benzoylecgonine in Blood, Plasma/Serum, Urine, Tissue by LC-MS/MS or GC-MS Clean Scren DAU Extraction Column. Clinical and Forensic Applications Manual, pg. 88-90.

20.11.3. Method File: Cocaine.M and Cocaine_U.M.

21. Opioids Confirmation by Gas Chromatography-Mass Spectrometry

21.1. Purpose

21.1.1. A targeted analysis is performed for confirmatory analysis of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, morphine, and 6-acetylmorphine using solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Drugs are isolated from the matrix using a basic extraction. The keto opioids react with hydroxylamine to form oxime derivatives to prevent tautomerism. Hydroxyl groups of all opioids are further derivatized with BSTFA containing 1% TMCS to form trimethylsilyl derivatives. Deuterated internal standards and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

21.2. Scope

21.2.1. This procedure describes a confirmatory analysis of opioids in biological specimens, including blood; 6-acetylmorphine is reported only qualitatively. Urine confirmations are reported only qualitatively.

21.3. Reagents and Solvents

21.3.1. 1 M Acetic acid
21.3.2. 100 mM Phosphate buffer, pH 6.0
21.3.3. 1% Hydroxylamine: weigh approximately 0.25 g of hydroxylamine and add to a 25 mL volumetric flask and bring to volume with deionized water. Prepare fresh daily.
21.3.4. Elution Solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol. Prepare fresh daily.
21.3.5. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS)
21.3.6. Methanol
21.3.7. Acetonitrile
21.3.8. Hexane
21.3.9. Deionized (DI) water
21.3.10. Ethyl acetate
21.3.11. Utak PM 100 Pain Management
21.3.12. Blank blood (preserved with potassium oxalate and sodium fluoride)
21.3.13. Blank urine

21.4. Equipment and Materials

21.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
21.4.2. Repeater pipette

21.4.3. UCT Clean Screen® DAU, 6 mL columns, 200 mg
21.4.4. Positive pressure SPE manifold
21.4.5. Analytical/Top-loading balance
21.4.6. pH meter
21.4.7. Evaporator
21.4.8. Vortex mixer
21.4.9. Centrifuge
21.4.10. Heating block

21.5. Instrumentation

21.5.1. Parameters

21.5.1.1. Capillary Column: 30 m HP-1MS Agilent J&W GC Column (or equivalent), 0.25 mm id X 0.25 µm film thickness. The flow rate is 1.1 mL/min for HYDOXY.M and MORPH.M and 1 mL/min for HYDOXY_U.M and MORPH_U.M. The injection volume is 2 µL for HYDOXY.M/HYDOXY_U.M and 1.5 µL for MORPH.M/MORPH_U.M in pulsed splitless mode.

21.5.1.2. GC-MS: Agilent GC 7890A-5975C MSD

HYDOXY.M and MORPH.M

Initial Temperature:	160 °C 35 °C/min to 195 °C 9 °C/min to 260 °C hold for 4 minutes 30 °C/min to 300 °C hold for 4.5 minutes 30 °C/min to 240 °C
Total Run Time:	20.05 minutes
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

HYDOXY_U.M and MORPH_U.M

Initial Temperature:	160 °C 35 °C/min to 195 °C 9 °C/min to 260 °C hold for 4 minutes 60 °C/min to 300 °C hold for 2.5 minutes (1.5 for MORPH_U.M)
Total Run Time:	15.38 minutes (13.38 MORPH_U.M)
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

21.5.1.3. SIM acquisition: HYDOXY.M/HYDOXY_U.M.

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
Hydrocodone-d3	300.1	389.2, 374.2	9.70	1/x
Hydrocodone	386.2	297.1, 371.2	9.73	
Hydromorphone-d3	447.3	432.0, 342.2	10.04	1/x-Lower unweighted-Upper
Hydromorphone	444.2	429.2, 339.1	10.06	
Oxycodone-d3	477.1	462.1, 420.2	10.78	1/x-Lower unweighted-Upper
Oxycodone	474.2	459.3, 385.2	10.81	
Oxymorphone-d3	535.2	520.3, 446.2	11.12	1/x-Lower unweighted-Upper
Oxymorphone	532.3	517.3, 443.3	11.15	
*Retention Time (RT) varies with column length.				

21.5.1.4. SIM acquisition: MORPH.M/MORPH_U.M.

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
Codeine-d3	374.2	346.2, 359.2	8.76	1/x
Codeine	371.2	343.1, 372.2	8.78	
Morphine-d6	435.3	420.2, 436.3	9.40	1/x
Morphine	429.2	430.2, 401.2	9.44	
6-acetylmorphine-d3	402.2	327.1, 290.1	9.85	1/x
6-acetylmorphine	399.2	340.2, 400.2	9.88	
*Retention Time (RT) varies with column length.				

21.5.2. Performance Check

21.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

21.5.2.2. Ensure that the tune verification and system suitability have been completed, all acceptance criteria met, and maintenance log filled out.

21.6. Standards and Solutions

21.6.1. Opioids Blood Working Standards for Calibrators

21.6.1.1. This analysis requires preparation of three solutions. The solutions contain hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine at 10 µg/mL, 2 µg/mL, and 0.5 µg/mL along with 6-acetylmorphine at 2.5 µg/mL, 0.5 µg/mL, and 0.125 µg/mL.

21.6.1.1.1. Preparation of 10/2.5 µg/mL Opioids Calibrator: add 100 µL of each 1 mg/mL CRM of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 25 µL of 1 mg/mL CRM of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol.

21.6.1.1.2. Preparation of 2/0.5 µg/mL Opioids Calibrator: add 20 µL of each 1 mg/mL CRM of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 50 µL of 100 µg/mL of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol. NOTE: Used for the High Calibrator for urine as well.

21.6.1.1.3. Preparation of 0.5/0.125 µg/mL Opioids Calibrator: add 500 µL of 10/2.5 µg/mL Opioids Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

21.6.2. Opioids Blood Working Standards for Quality Controls

21.6.2.1. This analysis requires preparation of two solutions. The solutions contain concentrations of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine at 10 µg/mL and 0.5 µg/mL along with 6-acetylmorphine at 2.5 µg/mL and 0.125 µg/mL.

21.6.2.1.1. Preparation of 10/2.5 µg/mL Opioids Control: add 100 µL of each 1 mg/mL CRM of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 25 µL of a 1 mg/mL CRM of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol.

21.6.2.1.2. Preparation of 0.5/0.125 µg/mL Opioids Control: add 500 µL of 10/2.5 µg/mL Opioids Control to a 10 mL volumetric flask and bring to volume with methanol.

21.6.3. Opioids Urine Working Standards for Calibrators

21.6.3.1. This analysis requires preparation of two solutions. The solutions contain hydrocodone, oxycodone, oxymorphone, codeine, and morphine at 10 µg/mL and 0.50 µg/mL; hydromorphone at 15 µg/mL and 0.75 µg/mL; and 6-acetylmorphine at 2.5 µg/mL and 0.125 µg/mL.

21.6.3.1.1. Preparation of 10/15/2.5 µg/mL Opioids Calibrator Stock Solution: add 100 µL of each 1 mg/mL CRM of hydrocodone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 150 µL of a 1 mg/mL CRM of hydromorphone and 25 µL of a 1 mg/mL CRM of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol.

21.6.3.1.2. Preparation of 0.5/0.75/0.125 µg/mL Opioids Calibrator: add 500 µL of 10/15/2.5 µg/mL Opioids Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

21.6.4. Opioids Urine Working Standards for Quality Controls

21.6.4.1. This analysis requires prepration of two solutions. The solutions contain hydrocodone, oxycodone, oxymorphone, codeine, and morphine at 10 µg/mL and 0.50

µg/mL; hydromorphone at 15 µg/mL and 0.75 µg/mL; and 6-acetylmorphine at 2.5 µg/mL and 0.125 µg/mL.

21.6.4.1.1. Preparation of 10/15/2.5 µg/mL Opioids Control Stock Solution: add 100 µL of each 1 mg/mL CRM of hydrocodone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 150 µL of a 1 mg/mL CRM of hydromorphone and 25 µL of a 1 mg/mL CRM of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol.

21.6.4.1.2. Preparation of 0.5/0.75/0.125 µg/mL Opioids Control: add 500 µL of 10/15/2.5 µg/mL Opioids Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

21.6.5. Opioids Internal Standard (for blood and urine)

21.6.5.1. This solution contains concentrations of hydrocodone-d3, hydromorphone-d3, oxycodone-d3, oxymorphone-d3, codeine-d3, and morphine-d6 at 5 µg/mL, and 6-acetylmorphine-d3 at 1.25 µg/mL.

21.6.5.1.1. Preparation of 5/1.25 µg/mL Opioids Internal Standard: add 50 µL of each 1 mg/mL CRM of hydrocodone, hydromorphone, oxycodone, oxymorphone, codeine, and morphine into a 10 mL volumetric flask. Add 125 µL of a 100 µg/mL CRM of 6-acetylmorphine to the same volumetric flask and bring to volume with methanol.

21.7. Fortification Guide

21.7.1. Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	20/5	0.5/0.125	40
1	40/10	0.5/0.125	80
1	100/25	2/0.5	50
1	300/75	10/2.5	30
1	500/125	10/2.5	50
1	800/200	10/2.5	80

21.7.2. Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	25/6.25	0.5/0.125	50
1	250/62.5	10/2.5	25
1	400/100	10/2.5	40

21.7.3. Urine Calibrators and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
1	20/30/5	0.5/0.75/0.125 (Calibrator)	40
1	40/60/10	0.5/0.75/0.125 (QC)	80
1	100/25	2/0.5 (Calibrator)	50

21.8. Extraction Procedure

21.8.1. Allow all biological specimens to come to room temperature before starting the procedure.

21.8.2. Label all round bottom screw cap tubes accordingly.

21.8.3. Add corresponding drug standard to calibrators and positive controls. Subsequently dry down at approximately 50 °C under nitrogen at 20 psi for 1 minute.

21.8.4. Pipet 1 mL of drug-free blood/urine for matrix blank/negative control/calibrators/positive controls and case samples into the appropriate labeled tubes. Vortex.

21.8.5. Using a repeater pipette, add 30 µL of internal standard to each sample to obtain the final concentration of 150 ng/mL (37.5 ng/mL for 6-acetylmorphine). Vortex.

21.8.6. Add 6 mL of acetonitrile immediately cap and shake each tube. Rotate for 20 minutes.

21.8.7. Centrifuge tubes at approximately 1204 rcf for 10 minutes to achieve separation.

21.8.8. Decant supernatant into appropriately labeled round bottom screw cap tubes.

21.8.9. Evaporate to approximately 1 mL at approximately 50 °C under nitrogen at 10 psi for 5 minutes followed by 20 psi for 10-12 minutes.

21.8.10. Add 2 mL of 100 mM sodium phosphate buffer, pH 6.

21.8.11. Add 500 µL of 1% hydroxylamine. Vortex.

21.8.12. Cover tubes and incubate at 60 °C for one hour. Allow tubes to cool to room temperature.

21.8.13. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.

21.8.14. Add 2 mL of hexane and aspirate.

21.8.15. Add 2 mL of methanol and aspirate.

21.8.16. Add 2 mL of deionized water and aspirate.

21.8.17. Add 2 mL of 100 mM sodium phosphate buffer, pH 6, and aspirate.

21.8.18. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

21.8.19. Add 1 mL of 1 M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.

21.8.20. (Urine ONLY) Add 2 mL of hexane and aspirate.

21.8.21. Add 3 mL of methanol and aspirate. Dry columns under full pressure for 6 minutes.

21.8.22. Elute opioids by adding 2 mL elution solvent, prepared fresh daily.

21.8.23. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi for 7-9 minutes.
21.8.24. Reconstitute in 30 µL of BSFTA with 1% TMCS and 30 µL ethyl acetate (acetonitrile for urine) to each tube. Cap and vortex.
21.8.25. Incubate for 20 minutes at 90 °C. Allow extracts to cool to room temperature.
21.8.26. Transfer derivatized extracts to appropriately labeled autosampler vials with inserts and cap tightly.
21.8.27. Inject 2 µL onto the GC-MS using HYDOXY.M/HYDOXY_U.M and 1.5 µL using the MORPH.M/MORPH_U.M methods.

21.9. Sequence Table

21.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, in-house quality controls, and an external Utak quality control (if available). Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC, or Utak).
21.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, high calibrator, and 10% positive controls that bracket case samples.

Opioids sequence example:

Blood	**Urine**
Matrix blank	Matrix Blank
20 ng/mL Calibrator	Cut-off Calibrator
40 ng/mL Calibrator	High Calibrator
100 ng/mL Calibrator	Negative Control
300 ng/mL Calibrator	Positive Control
500 ng/mL Calibrator	10 case samples
800 ng/mL Calibrator	Positive Control
Negative Control	10 case samples
LQC	Positive Control
Utak	10 case samples
HQC	Positive Control
MQC	
10 case samples	
MQC	
10 case samples	
MQC	
10 case samples	
MQC	
HQC	
Utak	
LQC	

21.10. Data Analysis

21.10.1. Blood

21.10.1.1. The calibration curve for opioids ranges from 20-800 ng/mL, with the exception of 6-acetylmorphine, which ranges from 5-200 ng/mL. 6-acetylmorphine will only be reported qualitatively.

21.10.1.1.1. The calibration range is split between 2 overlapping curves: the lower curve ranging 20-300 ng/mL and the upper curve ranging 100-800 ng/mL. The MQC (250 ng/mL) is quantified off of both curves to determine acceptability. Case samples will be initially quantified using the 20-300 ng/mL curve. If the initial concentrations are <250 ng/mL, the values will be reported. If the initial concentrations are ≥250 ng/mL, they will be re-analyzed and reported using the upper curve.

21.10.1.1.2. All case samples are first processed on the 20-300 ng/mL curve. If the value of a specific drug is above the 250 ng/mL threshold, it is re-processed on the 100-800 ng/mL curve.

21.10.1.2. The low quality control (LQC) for opioids is 25 ng/mL (6-acetylmorphine is 6.25 ng/mL).

21.10.1.3. The mid quality control (MQC) for opioids is 250 ng/mL (6-acetylmorphine is 62.5 ng/mL).

21.10.1.4. The high quality control (HQC) for opioids is 400 ng/mL (6-acetylmorphine is 100 ng/mL).

21.10.2. Urine

21.10.2.1. The urine cut-off calibrator is 20 ng/mL for hydrocodone, oxycodone, oxymorphone, codeine, and morphine; 30 ng/mL for hydromorphone; and 5 ng/mL for 6-acetylmorphine. The urine cut-off calibrator is forced through zero.

21.10.2.2. The high calibrator for opioids is 100 ng/mL. NOTE: This calibrator is not used as part of the calibration curve, but only to set the average for ion ratios.

21.10.2.3. The positive control (PQC) for opioids is 40 ng/mL for hydrocodone, oxycodone, oxymorphone, codeine, and morphine; 60 ng/mL for hydromorphone; and 10 ng/mL for 6-acetylmorphine.

21.10.3. General

21.10.3.1. Ion ratios for codeine (343.1 and 372.2) and oxycodone (459.3 and 385.2) use ±2 standard deviations for their acceptance criteria, based on the validation data.

21.11. Literature and Supporting Documents

21.11.1. Broussard, L. A., et al. Simultaneous Identification and Quantitation of Codeine, Morphine, Hydrocodone, and Hydromorphone in Urine as Trimethylsilyl and Oxime Derivatives by Gas Chromatography-Mass Spectrometry. Clinical Chemistry. 1997;43: 1029-1032.

21.11.2. Cremese, M., et al. Improved GC-MS Analysis of Opioids with use of Oxime-TMS Derivatives. Journal of Forensic Science. 1998:43:1220-1224.

21.11.3. Levine, Barry, ed. “Opioids.” Principles of Forensic Toxicology, 4th ed. Washington, DC: AACC Press, 2013, 271-292.

21.11.4. Ropero-Miller, Jeri D. Simultaneous Quantitation of Opioids in Blood by GC-EI-MS Analysis Following Deproteination, Detautomerization of Keto Analytes, Solid-Phase Extraction, and Trimethylsilyl Derivatization. Journal of Analytical Toxicology, 2002; 26:524-528.

21.11.5. Method file: HYDOXY.M/HYDOXY_U.M and MORPH.M/MORPH_U.M.

22. Phencyclidine Confirmation by Gas Chromatography-Mass Spectrometry

22.1. Purpose

22.1.1. A targeted analysis is performed for confirmatory analysis of phencyclidine (PCP) by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Drugs are isolated from the matrix using a basic extraction. Deuterated internal standard and selective ion monitoring (SIM) are used in electron ionization (EI) mode.

22.2. Scope

22.2.1. This procedure describes a confirmatory analysis of PCP in biological specimens including blood. Urine confirmations are reported only qualitatively.

22.3. Reagents and Solvents

22.3.1. 1 M Acetic acid
22.3.2. 100 mM Phosphate buffer, pH 6.0
22.3.3. Elution Solvent: 2% ammonium hydroxide in 80:20 methylene chloride/isopropanol. Prepare fresh daily.
22.3.4. Methanol
22.3.5. Hexane
22.3.6. Deionized (DI) water
22.3.7. Ethyl acetate
22.3.8. Blank blood (preserved with potassium oxalate and sodium fluoride)
22.3.9. Blank urine

22.4. Equipment and Materials

22.4.1. Air Displacement Pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
22.4.2. Repeater pipette
22.4.3. Cerex® Clin II, 6 mL columns, 50 mg
22.4.4. Positive pressure SPE manifold
22.4.5. Analytical/Top-loading balance
22.4.6. pH Meter
22.4.7. Evaporator
22.4.8. Vortex mixer
22.4.9. Centrifuge

22.5. Instrumentation

22.5.1. Parameters
22.5.1.1. Capillary Column: 30 m DB-5MS Agilent J&W GC Column (or equivalent), 0.25 mm

id X 0.25 µm film thickness. The flow rate is 1.3 mL/min with an injection volume of 2 µL in split mode (4:1).

22.5.1.2. GC-MS: Agilent GC 7890A-5975C MSD

Initial Temperature:	140 °C hold for 0.5 minutes 30 °C/min to 290 °C hold for 2.5 minutes 50 °C/min to 310 °C hold for 2.6 minutes
Total Run Time:	11.0 min
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quads:	150 °C
MS Source:	230 °C

22.5.1.3. SIM acquisition: PCP.M/PCP_U.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor
PCP-d5	205.2	190.1, 247.2	4.69	1/x
PCP	242.2	243.0, 200.2	4.70	
*Retention Time (RT) varies with column length.				

22.5.2. Performance Check

22.5.2.1. Wash solvents for autosampler: methanol and ethyl acetate are used as the wash solvents. A minimum of 6 pre and 6 post rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.

22.5.3. Ensure that the tune verification and system suitability have been completed, all acceptance criteria met, and maintenance log filled out.

22.6. Standards and Solutions

22.6.1. PCP Working Standards for Calibrators (for blood and urine)

22.6.1.1. This analysis requires preparation of two solutions. The solutions contain concentrations of PCP at 1.0 µg/mL and 0.1 µg/mL.

22.6.1.1.1. Preparation of the 1.0 µg/mL PCP Calibrator: add 25 µL of 1 mg/mL CRM of PCP to a 25 mL volumetric flask and bring to volume with methanol.

22.6.1.1.2. Preparation of the 0.1 µg/mL PCP Calibrator: add 1 mL of the 1.0 µg/mL PCP Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

22.6.2. PCP Working Standards for Quality Controls (for blood and urine)

22.6.2.1. This analysis requires preparation of two solutions. The solutions contain concentrations of PCP at 10 µg/mL and 1.0 µg/mL.

22.6.2.1.1. Preparation of the 10 µg/mL PCP QC Stock Solution: add 100 µL of 1 mg/mL CRM of PCP to a 10 mL volumetric flask and bring to volume with methanol.

22.6.2.1.2. Preparation of the 1.0 µg/mL PCP Control: add 1 mL of the 10 µg/mL PCP QC Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

22.6.3. PCP Internal Standard (for blood and urine)

22.6.3.1. This solution contains a concentration of 1 µg/mL of phencyclidine-d5.

22.6.3.1.1. Preparation of 1 µg/mL PCP Internal Standard: add 100 µL of 100 µg/mL CRM of phencyclidine-d5 to a 10 mL volumetric flask and bring to volume with methanol.

22.7. Fortification Guide

22.7.1. Blood Calibration Curve

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	5	0.1	50	50
1	10	0.1	100	--
1	25	1	25	75
1	50	1	50	50
1	75	1	75	25
1	100	1	100	--

22.7.2. Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	15	1	15	85
1	45	1	45	55
1	80	1	80	20

22.7.3. Urine Calibrator and Positive Control

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	10	0.1 (Calibrator)	100	--
1	20	1 (QC)	20	80

22.8. Extraction Procedure

22.8.1. Allow all biological specimens to come to room temperature before starting the

procedure.

22.8.2. Label all round bottom screw cap tubes accordingly.

22.8.3. Pipet 1 mL of drug-free blood/urine for matrix blank/negative control/calibrators/positive controls into the appropriate labeled tubes.

22.8.4. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.

22.8.5. Pipet 1 mL of case samples into appropriate labeled tubes followed by 100 µL methanol. Vortex.

22.8.6. Using a repeater pipette, add 50 µL of internal standard to each sample to obtain the final concentration of 50 ng/mL. Vortex.

22.8.7. Add 2 mL of 100 mM, pH 6.0 sodium phosphate buffer to each tube. Vortex.

22.8.8. Centrifuge tubes at approximately 3082 rcf for 10 minutes.

22.8.9. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent to dry, unless specified.

22.8.9.1. Pour sample into appropriate SPE column. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

22.8.9.2. Add 1 mL of deionized water and aspirate.

22.8.9.3. Add 1 mL of 1 M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.

22.8.9.4. Add 1 mL of hexane and aspirate.

22.8.9.5. Add 1 mL ethyl acetate and aspirate.

22.8.9.6. Add 1 mL methanol and aspirate. Dry columns under full pressure for 5 minutes.

22.8.9.7. Elute PCP by adding 1 mL of elution solvent, prepared fresh daily.

22.8.10. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi for 5 minutes.

22.8.11. Reconstitute in 30 µL of ethyl acetate to each tube using the repeater pipette. Vortex.

22.8.12. Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.

22.8.13. Inject 2 µL onto the GC-MS using the PCP.M/PCP_U.M method.

22.9. Sequence Table

22.9.1. Every blood batch must contain a matrix blank, negative control, calibrators, and in-house quality controls. Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC).

22.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.

PCP sequence example:

Blood	**Urine**
Matrix blank	Matrix blank
5 ng/mL Calibrator	Cut-off Calibrator
10 ng/mL Calibrator	Negative Control
25 ng/mL Calibrator	Positive Control
50 ng/mL Calibrator	10 Case Samples
75 ng/mL Calibrator	Positive Control
100 ng/mL Calibrator	10 Case Samples
Negative Control	Positive Control
LQC	10 Case Samples
HQC	Positive Control
MQC	
10 Case Samples	
MQC	
10 Case Samples	
MQC	
10 Case Samples	
MQC	
HQC	
LQC	

22.10. Data Analysis

22.10.1. Blood

22.10.1.1. The calibration curve for phencyclidine ranges from 5-100 ng/mL.

22.10.1.2. The low quality control (LQC) for phencyclidine is 15 ng/mL.

22.10.1.3. The mid quality control (MQC) for phencyclidine is 45 ng/mL.

22.10.1.4. The high quality control (HQC) for phencyclidine is 80 ng/mL.

22.10.2. Urine

22.10.2.1. The cut-off calibrator for phencyclidine is 10 ng/mL forced through zero.

22.10.2.2. The positive control (PQC) for phencyclidine is 20 ng/mL.

22.10.3. Limitations

22.10.3.1. Validation was performed using human blood without preservatives for the preparation of calibrators and controls. Evaluation of samples collected in grey top tubes indicates that one of the additives in these tubes causes an elevated m/z 200.2 ion. As a result, the qualifier ion ratio in samples containing <10 ng/mL does not fall within ±20% of the average calibrator ion ratio. The quantitative results are not significantly affected.

22.11. Literature and Supporting Documents

22.11.1. Baselt, Randall C. "Phencyclidine." Disposition of Toxic Drugs and Chemicals in Man, 11th ed. Seal Beach, CA: Biomedical Publications, 2017. 1680-1682.

22.11.2. Grieshaber, A., et al. Stability of Phencyclidine in Stored Blood Samples. Journal of Analytical Toxicology. 1998;22:515-519.

22.11.3. Kunsman, G.W., et al. Phencyclidine Blood Concentrations in DRE Cases. Journal of Analytical Toxicology. 1997;21:498-502.

22.11.4. Pestaner, J.P., et al. Sudden Death During Arrest and Phencyclidine Intoxication. American Journal of Forensic Medicine and Pathology. 2003;24:119-122.

22.11.5. Method File: PCP.M and PCP_U.M

23. Analysis of Alcohol and Other Volatiles by Headspace GC-FID

23.1. Purpose

23.1.1. This procedure outlines the steps for the qualitative or quantitative analysis of ethanol, methanol, acetone, and isopropanol in blood and liquid specimens using headspace sampling and dual column gas chromatography with flame ionization detection (GC-FID).

23.2. Scope

23.2.1. This procedure is used for the qualitative or quantitative analysis of ethanol, methanol, acetone, and isopropanol in blood and liquid specimens.

23.3. Reagents and Solvents

23.3.1. Ethyl Alcohol
23.3.2. n-Propanol
23.3.3. Isopropanol
23.3.4. Acetone
23.3.5. Methanol
23.3.6. Deionized (DI) Water
23.3.7. Blank Blood

23.4. Equipment and Materials

23.4.1. Instrumentation using a method that is approved and validated for use in the section
23.4.2. Compressed gas cylinders or equivalent (helium, hydrogen, air, and nitrogen)
23.4.3. Vials, caps/septa, stoppers, and crimpers
23.4.4. Hamilton pipettor-dilutor or equivalent
23.4.5. Volumetric flasks
23.4.6. Analytical balance
23.4.7. Homogenizer
23.4.8. Centrifuge
23.4.9. Vortex mixer or rocker

23.5. Calibration

23.5.1. Ethanol Calibration (0.010 – 0.500 g/100 mL)
23.5.1.1. Ethanol calibrators must be run with the first batch of case specimens prepared by an individual analyst each day. Subsequent batches prepared by the same analyst during the same work shift must contain controls prepared along with the case specimens.

23.5.1.2. External (e.g., Cerilliant, Lipomed, or equivalent) NIST certified reference ethanol standards or mixed volatile standards that include ethanol are used as calibrators for ethanol calibration. See manufacturer's Certificate of Analysis for storage and expiration information. The following calibrator concentrations are used for ethanol calibration unless otherwise specified in the case record:

Level 1: 0.010 g/100 mL
Level 2: 0.025 g/100 mL
Level 3: 0.050 g/100 mL
Level 4: 0.100 g/100 mL
Level 5: 0.200 g/100 mL
Level 6: 0.400 g/100 mL
Level 7: 0.500 g/100 mL

23.5.1.3. External calibrator solutions typically come in ampoules containing approximately 1.2 mL of solution. Once opened, the contents of the ampoule may be transferred to a labeled container, sealed, and stored in the refrigerator.

23.5.2. Methanol/Isopropanol/Acetone (M/I/A) Calibration (0.010 – 0.400 g/100 mL)

23.5.2.1. External (e.g., Cerilliant, Lipomed, or equivalent) NIST certified reference mixed volatile standards are used as calibrators to establish the M/I/A calibration. The following calibrator concentrations are used unless otherwise specified in the case record:

Level 1: 0.010 g/100 mL
Level 2: 0.025 g/100 mL
Level 3: 0.050 g/100 mL
Level 4: 0.100 g/100 mL
Level 5: 0.200 g/100 mL
Level 6: 0.400 g/100 mL

23.6. Internal Standard

23.6.1. 0.01% n-Propanol Internal Standard can be prepared using either Method 1 or Method 2.

23.6.2. 1.0% n-Propanol Internal Standard Stock Solution (%w/v) – Method 1

23.6.2.1. Weigh out 1.0 g of n-propanol in a 100 mL volumetric flask. Bring to volume with DI water. This will give a 1.0% I.S. stock solution (1.0 g/100 mL).
Storage: Store refrigerated. **Discard**: 6 months.

23.6.3. 0.01% n-Propanol Internal Standard Working Solution (%v/v) – Method 1

23.6.3.1. Add 10 mL of the 1.0% I.S. stock solution to a 1000 mL volumetric flask. Bring to volume with DI water. This will give a 0.01% I.S. working solution (0.010 g/100 mL). **Storage:** Store at room temperature. **Discard**: 6 months from the Stock Solution preparation date.

23.6.4. 0.01% n-Propanol Internal Standard Working Solution (%v/v) – Method 2

23.6.4.1. Add 0.1 mL of n-propanol in a 1000 mL volumetric flask. Bring to volume with DI water. This will give a 0.01% I.S. working solution (0.010 g/100 mL). **Storage**: Store at room temperature. **Discard**: 6 months.

23.7. Controls

23.7.1. Whole Blood Controls

23.7.1.1. Purchased whole blood ethanol controls at low (BQC1) and high (BQC2) concentrations will be used for quantitative ethanol analysis. **Storage:** Store refrigerated. **Discard:** Manufacturer expiration date; 45 days opened.

23.7.1.2. If quantitative M/I/A analysis is required, a purchased mixed volatile whole blood control (MQC) must also be used. **Storage:** Store refrigerated. **Discard:** Manufacturer expiration date; 45 days opened.

23.7.2. Low Aqueous Mixed Volatile Control (LMQC) (0.0192 g/100 mL)

23.7.2.1. The LMQC is a quality control diluted from CRM. Carefully transfer the entire contents (1.2 mL) of a 0.400 g/100 mL mixed volatile external control to a 25 mL volumetric flask and bring to volume with DI water. Cap and mix thoroughly. Carefully transfer into pre-labeled containers. Tightly cap each container. **Storage:** Store refrigerated. **Discard**: 1 year or CRM expiration date, whichever occurs sooner.

23.7.3. Aqueous Ethanol Control (EQC) – 0.080 g/100 mL

23.7.3.1. The EQC is a quality control from an external source. Once open, the contents of the vial may be transferred to a labeled container and tightly capped. **Storage:** Store refrigerated. **Discard:** CRM expiration date.

23.7.4. High Aqueous Ethanol Control (HEQC) – 0.400 g/100 mL

23.7.4.1. The HEQC is a quality control from an external source. Once open, the contents of the vial may be transferred to a labeled container and tightly capped. **Storage:** Store refrigerated. **Discard:** CRM expiration date.

23.7.5. Air Control

23.7.5.1. The air control is a quality control used to monitor any potential interference before and after analysis. This is an empty headspace vial, capped and crimped tightly.

23.7.6. Mixed Volatile System Suitability Control (SS)

23.7.6.1. The SS is a qualitative control at a 0.010 g/100 mL concentration used to confirm resolution prior to each calibration. This can be an external aqueous control or remaining calibration material. A higher concentration for the SS can be used if needed. **Storage:** Store refrigerated. **Discard:** CRM expiration date.

23.7.7. Dilution Control (DQC)

23.7.7.1. A dilution of the HEQC or other CRM with known concentration is analyzed in the batch, if necessary, to verify dilution results of an alcoholic beverage or other liquid case sample(s) employing the same dilution factor used in the case sample.

23.7.7.2. DQC can also be made in-house from chemical reagents to produce a final concentration of 10% Ethanol (v/v). Spike 1 mL of ethanol into a 10 mL volumetric flask filled partially with DI water. Bring to volume with DI water. Cap and mix thoroughly. Carefully transfer into pre-labeled containers. Tightly cap each container.

23.7.7.3. **Storage**: Store refrigerated. **Discard**: 1 year or CRM expiration date, whichever occurs sooner.

23.8. Sample Preparation

23.8.1. Allow calibrators, controls, and case samples to come to room temperature prior to sampling.

23.8.2. Mix all calibrators, controls, and case samples well prior to sampling by gentle inversion or rocking. Avoid shaking.

23.8.3. The DI Water Control, consisting of 100 µL deionized (DI) water, is left open for the duration of the aliquoting process. When all sampling is completed, add internal standard, cap, and crimp tightly.

23.8.4. Dilutions will be performed with DI water prior to aliquoting.

23.8.4.1. When alcoholic beverages or other liquids are analyzed, fluid from the container is diluted appropriately based on suspected sample prior to analysis. From the dilution, a 100 µL aliquot is analyzed. If the sample is unknown, start with a dilution of 1:50.

23.8.4.2. A DQC, using the same dilution as the case sample, must be included in the batch when diluted alcoholic beverages or other liquids are analyzed.

23.8.5. Commonly used dilutions are as follows:

Suspected Sample	Dilution	Beverage or liquid (µL)	Deionized Water (µL)
beer	1:20	50	950
wine or unknown	1:50	20	980
liquor	1:100	10	990

23.8.6. Before using the pipettor-dilutor, verify the appropriate pipette method is selected. Prior to aliquoting, transfer a portion of each case sample into a separate and labeled

transfer container. Transferring and aliquoting occurs one case sample at a time and the transfer container is discarded after each aliquot. Using the pipettor-dilutor, aliquot 100 µL of the calibrators, controls, or case samples. The pipettor-dilutor will deliver 1000 µL of IS working solution along with the 100 µL sample aliquot into a headspace vial. Label the headspace vial appropriately, cap, and crimp tightly.

23.8.7. Samples must be prepared and labeled appropriately in the same order they will be analyzed (see example sequence below).

23.8.8. Between each sample, rinse the pipettor-dilutor tubing at least two times by aliquoting DI water and dispensing the DI water along with the IS working solution. End the rinsing by wiping the tubing.

23.8.9. All case samples are analyzed in duplicate. A single aliquot will be taken from each case sample and recapped. Once all case samples have been singly prepared for analysis, they will be re-ordered prior to aliquoting a second time. This can be accomplished by preparing case samples in reverse order.

23.8.10. For cases that have multiple items submitted, one sample from each item will be analyzed, unless otherwise specified in the case record. Preference will be given in the following order based on type of blood tube: grey>lavender>pink>tan>royal blue (if it contains anticoagulant). See 3.11.2 of the Evidence Handling section for further details.

23.8.11. The condition of a case sample during aliquoting is deemed acceptable (normal) unless otherwise recorded (e.g., clotted). If a case sample is clotted, the sample must be homogenized using a tissue grinder or equivalent prior to aliquoting. See 3.9 of the Evidence Handling section for further details.

23.8.12. Transfer vials from the sampling rack to the autosampler using batch sequence.

23.9. Volatiles Sequence Example:

Air Control (x2)**
SS**
Calibrators (Low to High)
Negative Control (Carryover check)
HEQC
DQC[2]
MQC[1]
Up to 10 case samples
BQC2
Up to 10 case samples
BQC1
Up to 10 case samples
Continue to alternate 10 case samples with low (BQC1) and high (BQC2) controls

BQC1 or BQC2*
LMQC*
EQC*
DI Water Control*
Air Control (x2)*

[1] Only necessary if quantifying M/I/A.

[2] Only necessary if diluted case sample(s) are included in batch.

*NOTE: Each sample batch shall end with BQC1 or BQC2, LMQC, EQC, DI Water Control, and two Air Controls.

**NOTE: Prior to analysis, two air blanks and a SS must be run. The results will be documented in the maintenance log. This performance check does not need to be conducted more than once a day even if multiple analysts are running samples. On the other hand, it can be repeated as needed.

23.9.1. The order of samples on the autosampler must be verified by a second analyst prior to analysis. This must be documented by initialing and dating the sequence.

23.9.2. Batch size will accommodate all accessioned case specimens or a maximum of 30 case specimens unless determined otherwise by the supervisor or manager.

23.10. Calculation and Acceptance Criteria

23.10.1. Analyte concentrations are determined by linear regression (y = mx + b), based on the ratio of the peak area of the analyte divided by the peak area of the internal standard. The calibration curve is calculated by the instrument software.

23.10.2. Air Control and SS

23.10.2.1. The Air Control must be clear of significant (i.e., area counts >10% of the LOQ from the validation) peaks of interest. Presence of significant peaks of interest must be documented in the Headspace GC Maintenance Log (LAB-39 or equivalent form). Analyte(s) of significant interference will not be reported for case samples.

23.10.2.2. Resolution for the reporting analyte(s) in SS must be confirmed.

23.10.3. Calibrators and Controls (Ethanol and M/I/A)

23.10.3.1. Retention time of reporting analyte(s) must be within ±1% of the mean retention time of calibrators (i.e., “Default RT Windows” for “Other Peaks” under Calibration Settings was set at 2%).

23.10.3.2. Results are quantified using values obtained from FID1. FID2 is used as a semi-quantitative confirmation only.

23.10.3.3. For volatile concentrations of reporting analyte(s) >0.050 g/100 mL, obtained values from FID1 must be within ±5% of target value.

23.10.3.4. For volatile concentrations of reporting analyte(s) ≤0.050 g/100 mL, obtained values from FID1 must be within ±10% of target value.

23.10.3.5. Calibrator and control results from FID1 and FID2 per aliquot must be within ±10% of each other for the concentrations ≤0.050 g/100 mL and ±5% of each other for the concentrations >0.050 g/100 mL.

23.10.3.6. The calibration curve must yield an R^2 value of 0.99 or greater for reporting analyte(s).

23.10.3.7. If the concentration of a control or calibrator for reporting analyte(s) is not within the given range, the batch can still be accepted depending on analyte and/or condition.

23.10.3.7.1. HEQC, EQC, and LMQC must be within the allowable range for reporting analyte(s) to accept a batch.

23.10.3.7.2. LMQC and MQC must be within the allowable range for reporting analyte(s) to quantitatively report volatiles other than ethanol.

23.10.3.7.3. Case samples must be immediately bracketed by acceptable controls to report the positive results.

23.10.3.7.4. Any positive case samples bracketed by a control not within the acceptable range must be re-analyzed.

23.10.3.7.5. Negative results may be reported upon documented review of the data by the analyst and technical reviewer.

23.10.4. Internal Standard Recovery

23.10.4.1. For each batch, the consistency of internal standard signal must be evaluated.

23.10.4.2. The internal standard recovery, as indicated by the area counts of the integration, for case samples must be within 0.8 to 1.2 times the internal standard recovery average of the calibrators and controls within the batch.

23.10.5. Chromatography

23.10.5.1. All chromatography must be symmetric and well resolved from any interfering peaks.

23.10.5.2. Peak symmetry will be evaluated for every peak associated with a reporting analyte. The instrument software will generate and report the peak symmetry. A number between 0.5 and 2 indicates acceptable symmetry.

23.10.5.3. Peak resolution will be evaluated for every peak associated with a reporting analyte. The instrument software will generate and report the peak-to-peak valley ratio for peaks that have an apparent shoulder or interfering peak. A number greater than |10| indicates acceptable resolution.

23.10.5.4. Any samples with unresolved or asymmetric peaks for reporting analyte(s) will be re-analyzed.

23.10.6. Case specimens

23.10.6.1. Results from FID1 and FID2 per aliquot must be within ±10% of each other for the concentrations ≤0.050 g/100 mL and ±5% of each other for the concentrations >0.050 g/100 mL.

23.10.6.2. If the average, truncated to three decimals, of aliquot 1 and aliquot 2 from FID1 ≤0.050 g/100 mL, both FID1 aliquot values must be within ±10% of the average.

$$Acceptable\ Range = \frac{Aliquot\ 1\ + Aliquot\ 2}{2}\ x\ 0.9\ to\ \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 1.1$$

23.10.6.3. If the average, truncated to three decimals, of aliquot 1 and aliquot 2 from FID1 >0.050 g/100 mL, both FID1 aliquot values must be within ±5% of the average.

$$Acceptable\ Range = \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 0.95\ to\ \frac{Aliquot\ 1 + Aliquot\ 2}{2}\ x\ 1.05$$

23.10.6.4. If a case sample is not within the given range, that case sample must be re-analyzed. Documentation of the reason for reanalysis must be included in the case record. Case specific data must be included in the case record. If after two analyses the duplicate difference for each run exceeds the acceptable range, the lowest obtained blood alcohol value from all four results (FID1), after truncated to three decimals, will be reported. Any further attempts should be investigated and discussed with the section manager, supervisor, and/or Quality Director.

23.11. Reporting

23.11.1. Ethanol and M/I/A

23.11.1.1. Reported concentration will be the average of the results obtained from aliquot 1 and aliquot 2 on FID1, truncated to three decimal places. FID2 is used as a semi-quantitative confirmation only.

23.11.2. Reporting Statements

23.11.2.1. Blood sample results are reported in “grams per 100 milliliters of blood.”

23.11.2.2. The reported UM must be included on each report using the statement “The uncertainty of measurement associated with this value is ± x.xxx grams per 100 milliliters” or an equivalent statement.

23.11.2.3. For volatiles in alcoholic beverages or other liquid specimens, results are reported in “grams of ethanol/methanol/acetone/isopropanol per 100 milliliters of liquid.”

23.11.2.4. For serum/plasma samples, the report must indicate that the concentration is in serum or plasma using the statement, “Item x.x was found to contain x.xxx grams of ethanol per 100 milliliters of serum/plasma. The equivalent whole blood concentration

was calculated to be x.xxx - x.xxx grams of ethanol per 100 milliliters of blood using a conversion factor range of 1.15 to 1.2" or an equivalent statement.

23.11.2.5. For samples that required homogenization, the report should state "This sample was homogenized prior to analysis" or an equivalent statement.

23.11.2.6. If no volatiles are detected, or if results are below the lowest calibrator (0.010 g/100 mL), the report should state "no alcohol detected" or an equivalent statement.

23.11.2.7. If there is insufficient sample, the report should state "Insufficient sample for analysis" or an equivalent statement.

23.11.2.8. If the sample is unsuitable for analysis, the report should state "Sample unsuitable for analysis" or an equivalent statement.

23.12. Literature and Supporting Documents

23.12.1. Caplan, Yale H., Goldberger, Bruce A., eds. Garriott's Medicolegal Aspects of Alcohol, 6th ed. Tucson, AZ: Lawyers & Judges Publishing Company, Inc. 2015.

24. Validation of Quantitative Methods

24.1. Purpose

24.1.1. This procedure is intended to define the minimum parameters and sets of experiments to validate a quantitative method.

24.2. Scope

24.2.1. This procedure applies to all quantitative bio-analytical methods.

24.2.2. Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

24.2.2.1. Development of a new method.

24.2.2.2. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

24.2.2.3. Transfer of a validated method to a new instrument.

24.2.3. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

24.3. Validation Parameters

24.3.1. The following validation parameters are performed according to the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter. Some parameters may not be conducted or may be different from the described procedure. If so, the change(s) will be described in the validation documents.

- Calibration model/linearity
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Bias and imprecision
- Carryover
- Interference studies
- Matrix matching (if applicable, not included in SWGTOX guidelines)
- Dilution integrity (if applicable)
- Stability (if applicable)
- Ionization suppression/enhancement (if applicable)*

***Note:** At this time HFSC does not employ Liquid Chromatography-Tandem Mass spectrometry for any analysis. Ionization suppression/enhancement is not applicable to GC-MS testing.

- Extraction efficiency and matrix effect
- Blind sample analysis (if applicable; not included in the SWGTOX guidelines)

24.4. Validation Experiments

24.4.1. Calibration model and linearity

24.4.1.1. The most appropriate calibration model should be determined during method development. For methods using a detector that is theoretically linear over the covered concentration range, it is recommended that initially a straight line concentration-response model be adopted using weighted linear regression with inverse concentration weighting to estimate slope and intercept. Other procedures may be probed, preferably in the following sequence:

24.4.1.1.1. Straight line fit using weighted linear regression with inverse concentration-squared weights

24.4.1.1.2. Straight line fit forced through the origin for single point calibrations only

24.4.1.1.3. Quadratic fit with inverse concentration using a weighted 2nd order polynomial

24.4.1.1.4. There are three components to validation of the calibration model/linearity – verification of weighting scheme, validation of linearity across the analytical measurement range, and validation of calibration.

24.4.1.2. Procedure

24.4.1.2.1. Once the best calibration model has been determined, the following experiment is to be performed to evaluate the effectiveness of the model:

24.4.1.2.2. Prepare a series of standards covering the concentration range of interest with typically no more than a five-fold increase between sequential calibrators. At least six (nonzero) concentration levels should be used.

24.4.1.2.3. A minimum of five replicates per concentration is required. The replicates shall be in separate runs. The origin shall not be included as a calibration point.

24.4.1.3. Data analysis

24.4.1.3.1. Use data from all five runs to produce combined calibration curves with no weighting, 1/x weighting and $1/x^2$ weighting and process data calibration data using all three calibration curves. This can be done by exporting the data and using a validated worksheet in Microsoft Excel designed for this purpose.

24.4.1.3.1.1. These combined curves will be used to verify the weighting scheme.

24.4.1.3.2. Using the data from the appropriate weighting scheme, perform an unweighted linear regression of $C_{nominal}$ v. $C_{calculated}$ where $C_{nominal}$ is nominal (labeled or established) concentration of calibrator and $C_{calculated}$ is the concentration obtained from calibration curve.

24.4.1.3.2.1. These data will be used to evaluate the linearity of the analytical measurement range.

24.4.1.3.3. Process each individual run using the selected weighting scheme.

24.4.1.3.3.1. These data will be used to validate the calibration range.

24.4.1.3.4. If fewer calibrators will be used to analyze case samples than were used in validation, reprocess all data using only the calibrators selected to be used during case analysis. Perform an unweighted linear regression of C_{valcal} v. $C_{casecal}$ where C_{valcal} is the calculated concentrations of calibrators and controls using all calibrators included in the validation experiments and $C_{casecal}$ is the calculated concentrations of calibrators and controls using the calibrators that will be used during analysis of case samples.

24.4.1.4. Acceptance criteria

24.4.1.4.1. Verification of weighting scheme

24.4.1.4.1.1. Correct weighting must be verified by evaluating the $\sum|\%RE|$ for at least unweighted, 1/x and $1/x^2$. The least complex weighting scheme that minimizes $\sum|\%RE|$ should be used.

$$\%RE = \frac{C_{calculated} - C_{nominal}}{C_{nominal}} \text{ x } 100$$

24.4.1.4.2. Validation of linearity

24.4.1.4.2.1. Visual examination of combined calibration curve with appropriate weighting must verify linearity across analytical range

24.4.1.4.2.1.1. The linear regression of $C_{nominal}$ vs $C_{calculated}$ must meet the following requirements:

24.4.1.4.2.1.1.1. 95% CI of slope must include 1.

24.4.1.4.2.1.1.2. 95% CI of intercept must include 0.

24.4.1.4.3. Validation of calibration

24.4.1.4.3.1. Visual examination of calibration curve must verify linearity across analytical range.

24.4.1.4.3.1.1. %RE of each calibrator, as determined by the weighted linear regression of the individual run, must be ≤± 20% (25% at the lowest calibrator).

24.4.1.4.4. Validation of casework calibration

24.4.1.4.4.1. The linear regression of C_{valcal} vs $C_{casecal}$ must meet the following requirements:

24.4.1.4.4.1.1. 95% CI of slope must include 1.

24.4.1.4.4.1.2. 95% CI of intercept must include 0.

24.4.1.4.5. Note: All remaining validation experiments should be evaluated using results from the data analysis which includes only the calibrators which are to be used in the analysis of case samples.

24.4.2. Limit of detection (LOD)

24.4.2.1. Procedure

24.4.2.1.1. Analyze a minimum of three different sources of blank matrix fortified at decreasing concentrations in duplicate for three days.

24.4.2.2. Data analysis

24.4.2.2.1. Visually inspect the chromatograms to evaluate the retention time, peak shape, mass spectral ion ratios, and any other criteria used to identify the analyte of interest.

24.4.2.2.2. Use the instrument software to evaluate the signal-to-noise ratio in the blanks and the LOD samples.

24.4.2.3. Acceptance criteria

24.4.2.3.1. The LOD is the lowest concentration that:

24.4.2.3.1.1. Yields a reproducible instrument response greater than or equal to three times the noise level of the background signal from the negative samples.

24.4.2.3.1.2. Achieves acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).

24.4.3. Limit of quantitation (LOQ)

24.4.3.1. Procedure

24.4.3.1.1. Prepare a pooled fortified matrix sample at the same concentration as the lowest calibrator.

24.4.3.1.2. Analyze a minimum of three replicates a run in five different runs.

24.4.3.2. Data analysis

24.4.3.2.1. Use the established calibration curve to quantify the analyte of interest.

24.4.3.2.2. Calculate the bias

$$Bias = \frac{Average\ of\ calculated\ concentrations\ -\ Nominal\ concentration}{Nominal\ Concentration}\ x\ 100$$

24.4.3.2.3. Calculate the within-run and between-run imprecision

$$Within - Run\ Imprecision\ = \frac{Standard\ deviation\ of\ single\ run}{Average\ of\ single\ run}\ x\ 100$$

$$Between - Run\ Imprecision\ = \frac{Standard\ deviation\ of\ all\ runs}{Average\ of\ all\ runs}\ x\ 100$$

24.4.3.3. Acceptance criteria

24.4.3.3.1. Bias: %bias ≤25%

24.4.3.3.2. Within-run imprecision: %CV ≤25%

24.4.3.3.3. Between-run imprecision: %CV ≤25%

24.4.4. Bias and imprecision

24.4.4.1. Procedure

24.4.4.1.1. Prepare pooled fortified matrix samples using a minimum of three replicates per run at three control concentrations (low, medium, and high).

24.4.4.1.2. Analyze a minimum of three replicates per run in five different runs.

24.4.4.2. Data analysis

24.4.4.2.1. Use the established calibration curve to calculate the concentration of the analyte of interest.

24.4.4.2.2. Calculate bias, within-run and between-run precision.

24.4.4.3. Acceptance criteria

24.4.4.3.1. Bias: %bias ≤20%

24.4.4.3.2. Within-run imprecision: %CV ≤20%

24.4.4.3.3. Between-run imprecision: %CV ≤20%

24.4.5. Carryover

24.4.5.1. Procedure

24.4.5.1.1. Analyze blank matrix samples with IS (i.e., negative control) following the high calibrator in each validation run.

24.4.5.2. Data analysis

24.4.5.2.1. Use the established calibration curve to calculate the concentration of the analyte of interest.

24.4.5.3. Acceptance criteria

24.4.5.3.1. Quantitative result must be ≤25% of LOQ.

24.4.6. Interference studies

24.4.6.1. Procedure

24.4.6.1.1. Matrix interference – Analyze a minimum of ten different sources of blank matrix (without IS).

24.4.6.1.2. Interference from stable-isotope internal standards – Analyze a blank matrix fortified with IS but no analyte of interest in each validation run.

24.4.6.1.3. Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the low control concentration and potential interferences at high therapeutic or lethal concentrations.

24.4.6.2. Data analysis

24.4.6.2.1. Matrix interference – Evaluate response of any peak at the retention time of the analyte of interest.

24.4.6.2.2. Interference from stable-isotope internal standards – Evaluate response of any peak at the retention time of the analyte of interest.

24.4.6.2.3. Interference from commonly encountered exogenous analytes – Use the established calibration curve to calculate the concentration of the analyte of interest.

24.4.6.3. Acceptance criteria

24.4.6.3.1. Matrix interference – Response of blank matrix must be ≤10% of the average response of LOQ.

24.4.6.3.2. Interference from stable-isotope internal standards – Response of blank matrix must be ≤10% of the average response of LOQ.

24.4.6.3.3. Interference from commonly encountered exogenous analytes - Concentration of analytes of interest must be within ±20% of the average concentration obtained in the Bias and Imprecision studies.

24.4.7. Matrix matching

24.4.7.1. If matrices other than that used to create the calibration will be analyzed, the ability of the method to accurately quantify analytes in that matrix must be evaluated. Example: A method that uses blood calibrators but will be used to analyze serum samples.

24.4.7.2. Procedure

24.4.7.2.1. Perform bias and precision studies in each matrix that will be analyzed using the method.

24.4.7.2.2. Ideally multiple sources of each matrix should be used for these studies.

24.4.7.3. Data analysis

24.4.7.3.1. Use the established calibration curve to calculate the concentration of the analyte of interest.

24.4.7.3.2. Calculate bias, within-run and between-run precision.

24.4.7.4. Acceptance criteria

24.4.7.4.1. Bias: %bias ≤20%.

24.4.7.4.2. Within-run imprecision: %CV ≤20%.

24.4.7.4.3. Between-run imprecision: %CV ≤20%.

24.4.8. Dilution integrity (if applicable)
24.4.8.1. Procedure
24.4.8.1.1. Dilute the highly concentrated samples with blank matrix to evaluate dilution ratios that may be used on case specimens (e.g., high control, high calibrator, or sample fortified at a high concentration). Common dilutions are 2x (1 part sample + 1 part diluent), 5x (1 part sample + 4 parts diluent), and 10x (1 part sample + 9 parts diluent).
24.4.8.1.2. Analyze a minimum of two replicates of each dilution per run for at least three runs.
24.4.8.2. Data analysis
24.4.8.2.1. Calculate bias, between-run imprecision, and %RE (%difference) as described above.
24.4.8.2.2. Note: The average control concentrations from the bias and imprecision experiments can be used as the target for evaluating dilution integrity.
24.4.8.3. Acceptance criteria
24.4.8.3.1. All diluted samples must quantify within control acceptance criteria (typically 20%) of the average value obtained in the Bias and Imprecision studies if high controls are used and of the theoretical value if other samples are used.
24.4.9. Stability
24.4.9.1. Procedure
24.4.9.1.1. Storage stability – A literature search should be performed to determine if storage stability for the analyte of interest has been reported. If no information can be found, then follow the procedure below.
24.4.9.1.1.1. Determine what conditions will be evaluated. Stability should be investigated in gray-top tubes stored at room temperature, refrigerated temperature and frozen. If other types are expected to be received, they should also be investigated.
24.4.9.1.1.2. Note: If analytes are determined to be unstable at all storage conditions, an investigation of the effects of light protection should be considered.
24.4.9.1.1.3. Prepare enough control material at low and high control concentrations to complete entire stability experiment.
24.4.9.1.1.4. Store specimens at determined temperatures.
24.4.9.1.1.5. Analyze samples in triplicate after storage for 0, 7, 14, and 30 days and 6 months. Note: It is permissible to use Day 1 validation control results as “Day 0” for stability purposes.

24.4.9.1.2. In-Process stability – If it is expected that a break may need to take place during sample preparation, the stability of the analyte over that time period should be evaluated.

24.4.9.1.2.1. Determine the point in the procedure at which a break may occur and how long the break may be. In the case of procedures where solid phase or liquid extractions are performed, the studied step should be the final extraction or elution solvent.

24.4.9.1.2.2. Prepare a calibration curve and controls according to the normal procedure.

24.4.9.1.2.3. Prepare a second set of controls, in duplicate, but delay completion of preparation at the predetermined step and for the correct time.

24.4.9.1.3. Autosampler stability (two experiments) – Both the analyte(s) and the internal standard in processed control samples will be tested for stability. This will demonstrate that extracts are stable while in the autosampler awaiting analysis. In addition, determination of stability beyond 12 hours will allow for the re-injection of samples in the event of instrument malfunction.

24.4.9.1.3.1. Determine the maximum residence time of the extracts in the autosampler to be tested. Include the time a run may sit unanalyzed over a weekend in the auto-sampler due to an instrument malfunction. A different approach may be necessary for unstable molecules.

24.4.9.1.3.2. Autosampler stability of calibrators: Set aside a validation run that includes calibrators, and at least two low and two high controls to be re-injected. These samples will be added to another validation run with a freshly prepared calibrator.

24.4.9.1.3.3. Autosampler stability of controls: Prepare a calibration curve along with at least four low and four high controls.

24.4.9.1.3.3.1. Inject the calibration curve only and one set (at least two low and two high) controls.

24.4.9.1.3.3.2. Store the remaining vials in the autosampler for at least 12 hours. The interval chosen should represent the longest period that the processed extracts will remain at autosampler conditions during the course of an analytical run.

24.4.9.1.3.3.3. At the end of the predetermined time period inject all controls (half will be unanalyzed, half will be re-injects).

24.4.9.2. Data analysis

24.4.9.2.1. Use the established calibration curve to calculate the concentration of the analyte of interest.

24.4.9.3. Acceptance criteria

24.4.9.3.1. All storage stability samples must quantify within control acceptance criteria (typically 20%) of the method compared to the average concentration on Day 0.

24.4.9.3.2. All in-process and autosampler stability samples must quantify within control acceptance criteria of the average value obtained in the Bias and Imprecision studies for control samples; the target concentrations for calibrators are nominal values.

24.4.10. Extraction efficiency and matrix effect

24.4.10.1. Procedure

24.4.10.1.1. Prepare three groups of samples fortified with analyte at three different concentrations spanning the calibration curve (n = 5 at each concentration).

24.4.10.1.2. Fortify blank matrix samples with control solution before sample extraction (Group 1).

24.4.10.1.3. Fortify blank matrix samples with control solution after sample extraction (Group 2).

24.4.10.1.4. Fortify elution solvent with control solution (neat samples, Group 3).

24.4.10.2. Data Analysis

24.4.10.2.1. Determine a mean analyte area of samples in each group at each concentration.

24.4.10.2.2. Calculate the extraction efficiency:

$$Extraction\ Efficiency\ = \frac{Mean\ peak\ area\ of\ samples\ fortified\ before\ extraction}{Mean\ peak\ area\ of\ samples\ fortified\ after\ extraction}\ x\ 100$$

24.4.10.2.3. Calculate the matrix effect:

$$Matrix\ effect\ = (\frac{Mean\ peak\ area\ of\ samples\ fortified\ after\ extraction}{Mean\ peak\ area\ of\ neat\ samples} - 1)\ x\ 100$$

24.4.10.3. Acceptance Criteria

24.4.10.3.1. If a method achieves desired limit of quantification and/or detection for the analyte, its extraction efficiency and matrix effect are considered acceptable.

24.4.11. Blind sample analysis (if applicable)

24.4.11.1. Procedure

24.4.11.1.1. Prepare a total of 20 blind samples; the expected concentrations of these samples should be blinded (unknown) to the analyst performing the validation. These samples can be discarded/de-identified authentic samples known to be positive or negative for analyte(s) of interest or fortified samples. Ten of the

samples should have concentrations between 0% and 75% of LOD, and the other ten samples should have the analyte of interest at concentrations of ≥125% of LOD.

24.4.11.2. Data analysis

24.4.11.2.1. Determine the analyte concentration of each sample.

24.4.11.2.2. If positive, calculate the percent difference between expected concentration and determined concentration.

24.4.11.3. Acceptance criteria

24.4.11.3.1. Qualitative agreement must be 100%.

24.4.11.3.2. The percent difference between the expected and determined concentrations must be ≤20%.

25. Validation of Reportable Qualitative Methods

25.1. Purpose

25.1.1. This procedure is intended to define the minimum parameters and sets of experiments to validate a reportable qualitative method.

25.2. Scope

25.2.1. This procedure applies to all qualitative bio-analytical methods which produce reportable results.

25.2.2. Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

25.2.3. Development of a new method.

25.2.3.1. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

25.2.3.2. Transfer of a validated method to a new instrument.

25.2.4. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

25.3. Validation Parameters

25.3.1. The following validation parameters are performed according to the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter. Some parameters may not be conducted or different from the described procedure. If so, the change will be described in the validation documents.

25.3.2. Precision around reporting limit (RL)

25.3.3. Sensitivity and specificity

25.3.4. Interference studies

25.3.5. Stability (if applicable)

25.3.6. Ionization suppression/enhancement (if applicable)

25.3.6.1. Note: At this time HFSC does not employ Liquid Chromatography-Tandem Mass spectrometry for any analysis. Ionization suppression/enhancement is not applicable to GCMS testing.

25.3.7. Hydrolysis recovery (if applicable)

25.4. Validation Experiments

25.4.1. Precision around reporting limit (RL)

25.4.1.1. Procedure

25.4.1.1.1. Prepare a pooled fortified matrix sample at the qualitative negative control, cut-off calibrator, and positive control concentrations.

25.4.1.1.2. Analyze five samples a run at each concentration over a minimum of five runs (25 samples minimum at each concentration).

25.4.1.2. Data analysis

25.4.1.2.1. Determine if each sample is positive or negative.

25.4.1.3. Acceptance criteria

- At least 22 (90%) of the negative controls must be negative.
- At least 22 (90%) of the positive controls must be positive.
- 8-16 (35-65%) of the cut-off calibrators must be positive.

25.4.2. Sensitivity and specificity

25.4.2.1. Procedure

25.4.2.1.1. Prepare a total of 40 blind samples. These samples can be discarded/de-identified authentic samples known to be positive or negative for analyte(s) of interest or fortified samples. Twenty of the samples should have concentrations between 0% and 75% of the cut-off calibrator concentration, and the other 20 samples should have the analyte of interest at concentrations of ≥125% of the cut-off concentration. The expected result of these samples should be blinded to the analyst performing the validation.

25.4.2.2. Data analysis

25.4.2.2.1. Determine if each sample is positive or negative

25.4.2.2.2. Calculate sensitivity and specificity

$$Sensitivity = \frac{TP}{TP + FN} x\ 100$$

$$Specificity = \frac{TN}{TN + FP} x\ 100$$

25.4.2.3. Acceptance criteria

25.4.2.3.1. Sensitivity ≥90%

25.4.2.3.2. Specificity ≥90%

25.4.3. Interference studies

25.4.3.1. Procedure

25.4.3.1.1. Matrix interference – Analyze a minimum of 10 different sources of blank matrix (without IS).

25.4.3.1.2. Interference from stable-isotope internal standards – Analyze a blank matrix fortified with IS but no analyte of interest in each validation run.

25.4.3.1.3. Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the negative control concentration and potential interferences at high therapeutic or lethal concentrations.

25.4.3.2. Data analysis

25.4.3.2.1. Matrix interference – determine if sample is positive or negative.

25.4.3.2.2. Interference from stable-isotope internal standards – determine if sample is positive or negative.

25.4.3.2.3. Interference from commonly encountered exogenous analytes –determine if sample is positive or negative.

25.4.3.3. Acceptance criteria

25.4.3.3.1. All samples must test negative.

25.4.4. Stability

25.4.4.1. Procedure

25.4.4.1.1. Storage stability – A literature search should be performed to determine if storage stability for the analyte of interest has been reported. If no information can be found, then follow the procedure below.

25.4.4.1.2. Determine what conditions will be evaluated. At minimum, stability should be investigated in plastic urine cups stored at room temperature, refrigerated temperature and frozen. If other types are expected to be received, they should also be investigated.

25.4.4.1.2.1. Note: If analytes are determined to be unstable at all storage conditions, an investigation of the effects of light protection should be considered.

25.4.4.1.2.2. Prepare enough positive control material to complete entire stability experiment.

25.4.4.1.2.3. Store specimens at determined temperatures.

25.4.4.1.2.4. Analyze samples in triplicate after storage for 0, 7, 14, and 30 days and 6 months.

25.4.4.1.2.5. Note: It is permissible to use Day 1 validation control results as “Day 0” for stability purposes.

25.4.4.1.3. In-Process stability – If it is expected that a break may need to take place during sample preparation, the stability of the analyte over that time period should be evaluated.

25.4.4.1.3.1. Determine the point in the procedure at which a break may occur and how long the break may be. In the case of procedures where solid phase or liquid extractions are performed, the studied step should be the final extraction or elution solvent.

25.4.4.1.3.2. Prepare the cut-off calibrator, negative control and positive control according to the SOP.

25.4.4.1.3.3. Prepare a second set of positive controls, in duplicate, but delay completion of preparation at the predetermined step and for the correct time.

25.4.4.1.4. Autosampler stability – Both the analyte(s) and the internal standard in processed control samples will be tested for stability. This will demonstrate that extracts are stable while in the autosampler awaiting analysis. In addition, determination of stability beyond 12 hours will allow for the re-injection of samples in the event of instrument malfunction.

25.4.4.1.5. Determine the maximum residence time of the extracts in the autosampler to be tested. Include the time a run may sit unanalyzed over a weekend in the autosampler due to an instrument malfunction. A different approach may be necessary for unstable molecules.

25.4.4.1.6. Prepare a cut-off calibrator curve along with at least four positive controls and one negative control

25.4.4.1.7. Inject the calibration curve, negative control and one set of positive controls (at least two).

25.4.4.1.8. Store the remaining vials in the autosampler for at least 12 hours. The interval chosen should represent the longest period that the processed extracts will remain at autosampler conditions during the course of an analytical run.

25.4.4.1.9. At the end of the predetermined time period, inject all controls (half will be unanalyzed, half will be re-injects).

25.4.4.2. Data analysis

25.4.4.2.1. Determine if samples are positive or negative.

25.4.4.3. Acceptance criteria

25.4.4.3.1. All positive controls must remain positive.

25.4.5. Hydrolysis recovery

25.4.5.1. Procedure

25.4.5.1.1. Analyze a minimum of three positive controls and two glucuronide positive controls (hydrolysis controls) a run over a minimum of three runs.

25.4.5.2. Data analysis

25.4.5.2.1. Calculate the response ratios of controls and corresponding internal standards. Determine recovery of the hydrolysis process by dividing the average response ratio

of the positive controls by the average response ratio of the hydrolysis controls and multiplying it by 100.

25.4.5.3. Acceptance Criteria

25.4.5.3.1. If a method satisfies the requirement of hydrolysis controls being positive, its hydrolysis recovery is considered acceptable.

26. Validation of Immunoassay Using Commercial Kits

26.1. Purpose

26.1.1. This procedure is intended to define a series of experiments that are relevant elements of a qualitative immunoassay method validation.

26.2. Scope

26.2.1. This procedure applies to immunoassay based methods using commercially available kits.

26.2.2. Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

26.2.2.1. Development of a new method.

26.2.2.2. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

26.2.2.3. Transfer of a validated method to a new instrument.

26.2.3. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

26.3. Validation Parameters

26.3.1. The following validation parameters are performed according to the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Please refer to the document published May 20, 2013 for detailed information on each parameter. Some parameters may not be conducted or different from the described procedure. If so, the change will be described in the validation documents.

26.3.2. Limit of detection (LOD)

26.3.3. Precision at the decision point

26.3.4. Cross-reactivity (if applicable) – Note: Cross-reactivity will not be evaluated, unless otherwise specified in the validation document, if cross-reactivity data are provided for the commercial immunoassay kits by the manufacturer.

26.3.5. Dilution integrity (if applicable)

26.3.6. Stability (if applicable) – Note: Stability will not be evaluated unless otherwise specified in the validation document. Stability could have been evaluated during validation of confirmation assays and/or indicated in literature.

26.4. Validation Experiments

26.4.1. Limit of detection (LOD)

26.4.1.1. Typically, the LOD of immunoassay based methods is defined as the decision point concentration.

26.4.2. Precision at the decision point

26.4.2.1. Procedure

26.4.2.1.1. Negative (no more than 50% below decision point) and positive controls (no more than 50% above decision point) are prepared and tested for each qualitative method validation. In addition, a material of known concentration near the cut-off limit is tested.

26.4.2.1.2. Perform a daily assay testing each of the three materials (negative, positive and near cut-off) over a period of five days, with five replicates per day. Thus, 25 data points are generated for each level. The same lots of reagents and cut-off material are used during the test period.

26.4.2.2. Data analysis

26.4.2.2.1. Determine if each sample is positive or negative using the average absorbance of the five replicates of cut-off calibrator.

26.4.2.3. Acceptance criteria

26.4.2.3.1. Expected results for the negative and positive controls.

26.4.2.4. Monitored data

26.4.2.4.1. These data are calculated as part of method validation to identify any potential issues which may be encountered during routine use. These are not criteria for acceptance or rejection of the immunoassay validation.

26.4.2.4.1.1. Overall %RSD of the absorbance <20% at each of the three levels.

26.4.2.4.1.2. Mean ± two standard deviations of the absorbance should not overlap the decision point.

27. Validation of Screening Methods

27.1. Purpose

27.1.1. This procedure is intended to define the minimum parameters and sets of experiments to validate a screening method.

27.2. Scope

27.2.1. This procedure applies to all qualitative bio-analytical methods whose results need to be confirmed by a confirmatory method.

27.2.2. Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

27.2.3. Development of a new method.

27.2.3.1. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

27.2.3.2. Transfer of a validated method to a new instrument.

27.2.4. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. Determination of validation parameters which must be re-evaluated will be dependent upon the changes made to the method.

27.3. Validation Parameters

27.3.1. Positive, carryover, and negative control percent match

27.3.2. Sensitivity and specificity

27.3.3. Interference studies

27.3.4. Stability (if applicable)

27.3.5. Ionization suppression/enhancement (if applicable)

27.3.5.1. Note: At this time HFSC does not employ Liquid Chromatography-Tandem Mass spectrometry for any analysis. Ionization suppression/enhancement is not applicable to GCMS testing.

27.4. Validation Experiments

27.4.1. Positive, carryover, and negative control percent match

27.4.1.1. Procedure

27.4.1.1.1. Prepare a pooled fortified matrix sample at the qualitative positive control, carryover control, and negative control concentrations.

27.4.1.1.2. Analyze three samples a run at each concentration over a minimum of three runs (9 samples minimum at each concentration).

27.4.1.2. Data analysis

27.4.1.2.1. Determine if each sample is positive or negative.

27.4.1.3. Acceptance criteria

- All (100%) of the positive controls must be positive.
- All (100%) of the carryover controls must be positive.
- All (100%) of the negative controls must be negative.

27.4.2. Sensitivity and specificity

27.4.2.1. Procedure

27.4.2.1.1. Prepare a total of 40 blind samples. These samples can be discarded/de-identified authentic samples known to be positive or negative for analyte(s) of interest or fortified samples. Twenty of the samples should have concentrations between 0% and 75% of the cut-off calibrator concentration, and the other 20 samples should have the analyte of interest at concentrations of ≥125% of the cut-off concentration. The expected result of these samples should be blinded to the analyst performing the validation.

27.4.2.2. Data analysis

27.4.2.2.1. Determine if each sample is positive or negative

27.4.2.2.2. Calculate sensitivity and specificity

$$Sensitivity = \frac{TP}{TP + FN} x\ 100$$

$$Specificity = \frac{TN}{TN + FP} x\ 100$$

27.4.2.3. Acceptance criteria

27.4.2.3.1. Sensitivity ≥90%

27.4.2.3.2. Specificity ≥90%

27.4.3. Interference studies

27.4.3.1. Procedure

27.4.3.1.1. Matrix interference – Analyze a minimum of 10 different sources of blank matrix (without IS).

27.4.3.1.2. Interference from stable-isotope internal standards – Analyze a blank matrix fortified with IS but no analyte of interest in each validation run.

27.4.3.2. Data analysis

27.4.3.2.1. Matrix interference – determine if sample is positive or negative.

27.4.3.2.2. Interference from stable-isotope internal standards – determine if sample is positive or negative.

27.4.3.3. Acceptance Criteria

27.4.3.3.1. All samples must test negative.

27.4.4. Stability

27.4.4.1. Procedure

27.4.4.1.1. Storage stability – A literature search should be performed to determine if storage stability for the analyte of interest has been reported. If no information can be found, then follow the procedure below.

27.4.4.1.2. Determine what conditions will be evaluated. At minimum, stability should be investigated in plastic urine cups stored at room temperature, refrigerated temperature and frozen. If other types are expected to be received, they should also be investigated.

27.4.4.1.2.1. Note: If analytes are determined to be unstable at all storage conditions, an investigation of the effects of light protection should be considered.

27.4.4.1.2.2. Prepare enough positive control material to complete entire stability experiment.

27.4.4.1.2.3. Store specimens at determined temperatures.

27.4.4.1.2.4. Analyze samples in triplicate after storage for 0, 7, 14, and 30 days and 6 months.

27.4.4.1.2.5. Note: It is permissible to use Day 1 validation control results as “Day 0” for stability purposes.

27.4.4.1.3. In-Process stability – If it is expected that a break may need to take place during sample preparation, the stability of the analyte over that time period should be evaluated.

27.4.4.1.3.1. Determine the point in the procedure at which a break may occur and how long the break may be. In the case of procedures where solid phase or liquid extractions are performed, the studied step should be the final extraction or elution solvent.

27.4.4.1.3.2. Prepare the cut-off calibrator, negative control and positive control according to the SOP.

27.4.4.1.3.3. Prepare a second set of positive controls, in duplicate, but delay completion of preparation at the predetermined step and for the correct time.

27.4.4.1.4. Autosampler stability – Both the analyte(s) and the internal standard in processed control samples will be tested for stability. This will demonstrate that extracts are stable while in the autosampler awaiting analysis. In addition, determination of stability beyond 12 hours will allow for the re-injection of samples in the event of instrument malfunction.

27.4.4.1.5. Determine the maximum residence time of the extracts in the autosampler to be tested. Include the time a run may sit unanalyzed over a weekend in the autosampler due to an instrument malfunction. A different approach may be necessary for unstable molecules.

27.4.4.1.6. Prepare at least four positive controls and one negative control.

27.4.4.1.7. Inject the negative control and one set of positive controls (at least two).

27.4.4.1.8. Store the remaining vials in the autosampler for at least 12 hours. The interval chosen should represent the longest period that the processed extracts will remain at autosampler conditions during the course of an analytical run.

27.4.4.1.9. At the end of the predetermined time period, inject all controls (half will be unanalyzed, half will be re-injects).

27.4.4.2. Data analysis

27.4.4.2.1. Determine if samples are positive or negative.

27.4.4.3. Acceptance criteria

27.4.5. All positive controls must remain positive.

28. Retrograde Extrapolation and the Widmark Equation

28.1. Purpose

28.1.1. This procedure outlines the process of performing retrograde extrapolations and calculations using the Widmark equation.

28.2. Scope

28.2.1. This procedure is used for performing retrograde extrapolations and calculations using the Widmark equation based on facts given by a requestor for case samples involving blood alcohol concentrations.

28.3. Equations and Calculations

28.3.1. Retrograde Extrapolation

$BAC_E = [(BAC_K – UM) + (t \times e_{low})]$ to $[(BAC_K + UM) + (t \times e_{high})]$

Where: BAC_E = the estimated blood ethanol concentration range at the time of the stop (g/100 mL); BAC_K = the known blood ethanol concentration at the time of the blood draw (g/100 mL); UM = the uncertainty of measurement associated with the BAC_K (g/100 mL); t = time difference between the stop and the blood draw (hour); e_{low} = elimination rate range low limit (0.01 g/100 mL/hour); and e_{high} = elimination rate range high limit (0.03 g/100 mL/hour).

28.3.2. The Widmark Equation

$a = BAC \times \rho \times V_d$

OR

$BAC = a/(\rho \times V_d)$

Where: a = estimated amount of alcohol in body (g); BAC = blood ethanol concentration, either known, estimated, or hypothetical (g/100 mL); ρ = weight (g); and V_d = volume of distribution (0.7 L/kg = 0.007 100 mL/g for males and 0.6 L/kg = 0.006 100 mL/g for females).

28.3.3. Number of standard drinks (N)

$N = a/14$

OR

$N = (BAC \times \rho \times V_d)/14$

28.3.4. Conversions and Constants

- 1 fluid ounce (oz) = 29.6 mL
- 1 pound (lb) = 454 g
- 1 mL ethanol = 0.789 g ethanol (using the ethanol density at 20 °C)
- 1 standard drink = 14 g ethanol (rounded to the whole number) = 5 oz wine (12% alcohol) = 12 oz beer (5% alcohol) = 1.5 oz liquor (80 proof or 40% alcohol)

28.3.5. Facts

28.3.5.1. Facts needed in order to perform a retrograde extrapolation:

- BAC_K
- Time of the blood draw
- Time of the stop
- Time of the last drink

28.3.5.2. Facts needed in order to use the Widmark equation:

- BAC or Number of drink(s)
- Weight (lb)
- Male or Female

28.3.5.3. Additional facts such as time of the last meal, number of drink(s), height, age, type of food, type of drink(s), and other facts not listed above are NOT included in retrograde extrapolation or calculations using the Widmark equation.

28.3.6. Assumptions and Limitations

28.3.6.1. Retrograde extrapolation may only be calculated if the individual is undergoing elimination of ethanol at the time of the stop.

28.3.6.1.1. The time between the last drink and the stop must be at least 2 hours in order to assume elimination at the time of the stop unless it is a hypothetical scenario assuming elimination.

28.3.6.2. A retrograde extrapolation will not be conducted if the facts in 28.3.5.1 are not given by the requestor unless it is a hypothetical scenario with disclosed assumptions.

28.3.6.3. A retrograde extrapolation will not be conducted if the BAC_K is less than 0.02 g/100 mL.

28.3.6.4. The Widmark equation will not be used and the number of standard drinks cannot be calculated if the facts in 28.3.5.2 are not given by the requestor.

28.3.6.5. It is assumed the individual falls within the elimination rates 0.01 and 0.03 g/100 mL/hr. The range includes the majority of the population except alcoholics after a period of chronic, heavy drinking and ultra-rapid metabolizers (28.4.2 and 28.4.3).

28.3.6.6. It is assumed that a male has V_d of 0.7 L/kg and a female has V_d of 0.6 L/kg.

28.3.6.7. First pass metabolism will not be considered.

28.3.6.8. Gastroesophageal Reflux Disease (GERD) will not be considered, but an individual with GERD may absorb alcohol for more than 2 hours (28.4.6).

28.3.6.9. Food in the stomach slows the rate of absorption of ethanol and delays the peak BAC. To account for this factor, retrograde extrapolation will not be performed if the time between the last drink and the stop is shorter than 2 hours. The range of elimination rates encompasses fasting and non-fasted individuals. However, the ethanol absorption phase can last more than 2 hours in certain individuals as documented in 28.4.6 and 28.4.7.

28.3.6.10. Type of food: carbohydrates (fructose) and amino acids (glycine) may slow the ethanol absorption rate.

28.3.6.11. Type of drink: drinks with high ethanol concentrations (liquor) have faster rate of absorption compared to wine or beer.

28.3.6.12. Trauma, shock, and massive blood loss decrease the ethanol absorption rate.

28.3.6.13. Gastric surgery increases the ethanol absorption rate.

28.3.6.14. Drugs affecting gastric emptying may change the ethanol absorption rate.

28.3.7. Performing calculations

28.3.7.1. A requestor may submit facts for a specific case using LAB-88 (Retrograde Extrapolation and Widmark Calculation Request), or an equivalent method. The analyst will use LAB-87 (Retrograde Extrapolation and Widmark Calculation Worksheet), or an equivalent method, to conduct the calculations. LAB-88 or other retrograde extrapolation or the Widmark calculation-related documents provided by the requestor will become part of the case record.

28.3.7.2. When calculating retrograde extrapolation estimations, the lower and upper range will be rounded down to two decimals.

28.3.7.3. When calculating the estimated amount of alcohol in the body (in number of drinks) using the Widmark equation, the result will be rounded to one decimal.

28.3.7.4. When calculating the estimated BAC using the Widmark equation, the result will be rounded down to two decimals.

28.3.7.5. When calculating the estimated number of standard drinks using the Widmark equation, the result will be in a whole number range, encompassing the calculated number (e.g., if N = 1.6, the range will be reported as 1-2).

28.4. Literature and Supporting Documents

28.4.1. Jones, AW. Body Mass Index and Blood-Alcohol Calculations. Journal of Analytical Toxicolology. 2007;31:177-178.

28.4.2. Jones, AW. Evidence-based survey of the elimination rates of ethanol from blood with application in forensic casework. Forensic Science International. 2010;200:1-20.

28.4.3. Jones, AW and Andersson, L. Influence of Age, Gender, and Blood-Alcohol Concentration on the Disappearance Rate of Alcohol from Blood in Drinking Drivers. Journal of Forensic Sciences. 1996;41:922-926.

28.4.4. Maudens, KE, Patteet, L, van Nuijs, ALN, Van Broekhoven, C, Covaci, A, and Neels, H. The Influence of the Body Mass Index (BMI) on the Volume of Distribution of Ethanol. Forensic Sci International. 2014;243:74-78.

28.4.5. Mitchell Jr, MC, Teigen, EL, and Tamchandani, VA. Absorption and Peak Blood Alcohol Concentration After Drinking Beer, Wine, or Spirits. Alcoholism, Clinical and Experimental Research. 2014;38:1200-1204.

28.4.6. Booker, JL and Renfroe, K. The Effects of Gastroesophageal Reflux Disease on Forensic Breath Alcohol Testing. Journal of Forensic Sciences. 2015;60:1516-1522.

28.4.7. Caplan, YH and Goldberger, BA., eds., Garriott's Medicolegal Aspects of Alcohol, 6th ed. Tucson, AZ: Lawyers & Judges Publishing Company, Inc., 2015.

29. Estimation of Uncertainty of Measurement (UM)

29.1. Purpose

29.1.1. This procedure describes an estimation of UM, a statistical calculation of all known variables that contribute to the inherent variance of the overall result at a desired confidence level.

29.2. Scope

29.2.1. The UM is calculated for quantitative methods to ensure the reported quantitative results can be interpreted within the context of accuracy and precision of the analytical methods.

29.2.2. The UM is not calculated for qualitative methods whose results are reported positive or negative rather than numerical values.

29.3. Calculating and Reporting of UM

29.3.1. For the Volatiles analysis, the confidence level used is 99.73%, k = 3.

29.3.2. For the GC-MS analysis, the confidence level used is 95.45%, k = 2.

29.3.3. UM is reported in the same units and decimal places as the test results.

29.3.4. Reported UM is calculated using the following formula:

Reported UM = ± (Reported concentration x UM%)

29.3.4.1. For the Volatiles analysis, reported UM is rounded to three decimal places for blood alcohol samples and to three significant figures for liquid samples.

29.3.4.2. For the GC-MS analysis, reported UM is rounded to one decimal place if the value is < 10 and to the whole number if the value is ≥ 10.

29.3.5. The uncertainty of measurement is evaluated yearly.

29.3.6. The Uncertainty of Measurement Spreadsheets are available in the laboratory in a retrievable format.

29.4. Uncertainty Components

29.4.1.1. Measurement Reproducibility – accounts for the control with the largest Percent Relative Standard Deviation or pooled %RSD based on historical control data.

$$\%RSD_{pooled} = \sqrt{\frac{(n1-1)\%RSD_1^2 + (n2-1)\%RSD_2^2 + \ldots + (nk-1)\%RSD_k^2}{n1 + n2 + \ldots + nk - k}}$$

k is the number of pooled control lots and n is the number of measurements obtained from a specific lot.

29.4.1.2. CRM Uncertainty – accounts for the CRM used as a calibrator with the largest reported uncertainty.

29.4.1.3. Pipettor-dilutor (Volatiles) – accounts for the variability of the two syringes used by the Hamilton pipettor-dilutor for creating samples for analysis. Values are obtained from calibration certificates.

29.4.1.4. Duplicates (Volatiles) – accounts for the maximum allowed difference between analytically-obtained values of duplicate case samples.

29.4.1.5. Pipette – accounts for the variability in transferring drug standards to prepare calibration working solutions and calibrators and in aliquoting blood samples. Highest %RSD reported on external calibration certificates across all pipettes with relevant volume range is used.

29.4.1.6. Volumetric flask – accounts for the variability in diluting CRM to prepare calibration working solutions. Highest uncertainty reported on certificates of calibration of NIST traceable flasks with the volume as described in the relevant analytical SOP.

29.5. Re-calculation

29.5.1. UM will be reviewed and if needed, recalculated every two years at the minimum.

29.6. Literature

29.6.1. National Institute of Standards and Technology. SOP No. 29. Standard Operating Procedure for the Assignment of Uncertainty. 2014.

29.6.2. ASCLD/LAB Guidance on the Estimation of measurement Uncertainty - Annex A. Details on the NIST 8 Step Process. 2011.

30. Appendix 1. Abbreviations

=	equal
+/- or ±	plus or minus
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
6-AM or 6-MAM	6-acetyl morphine or 6-monoacetyl morphine
11-OH-THC	11-hydroxy-THC
Abs	absolute absorbance
ADA	assistant district attorney
ALC	test for the presence of alcohol
ALP	alprazolam
AMP	amphetamines confirmation or immunoassay
AN	acid-neutral
AND	acidic and neutral drug qualitative confirmation
Avg. Abs	average absorbance
BAC	blood alcohol concentration
BAN	basic, acidic, and neutral drug screen
BARB	barbiturates
BCR	blood collection report located on inner plastic box
BE or BZE	benzoylecgonine
BH	biohazard
BNZ	benzodiazepines confirmation
BQC	whole blood ethanol control
BSD	basic drug qualitative confirmation
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BZ or BENZO	benzodiazepines
c	concentration
CAR or CARISO	carisoprodol
CE	cocaethylene
C/M	carisoprodol/meprobamate
CI	confidence interval
CNS	central nervous system
COA	certificate of analysis
COC	cocaine or cocaine confirmation
COQC	carryover quality control

CRM	certified reference material
CV	coefficient of variation
DI	deionized
DOB	date of Birth
DQC	dilution control
DRS	deconvolution reporting software
DUID	driving under the influence of drugs
DWI	driving while intoxicated
EI	electron ionization
ELISA, EIA	enzyme-linked immunosorbent assay
EME	ecgonine methyl ester
ENV	envelope
EXP	expiration
EQC	aqueous ethanol control
EtAc	ethyl acetate
EtOH	ethanol (ethyl alcohol)
Evid	evidence
FN	false negative
FP	false positive
g	grams
GC	gas chromatograph
GC-FID	gas chromatograph-flame ionization detector
GC-MS	gas chromatograph-mass spectrometer
H_2O	water
HEQC	high aqueous ethanol control
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HFSC	Houston Forensic Science Center
HHC	higher than the highest calibrator
HQC	high quality control
hr(s)	hour(s)
HS	headspace
HSA	hexane saturated with acetonitrile
IB	inner box
Incl/	including
I.S., IS, ISTD	internal standard
kg	kilogram
L	liter
lb	pound

LIMS	Laboratory Information Management System
LLC	lower than the lowest calibrator
LLE	liquid-liquid extraction
LMQC	low aqueous mixed volatile control
LOD	limit of detection
LOQ	limit of quantitation
LQC	low quality control
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
METH	methamphetamine
MeOH	methanol (methyl alcohol)
mcg, µg, or ug	microgram
mg	milligram
M/I/A	methanol, isopropanol and acetone
min(s)	minute(s)
MQC	middle quality control or mixed volatile whole blood control
MRM	multiple reaction monitoring
MS	mass spectrometer
MTDN	methandone
MU, UM	measurement uncertainty or uncertainty of measurement
N/A, NA, or na	not applicable
NAM	not acceptable match
Neg	negative
ND	not detected
NF	not found
ng	nanogram
OB	outer box
OFC or Ofc	officer
OPI	opioids confirmation or immunoassay
OXY	oxycodone
oz	ounce
PBS	phosphate buffer saline
PCP	phencyclidine, PCP confirmation, or PCP immunoassay
PFPA (PFAA)	pentafluoropropionic anhydride (pentafluoropropionic acid anhydride)
Pkg	package
Pos	positive
PQC	positive quality control
PWP	package with parent

QC	quality control
QNS	quantity not sufficient
QS	quantum satis (bring to volume)
RCF	relative centrifugal force
RE	relative error
RL	reporting limit
RRT	relative retention time
RT	retention time
RSD	relative standard deviation
SD	standard deviation
SDS	safety data sheets
SIM	selective ion monitoring
SN	serial number
SOP	standard operating procedure
SPE	solid phase extraction
SS	system suitability control
SSRI	selective serotonin reuptake inhibitor
SWGTOX	scientific working group for forensic toxicology
THC	delta9-tetrahydrocannabinol or cannabinoids confirmation or immunoassay
THC-COOH or THCA	11-nor-9-carboxy-THC
TMCS	trimethylchlorosilane
TN	true negative
TP	true positive
ULOQ	upper limit of quantification
UM	uncertainty of measurement
UNK or unk	unknown
V_d	volume of distribution
w/	with
w/out	without
ZOL or ZOLP	zolpidem

31. Appendix 2. Terms and Definitions

Blind Sample:	Matrix matched sample fortified with the analyte(s) of interest by an individual other than the assigned analyst. It can also be a negative sample. The expected concentration is blind (unknown) to the assigned analyst.
Calibration Protocol:	A written procedure, which describes the preparation of calibration samples, the processing of these samples and the method-specific calculation model that is to be used.
Calibration Sample:	Analytical standard used to fix, set or check the graduations or scale of an analytical procedure.
High Calibrator:	A calibrator used in SIM qualitative assays to account for ion ratios that show concentration-dependent variability during validation. This calibrator is ONLY used to establish the acceptance criteria for ion ratios and is not included in the calibration curve.
Carryover:	An analyte that is retained from one sample into another sample, usually the sample immediately following that contains an elevated concentration of the analyte of interest.
Certified Reference Material:	Drug standard purchased from an approved vendor which includes a certificate of analysis verifying the concentration.
Cut-off Calibrator:	(Qualitative analysis) Matrix sample fortified with the analyte of interest at the reporting limit of the assay.
Drug Standard:	Any chemical other than the sample used in the preparation of standard solutions for calibrators, controls or internal reference. CRM should be used as drug standards whenever possible.
False Negative:	(Qualitative analysis) Sample containing the analyte of interest above the cut-off concentration that gives a negative result.
False Positive:	(Qualitative analysis) Sample containing the analyte of interest below the cut-off concentration that gives a positive result.
Fortified Quality Control Sample:	A sample of similar matrix to the unknown case sample, which has been spiked with a predetermined amount of the analyte(s) of interest. Control samples can be prepared in-house or purchased from an approved vendor.
Qualitative Negative Control:	(Qualitative analysis) Matrix sample fortified with the analyte of interest at a concentration no less than 50% of the cut-off calibrator.

Qualitative Positive Control:	(Qualitative analysis) Matrix sample fortified with the analyte of interest at a concentration no more than 200% of the cut-off calibrator
Internal Standard:	An analyte (generally of similar chemical structure to an analyte being measured) that is added, in a known concentration, to all samples (calibrators, controls and unknowns) in an analytical method, and that functions as a reference marker for that sample, against which the analyte of interest can be measured.
Linear Range:	Typically the Limit of Quantification (LOQ) to the Upper Limit of Quantification (ULOQ) are administratively defined as the concentration of the lowest and highest calibrator used in preparation of the calibration curve.
Matrix:	The material into which is spiked known amounts of an analyte(s) of interest in order to calibrate the method or to track method performance.
Neat:	A systematic representative of an analyte of interest that is free from a mixture or dilution.
Negative Control:	Matrix fortified with internal standard. The negative control may also contain the analyte of interest at a concentration below the LOQ or cut-off of the assay.
Positive Control:	Matrix fortified with the analyte of interest at a concentration above the LOQ or cut-off of the assay. It can be stated as positive control (PQC), low control (LQC), mid-control (MQC), high control (HQC), carry-over control (COQC), and Utak.
Not Detected:	(Reporting) the analyte of interest does not meet the acceptance criteria described in the appropriate SOP including the limit of detection or the limit of quantification.
Reagent:	A chemical, chemical mixture or dilution of a chemical substance used in toxicological analysis.
True Negative:	(Qualitative analysis) Sample containing the analyte of interest below the cut-off concentration that gives a negative result
True Positive:	(Qualitative analysis) Sample containing the analyte of interest above the cut-off concentration that gives a positive result
Working Standard Solution:	Solution prepared by diluting a drug standard to a pre-determined concentration and used to prepare calibration or control samples.