

**Toxicology Section**  
**Analytical Manual - Standard Operating**  
**Procedures (Version 3.9)**  
Comparative and Analytical Division



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## **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 include 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 HFSC facility. Freezer storage is an acceptable alternative. All refrigerators and freezers in the toxicology section and containing toxicology evidence are monitored using a temperature monitoring system, such as DicksonOne, which is further detailed in the Quality Manual, or an equivalent system.

- Acceptable refrigerator temperature range:  $>0 - 8\text{ }^{\circ}\text{C}$
- Acceptable freezer temperature range:  $\leq 0\text{ }^{\circ}\text{C}$

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 **or designee** 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. **A comment on the chain of custody is required for each analysis.** 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 consumption order or similar court order regarding testing to proceed with analysis. This applies to instances where (1) evidence consists of one blood containing less than 3 mL, (2) the evidence consists of two or more tubes of blood, all of which must be opened to complete the analysis or (3) the section determines the need for a court order (e.g., outsourcing a case that includes only one blood tube). The second instance includes cases with a broken tube; in order for HFSC to analyze these cases, the remaining tube must be opened and thus a court order is required. A request to consume will be communicated to the submitting agency by email or in a report.
- 3.6.4.1. When a consumption order is required, the defense should have an opportunity to timely object before moving forward with analysis. “An opportunity to timely object” means either the defense had an opportunity to object with the court before the order was signed, or a written objection by the defense is received within the number of business days (e.g., 3 days) specified in the notice of a consumption order provided by HFSC to the defense.
- 3.6.5. The section will reserve at least one unopened tube for additional testing if the evidence consists of two or more tubes of blood. If the evidence consists of just one tube containing  $\geq$  3 mL blood or has a court order that meets the requirements in 3.6.4 or other instances meeting the requirements described in Quality Manual on evidence with an insufficient quantity, the section will open the tube for analysis. The sample volume in the reserved tube will vary. For cases with limited sample volume, the section management **or designee** will determine the sample volume to be reserved on a case-by-case basis.

### **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. Blood tubes for analysis are selected based on the consideration of multiple factors such as the sample volume, biological fluid type, tube type, and collection date/time. As a result, the tube selected for analysis may not be collected at the earliest date/time. For non-HFSC DWI Collection kits that contain one or more non-gray top tubes, a reason for selecting a



particular tube will be noted in the case records. Because gray top tubes in HFSC DWI Collection kits and hospital toxicology kits are typically collected on the same day, gray-top tubes collected in these kits are selected based on the sample volume and thus will not have a reason for selection noted in the case records.

- 3.7.3. For cases that have multiple color top tubes, the best suited one will be analyzed as follows: gray>lavender>pink>tan>royal blue (if it contains anticoagulant). The following color top tubes and other container types may require additional attention to ensure the sample matrix is appropriate for the required testing (e.g., blood, serum, urine, liquid): gold or red/gray, orange, light green or green/gray, white, red, royal blue (if it contains a clot activator), green, light blue, yellow, and replacement top. A consultation with the manager/supervisor and potentially the requester could occur to decide the appropriateness of testing, as needed.

### 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.**

### 3.9. Analytical Requests

- 3.9.1. Based on **the** type of offense and type of evidence submitted, the following analytical requests will be added to LIMS, unless otherwise requested or specified in the case record. For cases with multiple subjects associated with separate evidence items, a request 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/DUID

Blood specimens – alcohol analysis, if ethanol <0.100 g/100 mL → Drug Screen, if positive → Confirmation

Urine specimens (if submitted and if blood specimen is not available) – Drug Screen, if positive → Confirmation; if a urine specimen is submitted as a separate item and there is a blood specimen for the same case, collected on the same day, only the blood specimen will be analyzed.

#### 3.9.3. Fatalities

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 by an authorized entity

Blood Specimens – alcohol analysis

Urine Specimen – Drug Screen, if positive → Confirmation per request by an authorized entity

3.9.5. Others – Assignment will be made following the DWI/DUID workflow or based on client request or consult.

3.9.6. Immunoassay offers cross-reactivity with multiple analytes. A positive immunoassay finding leading to confirmation of multiple analytes that cross-react with the immunoassay kit (e.g., a positive methamphetamine EIA leading to amphetamine and methamphetamine confirmed results; a positive cocaine/metabolite EIA leading to a cocaine confirmed result) is considered acceptable.

3.9.7. **If routine confirmatory analysis (e.g., BNZ) does not detect any analyte(s) screened positive or upon request by an authorized entity, an add-on confirmatory analysis (e.g., NBZ) will be performed if available and applicable. Add-on confirmatory analyses will not be performed routinely.**

3.9.8. Deviations from SOP affecting an analytical batch or analysis of a case are documented in the SOP Deviation Request Form (LAB-093) or equivalent, which is approved by the section management **or designee**. The document is placed in the case or batch records depending on the type of **deviation**.

3.9.8.1. Deviations related to analytical requests (e.g., inadvertently performing confirmatory analysis of a sexual assault case without receiving a request or analyzing both urine and blood samples instead of blood only for a DWI case), however, do not require the SOP Deviation Request Form. Because prioritization of requests has been established based on the section's capacity, performing additional analysis will not be considered a nonconformance as they provide additional information for requesters.

### **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 **the** submitting agency.
- 3.12.2. **Before evidence is sealed, the contents will be checked for proper labeling.**
- 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.

### **3.13. References**

- 3.13.1. BD Vacutainer® blood collection tubes in-service poster. <https://www.bd.com/en-us/offering/capabilities/specimen-collection/specimen-collection-resource-library?products=3820> (accessed on July 24, 2020).



## 4. Technical and Administrative Reviews

### 4.1. Purpose

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

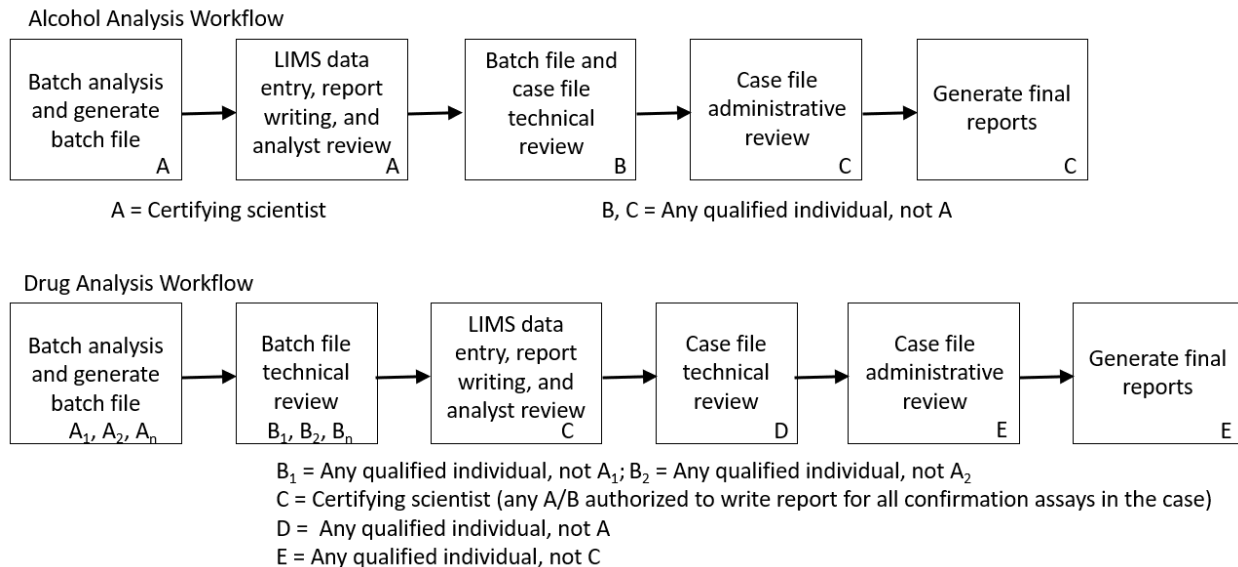
### 4.2. Scope

4.2.1. This procedure is used to manage the technical and administrative reviews 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/batch reviewers 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 (if applicable) and administratively reviewed once more.
- 4.3.4. Calculations regarding case sample data (e.g., dilution, averaging of multiple valid results) will be checked during the batch review and case review processes to verify appropriate math.
- 4.3.5. 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.
  - 4.3.5.1. 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.
  - 4.3.5.2. 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 printed 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 analysis (LAB-070), immunoassay (LAB-075), and GC-MS full scan analysis (LAB-077) and/or GC/LC-MS targeted analysis (LAB-073). 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-074). Equivalent electronic checklists may also be used.

**4.8. Paperless Batch and Case Files**

4.8.1. Electronic batch records do not require the analyst's initials on each page. The analyst processing the batch will apply his/her password-protected electronic signature on the designated page of the batch file (e.g., Batch Materials sheet).

4.8.2. Electronic case records do not require the analyst's initials on each page. The password-protected login of LIMS and the analyst's name applied as an annotation on each case record uploaded to LIMS is considered proper authentication.

4.8.3. Electronic records do not count toward the total number of examination documents.

4.8.4. Locations of the analyst and reviewer on the batch and case review forms denote their locations at the time when they apply their signatures signifying completion of the review. In other words, the review could have been performed off-site for some portion but if it is completed with application of the reviewer's signature on-site, the location will be noted as on-site.

**4.9. Administrative Review Checklist**

4.9.1. The following items denote what constitutes a case file administrative review. All items are first completed by the certifying scientist during report writing, and the administrative review constitutes a second check:

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

4.9.1.2. All pages have correct unique case identifier



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

4.9.1.4. Evidence Description and Review Form:

4.9.1.4.1. Initials/signature of individual that accessioned and dated

4.9.1.4.2. Appropriate identifiers consistent with submission information

4.9.1.4.3. Outer evidence container and sealed sections completed

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

4.9.1.5. LIMS:

4.9.1.5.1. Toxicology request added, if needed

4.9.1.5.2. Representative images of evidence uploaded

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

4.9.1.5.4. Report ready for administrative review

4.9.1.6. Report:

4.9.1.6.1. Review forensic case number

4.9.1.6.2. Review appropriate identifiers (i.e., name, DOB, ACN)

4.9.1.6.3. Review results are correct and consistent with examination documentation

4.9.1.6.4. Review all items listed and descriptions are consistent with documentation

4.9.1.6.5. Statement included for each item untested/requiring additional testing

4.9.1.6.6. Any comments/discrepancies are clear and consistent with documentation

4.9.1.6.7. Reviewed for clerical errors

4.9.1.6.8. Review final report



## 5. Reporting

### 5.1. Purpose

5.1.1. This document outlines the process and requirements for reporting toxicological test results.

### 5.2. Scope

5.2.1. This procedure is used to report none detected/not detected, preliminary, qualitative and quantitative results of alcohol and drug analyses.

### 5.3. Toxicological Report Content

5.3.1. Laboratory reports include results or a summary of all analytical testing results that meet the laboratory's defined reporting criteria.

5.3.2. The following elements will be included in the report or report appendix:

5.3.2.1. name, address, and contact information of the laboratory where analytical testing was performed;

5.3.2.2. agency name and case number of the specimen submitter;

5.3.2.3. subject's name;

5.3.2.4. listing of specimen(s) tested to include type;

5.3.2.5. date of specimen receipt;

5.3.2.6. date of report issuance;

5.3.2.7. laboratory results;

5.3.2.8. name of the person responsible for the report; and

5.3.2.9. page numbers and case number (or other identifying information) on each page.

5.3.3. The following elements will be included in the report or documented elsewhere in the case record, when known:

5.3.3.1. subject's sex;

5.3.3.2. subject's date of birth or age;

5.3.3.3. specimen container type(s);

5.3.3.4. estimate of the volume, weight, or amount of specimen received;

5.3.3.5. date and time of specimen collection; and

5.3.3.6. interpretive information.

5.3.4. If the condition of the specimen(s) was unsuitable for analysis and/or may have compromised the results of the testing, this will be noted in the report.

5.3.5. If low specimen amount precluded any or all testing, it will be noted.

5.3.6. The section has a list of analytes covered in the analysis schemes. This list is included in the report as an appendix and also available in this analytical manual posted online. This list will be updated after completion of the validation and before a method is used in casework.



#### 5.4. Reporting Laboratory Results

- 5.4.1. Results may be none detected/not detected, preliminary, positive (qualitative or quantitative), or inconclusive. In all instances, the following elements shall be included with each type of analytical result reported:
  - 5.4.1.1. name or class of analyte, the result obtained, and the specimen(s) tested;
  - 5.4.1.2. technique(s) of analysis; and
  - 5.4.1.3. reporting limit or limit of detection for the analyte (in the report body, in an appendix, or made available elsewhere to the customer).
- 5.4.2. None detected/not detected results
  - 5.4.2.1. The laboratory report will state the analytes or classes of analytes that were tested for but not detected or confirmed. As an example, results may be described as “none detected” or “not detected.” It will not be reported as “negative.”
- 5.4.3. Preliminary results
  - 5.4.3.1. It is sometimes necessary to report preliminary analytical results (e.g., caffeine). In these instances, it will be noted in the report that confirmatory testing is pending or will be performed upon request.
  - 5.4.3.2. 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, LC-MS/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 confirmatory analysis is needed, please contact HFSC at [toxicology@hfsctx.gov](mailto:toxicology@hfsctx.gov).”).
  - 5.4.3.3. 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.
- 5.4.4. Positive results - qualitative
  - 5.4.4.1. Qualitative toxicological results will be reported as “detected”, “identified”, “present”, “confirmed”, or “positive”, with no numerical value assigned when meeting the laboratory’s requirements for detection or identification of analytes.
- 5.4.5. Positive results – quantitative
  - 5.4.5.1. Quantitative toxicological results will include both the amount and the units of measurement for each analyte meeting the laboratory’s requirements





for identification along with an estimated uncertainty of measurement. It is acceptable to report analytical results as “greater than” or “less than.”

#### 5.4.6. Inconclusive results

5.4.6.1. Inconclusive analytical results are results that do not meet criteria for reporting or were unsuitable due to analytical interferences or condition of the sample. Those will be marked as such.

5.4.6.2. If the sample is unsuitable for analysis, the report should state “Sample unsuitable for analysis” or an equivalent statement.

5.4.6.3. If there is insufficient sample, the report will state “Insufficient sample for analysis” or an equivalent statement.

### 5.5. Results of the alcohol and other volatiles analysis

5.5.1. Tested sample results are reported in g/100 mL.

5.5.1.1. For serum/plasma samples, the equivalent whole blood concentration is calculated **first by applying the UM and then** a conversion factor range of **1.04-1.26 (5.14.2)**. **The lower end of the concentration range will be divided by the higher conversion factor 1.26 and the higher end of the concentration range will be divided by the lower conversion factor 1.04 to be conservative.**

5.5.1.1.1. **The conversion factor is to account for the higher water content of serum and plasma compared to whole blood.**

5.5.1.1.2. The report will have a comment disclosing the sample type and the equivalent whole blood concentration range, “e.g., Item x.x was serum/plasma. The equivalent whole blood concentration was calculated to be x.xxx - x.xxx grams of ethanol per 100 milliliters of blood **by applying the uncertainty of measurement and a conversion factor range of 1.04 to 1.26**”.

5.5.2. Reported concentration will be the average of the four results obtained from aliquot 1 and aliquot 2 on FID1 and FID2, truncated to three decimal places. The concentration will be reported when the average value is  $\geq$  the method LOQ of 0.010 g/100 mL.

5.5.3. For samples that required homogenization, the report should state “This sample was homogenized prior to analysis” or an equivalent statement.

### 5.6. Results of GC/LC-MS analysis

5.6.1. Tested sample results are reported in ng/mL or  $\mu\text{g/mL}$ .

5.6.2. Reported result is truncated to one decimal place if the concentration is  $< 10$  and to the whole number if the concentration is  $\geq 10$ .

5.6.3. Reported result is truncated and limited to two significant digits at most (e.g., 510 instead of 516 ng/mL; 1300 instead of 1387 ng/mL).

### 5.7. Dilution

5.7.1. Water is used as a diluent for alcohol analysis whereas blank test matrix (blood or urine) is used as a diluent for GC and LC analyses of highly concentrated samples.

5.7.2. Dilution factor used must have been validated.



5.7.3. The dilution factor used will be incorporated before truncating the original result to the appropriate decimal place as described in 5.5 and 5.6.

5.7.3.1. Alcohol analysis: Reported result is truncated to two decimal places if the concentration is < 10 and to the one decimal place if the concentration is ≥ 10.

5.7.3.2. GC/LC analyses: Reported result is truncated to one decimal place if the concentration is < 10 and to the whole number if the concentration is ≥ 10. The value is limited to two significant digits at most (e.g., if the chromatogram of an aliquot diluted by 2 had a benzoylecgonine concentration of 523.57 ng/mL, the reported value would be 1000 ng/mL:  $523.57 \times 2 = 1047.14$ , truncating to 1000 ng/mL to make it an acceptable significant figure of one).

5.7.4. When test results after application of more than one dilution factor are obtained, valid test results with the lowest dilution factor will be used (e.g., if cocaine was quantified at 31 ng/mL in an undiluted aliquot and at 35 ng/mL in an aliquot diluted by 2, the reported value would be 31 ng/mL).

## 5.8. Multiple valid results for quantitative assays

5.8.1.1.1. 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.

5.8.1.1.2. For such analytes, the average of the valid results will be reported. Each valid result should be within ±20% of the average for GC/LC-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.

5.8.1.1.3. For example, a case sample was analyzed for cocaine/metabolites 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.

5.8.1.1.4. If the valid results do not acceptably agree with each other as stated [5.8.1.1.2.](#), analysis may be performed for the third time unless there is an acceptable justification noted in the case record (e.g., analyte instability, concentration around LOQ). The decision will be based on the consensus among the analyst, technical reviewer, and management **or designee** and will be documented in the case record.

## 5.9. Multiple valid results for qualitative assays

5.9.1. The concentrations around the cutoff values are expected to be either positive or negative. If multiple analyses were performed producing valid qualitative results of both positive and negative findings because the responses are around the cutoff, the type of analysis will dictate which result the section uses for reporting or proceeding to the next step of the analysis.



5.9.1.1. Screening results: the positive finding will be selected for reporting of preliminary results as described in 5.4.3 or performing confirmation analysis as needed. This is to ensure the screening result is confirmed.

5.9.1.2. Confirmation results: the negative finding will be selected for reporting to be conservative.

5.9.2. If the discrepant results are deemed not related to the responses around the cutoff, the issue will be investigated, and the analysis may be performed for the third time.

### **5.10. Biological matrices for testing**

5.10.1.1. 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.

5.10.1.2. 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

### **5.11. Results from a Reference Laboratory**

5.11.1. When all or part of the testing has been performed by an outside reference laboratory, the outside laboratory's testing will be indicated in the report issued by the primary laboratory or the outside laboratory's report will be provided to the client. A complete copy of the reference laboratory's report will be retained in the case record.

### **5.12. Supplemental and Amended Reports**

5.12.1. Supplemental and amended reports will be marked as such.

5.12.2. Supplemental reports

5.12.2.1. The section may follow the two options for issuing supplemental reports depending on the situation:

5.12.2.1.1. A report containing only the supplemental findings may be issued. The report will follow the requirements in the sections 5.3-5.5.

5.12.2.1.2. Supplemental findings may be added to the original report, but the supplemental information will be indicated. The report will follow the requirements in the sections 5.3-5.5.



### 5.12.3. Amended reports

5.12.3.1. Modifications to an original report will be indicated. The report will follow the requirements in the sections 5.3-5.5 and reference the date of the original report. Quality Manual contains more detailed requirements.

## 5.13. Interpretive Information

5.13.1. Factual statements concerning detected analytes that may include, but not be limited to information regarding drug scheduling, pharmacokinetics, pharmacodynamics, or factors that can affect detection or quantitation of the analyte.

5.13.2. It may be appropriate to include interpretive information to help the reader understand the meaning of detected analytes. Interpretive information is not considered a mandatory part of the toxicological report but is based on jurisdictional or laboratory preference to include such. This information may be included in the body of the report.

5.13.3. It should be made clear to the reader that the interpretive information provided is not exhaustive or meant to encompass all scenarios where toxicological results are reported. It should be further noted that the interpretive information provided is meant to serve as a general guide for the reader and that for any given case, consultation with a forensic toxicologist is recommended.

5.13.4. The interpretive information provided shall:

5.13.4.1. be factual;

5.13.4.2. be clear and understandable by the intended audience;

5.13.4.3. be supported by scientific data (note: the citations of the reference(s) used to generate the information need not be in the body of the report, but must be available upon request);

5.13.4.4. note any specific limitations to the provided information; and

5.13.4.5. be appropriate to the analyzed matrix.

5.13.5. See the section 31, Opinions and Testimony, for more details.

## 5.14. References

5.14.1. ANSI/ASB Standard 053, First Edition 2020. Standard for Report Content in Forensic Toxicology.

5.14.2. Charlebois, RC, Corbett, MR, and Wigmore, JG. Comparison of ethanol concentrations in blood, serum, and blood cells for forensic application. *Journal of Analytical Toxicology*. 1996;20:171-178.



## 6. Preparation and Verification of Drug-Free Matrix

### 6.1. Purpose

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

### 6.2. Scope

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

6.2.2. 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.

### 6.3. Safety/Quality Assurance

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

### 6.4. Reagents

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

### 6.5. Equipment

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

### 6.6. Procedure

#### 6.6.1. Drug-Free Blood:

6.6.1.1. 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).

#### 6.6.2. Drug-Free Urine:

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

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



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

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

#### 6.6.3. Verification

6.6.3.1. All drug-free matrices are appropriately tested by immunoassay as blank and/or GC-MS screen/basic qualitative confirmation as **a negative control and at the calibrator level** to ensure they are drug-free prior to being used in casework.

6.6.3.1.1. **Negative control should be clear of analytes of interest (i.e., no analytes of interest meeting identification criteria).**

6.6.3.1.2. **Calibrator level control should have all analytes of interest identified.**

6.6.3.2. For LC-MS/MS analyses, the same blank matrix is used for preparing negative controls, positive controls, and calibrators in a batch so that the absence of the target analyte(s) or interferences can be evaluated concurrently with use.

6.6.3.3. Documentation is maintained in a retrievable format.



## 7. Verification of Relative Concentrations of Working Standard Solutions

### 7.1. Purpose

7.1.1. 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.

### 7.2. Scope

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

### 7.3. Procedure

7.3.1. Working standard solutions are the solutions used to fortify a calibration curve and controls or the solution used to prepare 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. Or certain fortifying solutions can be selected as needed.

7.3.2. 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.

7.3.2.1. For example, if the compound of interest is to be analyzed by GC-MS, dilutions will be prepared from each working solution by diluting each working 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 should 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 [AMP method as an example]:

7.3.2.1.1. Prepare triplicate dilutions of each of the working standard solutions as listed in the Fortification Guide (e.g., 0.2, 2.5 and 10  $\mu\text{g}/\text{mL}$  for the calibration solutions and 0.5 and 5/10  $\mu\text{g}/\text{mL}$  for control solutions) by adding 25  $\mu\text{L}$  of the working standards into conical tubes.

7.3.2.1.2. Follow the procedure for preparing System Suitability samples.

7.3.2.1.2.1. Add 50  $\mu\text{L}$  of acidic methanol and evaporate to dryness under nitrogen.

7.3.2.1.2.2. Add 200  $\mu\text{L}$  reconstitution solvent to each tube and vortex briefly to mix.

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



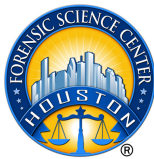
7.3.2.1.4. Note: Aliquot volumes may change. However, each method must be evaluated to ensure the final sample has an appropriate concentration. It is important that both working standard solutions are prepared using the same volumes.

#### **7.4. Data Analysis**

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

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





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

### 8.1. Purpose

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

### 8.2. Scope

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

8.2.2. Verification of calibration, control, and internal standard solutions and reagents can be performed as part of validation.

### 8.3. Sources and Forms

8.3.1. Drug standards used for calibration and control solutions are certified reference materials (CRM) from approved vendors.

8.3.2. Organic solvents and inorganic reagents (e.g., salts) should be ACS grade or higher. Deionized (DI) 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.

8.3.3. All drug standards, working standard solutions, and quality control preparations must be documented on the Reagent and Quality Control (Volatile) Preparation Log (LAB-068), the Working Stock/Standard Preparation Log (LAB-027), 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.

### 8.4. Purchasing, Storage, and Expiration

8.4.1. 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.

8.4.2. Storage conditions follow the vendor's recommendations if possible. If a storage condition of a CRM does not follow the vendor's recommendations (e.g., 7-aminoclonazepam stored at a freezing temperature), its acceptability will be verified during the validation process.

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

8.4.4. 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.



- 8.4.5. If a drug/internal standard is transferred, the expiration date follows the CRM expiration date, unless otherwise specified in the analysis-specific procedures.
- 8.4.6. 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.
- 8.4.6.1. 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 **except for those containing acid or base.**
  - 8.4.6.2. **If a reagent contains acid or base (e.g., 1M acetic acid, 1M ammonium hydroxide, acidic methanol) and expiration date of acid or base is available, the expiration date from the vendor will be considered when establishing an expiration date for the reagent.**
  - 8.4.6.3. 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.

#### 8.5. Use of Calibration, Control, and Internal Standard Solutions and Reagents

- 8.5.1. Assay calibration must be performed as validated and described in the analytical method.
- 8.5.2. Matrix-matched samples will be used as calibrators unless the use of non-matrix-matched calibrators has been validated.
- 8.5.3. Control Samples: Types of controls, in order of preference:
- 8.5.3.1. Commercial controls
  - 8.5.3.2. In-house controls prepared in bulk
  - 8.5.3.3. Controls prepared at the time of analysis using a working standard solution
- 8.5.4. If CRM is used directly as calibrator or control sample, verification is not required.
- 8.5.5. Calibration, control, and internal standard solutions used for both blood and urine samples will be verified **using quantitative** assays.
- 8.5.6. Reagents verified during any applicable assay are considered acceptable for all the assays that use the reagents.

#### 8.6. Preparation of Calibration, Control, and Internal Standard Solutions and Reagents

- 8.6.1. Standards must be made and stored in accordance with the SOP. An equivalent procedure may be used if it is documented **in** the appropriate preparation log.
- 8.6.2. 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.
- 8.6.3. 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.

8.6.3.1. There could be instances where the same manufacturer CRM for both calibration and control solutions is used even though CRM from a different manufacturer is available due to difference in analyte purity, solvent (e.g., methanol vs. acetonitrile), or quality; lot expiring sooner than a year from the preparation date; or other reasons documented in the preparation log.

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

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

8.6.5.1. LAB-027: Working Stock/Standard Preparation Log;

8.6.5.2. LAB-068: Reagent and Quality Control (Volatiles) Preparation Log

8.6.6. 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.

8.6.7. 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.

8.6.8. 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 are kept in a retrievable format in the laboratory.

## **8.7. Verification of New Lots of Calibration Solutions in Quantitative Assays**

8.7.1. Verification batch must include:

8.7.1.1. Current calibrator set

8.7.1.2. New calibrator set

8.7.1.3. Control samples

8.7.2. Evaluation of new calibration solutions

8.7.2.1. Calculate a run as normal, using current calibrator set as “calibrators”

8.7.2.2. Treat the new calibrators and quality controls as unknowns and determine their calculated values

8.7.2.3. If possible, repeat data analysis steps using new calibrator set as “calibrators”



8.7.2.4. Evaluate the data using Calibrator Verification Excel Spreadsheet Template

#### 8.7.3. Acceptance Criteria

8.7.3.1. 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.

8.7.3.2. [Linear curves] The slope of best-fit line is acceptable if:

8.7.3.2.1. It is between 0.85 and 1.15, and either

8.7.3.2.2. It is between 0.95 and 1.05, or

8.7.3.2.3. [Linear and quadratic curves] The uncertainty range (95% confidence interval) contains 1.

8.7.3.3. The y-intercept of the best fit line is acceptable if:

8.7.3.3.1. The uncertainty range (95% confidence interval) contains 0.

8.7.3.4. [Quadratic curves] the  $r^2$  value of the curve combining the current calibrators (x) and new calibrators (y) is at least 0.99 and there is no outlier observed during visual inspection.

8.7.3.5. The control values when calculated vs. the new calibrator must be within the percent acceptance used when validating the particular control (typically 20%)

8.7.3.6. If criteria are met, the pass/review fields will read "Pass".

8.7.3.7. If the criteria are not met, the pass/review fields will read "Review".

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

8.7.3.8.1. The percent difference between the two calibrator results will be calculated and will be **evaluated** if the new calibrator is more than  $\pm 15\%$  different than the current calibrator and will be back calculated to within  $\pm 20\%$  of the target value.

#### 8.7.4. Review/Approval

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

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

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

8.7.4.4. 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.

8.7.4.5. 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.

## 8.8. Verification of New Lots of Calibration Solutions in Qualitative Assays

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

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

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

8.8.1.1.2. For GC-MS and LC-MS/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  $\pm 20\%$  of the target value.

## 8.9. Verification of New Lots of Internal Standard Solutions

8.9.1. Volatiles: Prepare two System Suitability samples, one using the current internal standard and the other using the new internal standard. Additionally prepare one Negative control using the new internal standard.

8.9.1.1. Compare the average area of the new internal standard to the area of the current internal **standard**. The **average** of the new internal standard area counts should match within  $\pm 20\%$  of the current internal standard area count. For results outside this range, consult a supervisor or manager.

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

8.9.2.1. Compare the area of the new internal standard to the area of the current internal standard. The result should match within  $\pm 30\%$  of the current internal standard area count. **For results outside this range, consult a supervisor or manager.**

## 8.10. Verification of New Lots of In-House Control Solutions

8.10.1. Control Solutions in Quantitative Assays

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

8.10.1.1.1. The results of the newly prepared control samples should be within  $\pm 20\%$  of the target concentration for drug analysis and within  $\pm 10\%$  of the target concentration for alcohol analysis if BAC  $\leq 0.05$  g/100 mL and  $\pm 5\%$  if BAC  $> 0.05$  g/100 mL.

8.10.2. Control Solutions in Qualitative Assays

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



- 8.10.2.1.1. The results of the newly prepared control samples should be qualitatively acceptable (i.e., positive control should be positive).
- 8.10.2.1.2. For immunoassay, relative absorbance of the newly prepared control samples should be acceptable: Blank QC > Negative QC > Cut-off Calibrator > Positive QC.
- 8.10.2.1.3. For GC-MS and LC-MS/MS analysis, relative response of the newly prepared control samples should be acceptable: matrix blank/negative control < cut-off calibrator < positive control.

### **8.11. Establishing Target Concentration and Acceptance Range for Control Solutions in Quantitative Assays**

#### 8.11.1. Commercial controls

8.11.1.1. 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. An acceptable criterion is that the mean value is within  $\pm 30\%$  ( $\pm 20\%$  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.

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

8.11.2.1. 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  $\pm 15\%$  of the nominal value.

### **8.12. Establishing Performance for Control Solutions for Qualitative Assays**

#### 8.12.1. Commercial controls

8.12.1.1. 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.

#### 8.12.2. In-house controls

8.12.2.1. 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.



### **8.13. Concurrent Verification of Calibration, Control, and Internal Standard Solutions**

- 8.13.1. 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.
- 8.13.2. 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.
- 8.13.3. 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.
- 8.13.4. Concurrent verification process
  - 8.13.4.1. ELISA: perform three runs, each consisting of Negative QC, Cut-off Calibrator, and Positive QC samples in triplicate.
  - 8.13.4.2. GC-MS screen: perform three runs, each consisting of **one Negative QC sample, one Cut-off calibrator, and Positive QC** samples in triplicate.
  - 8.13.4.3. 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.
  - 8.13.4.4. GC-MS and LC-MS/MS quantification: perform three runs, each consisting of control samples in triplicate at each concentration and one calibration curve.
  - 8.13.4.5. The results will be evaluated according to the acceptance criteria of the method.

### **8.14. Verification of Newly Prepared Reagents**

- 8.14.1. 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 will be verified using two hydrolysis controls and the 1% HCl in methanol solution will be verified using two positive controls.
- 8.14.2. The reagent will be considered verified if controls meet the acceptance criteria defined by the method.
- 8.14.3. 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 and following the normal method sequence; in this case, all controls in the batch will be reviewed.



8.14.4. This requirement does not apply to additives to mobile phases (e.g., 5 M ammonium formate). Additives do not require a verification.





## 9. In-Process Calibration and Quality Controls for Drug Screening/Confirmation Testing

### 9.1. Purpose

9.1.1. 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.

### 9.2. Scope

9.2.1. 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.

### 9.3. Calibration of Qualitative Assays

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

9.3.2. Immunoassay

9.3.2.1. 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.

9.3.3. GC-MS and LC-MS/MS

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

9.3.3.2. 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.

9.3.3.3. 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  $\pm 20\%$  relative to the average ion ratio to meet acceptance criteria.

### 9.4. Calibration of Quantitative Assays

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

9.4.2. Unless otherwise specified in the analytical procedure, no fewer than four **and six calibration levels may be used for linear and quadratic calibration curves, respectively**. The **calibrator** concentrations must span the range of the assay.

9.4.3. For the calibration curve to be accepted, the back-calculated results for each calibrator must calculate to within  $\pm 20\%$  of its target value.



9.4.3.1. For selective ion monitoring (SIM) and multiple reaction monitoring (MRM) analyses, ion ratios for all calibrators must be within  $\pm 20\%$  relative to the average ion ratio from all calibrators used in the calibration curve, as validated to be appropriate for the ion ratio. Any calibrators that do not meet the requirement must be excluded from the curve.

9.4.4. **While a calibrator is** eliminated from the calibration curve when it does not meet the aforementioned acceptance criteria, elimination of two **calibrators** 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.

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

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

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

## 9.5. Controls of Qualitative Assays

9.5.1. 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.

### 9.5.2. Immunoassay

9.5.2.1. 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 12.

9.5.2.2. 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 12.

### 9.5.3. GC-MS and LC-MS/MS

9.5.3.1. 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.

9.5.3.1.1. GC-MS and LC-MS/MS analyses contain 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.

9.5.3.2. Negative control: sample fortified with internal standard.

9.5.3.3. Matrix blank control: sample without internal standard.

9.5.3.4. Hydrolysis control: urine sample fortified with a conjugated analyte for



methods that require hydrolysis. It is used to check that the hydrolysis step has been performed acceptably. If the hydrolysis control fails, the case samples that are negative for the target analyte to be hydrolyzed (e.g., oxazepam and THC-COOH) will be re-analyzed.

9.5.3.5. 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.

9.5.3.6. The matrix blank and the negative control must have an analyte response no greater than 10% of the cut-off calibrator (not applicable for GC-MS full scan analyses), and all case samples must be bracketed by acceptable positive controls to report positive results.

9.5.3.7. 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.

## 9.6. Controls of Quantitative Assays

9.6.1. Each analytical run must contain at least one matrix blank, one negative control sample, **low** positive control samples and **high 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.

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

9.6.1.2. Mid control (MQC) concentration should be in the middle of the calibration range, between LOQ and HQC.

9.6.1.3. High control (HQC) concentration should be no less than 80% of the upper limit of quantification (ULOQ).

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

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

9.6.2. An LQC sample should be injected after the last case sample for each run.

9.6.3. Control samples must be included for every analyte being quantified by the method.

9.6.4. 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.

9.6.5. 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.
- 9.6.5.1. The matrix blank and the negative control must have an analyte response or concentration no greater than 10% of the lower LOQ and all case samples must be bracketed by acceptable positive controls to report positive results.
  - 9.6.6. 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. Passing bracketing controls count toward this 2/3 rule.
    - 9.6.6.1. If both low-quality controls fail, the results below the target concentration of the next level quality controls (MQC or UTAK) cannot be reported.
    - 9.6.6.2. If both high-quality controls fail, the results above the target concentration of the next level quality controls (MQC or UTAK) cannot be reported.



## 10. General Guidelines for Instruments and Equipment

### 10.1. Purpose

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

### 10.2. Scope

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

### 10.3. Headspace GC-FID

#### 10.3.1. Method of Use

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

#### 10.3.2. Maintenance

10.3.2.1. Conduct the routine maintenance tasks including water level checks of hydrogen generators, pressure checks of nitrogen, hydrogen, air, and helium as well as performance checks of air **blanks** and system suitability sample (11.5.2). Documentation of the maintenance performed is on the Headspace GC Maintenance Log (LAB-039) or an equivalent method.

10.3.2.2. Other maintenance tasks or repairs can be performed as needed. A preventive maintenance is performed annually by an external party.

#### 10.3.3. Verification

10.3.3.1. Verification is performed after significant maintenance, repair, or changes in the method parameters other than data acquisition. A validation will be performed for changes in data acquisition. The verification run must meet acceptance criteria outlined in 10.3.3.4. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification runs can be documented in the maintenance log or equivalent form or a separate PDF file. Data are kept in a retrievable format in the laboratory.

10.3.3.2. 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.

10.3.3.2.1. As part of verification following annual preventative maintenance, the calibrator data should be used to update the retention time for each analyte in the method.

#### 10.3.3.3. Procedure

10.3.3.3.1. A major verification consists of three analyses of calibrators and at least three replicates of MQC, BQC, EQC and LMQC; MQC1 + BQC2 or MQC2 + BQC1 can be used to cover low, mid, and high ethanol concentrations.



- 10.3.3.3.2. A minor verification consists of one analysis of calibrators and at least three replicates of MQC, BQC, EQC, and LMQC; MQC1 + BQC2 or MQC2 + BQC1 can be used to cover low, mid, and high ethanol concentrations.
- 10.3.3.3.3. Whether a major or minor verification is performed depends on the extent of the maintenance/repair/changes in the method. Minor maintenance/repair/changes may not warrant a verification.
- 10.3.3.3.4. Modifications to the aforementioned verification designs and/or additional experiments can be performed depending on the nature and purpose of a verification.
  - 10.3.3.4. Acceptance criteria
  - 10.3.3.4.1. Bias: %bias  $\leq 5\%$  if target concentration is  $>0.05$  g/100 mL;  $\leq 10\%$  if target concentration is  $\leq 0.05$  g/100 mL.
  - 10.3.3.4.2. Within-run precision: %CV  $\leq 10\%$
  - 10.3.3.4.3. Between-run precision (for major verifications): %CV  $\leq 10\%$
- 10.3.4. References
  - 10.3.4.1. Agilent Technologies. Agilent 7697A Headspace Sampler: Getting Started, 1st Edition. Part Number G4556-90010, 2011.
  - 10.3.4.2. Agilent Technologies. Agilent 7890B Gas Chromatograph: Getting Started, 1st Edition. Part Number G3430-90055, 2013.
  - 10.3.4.3. Agilent Technologies. Agilent GC & GC/MS: User Manuals and Tools CD, Part Number G4600-64006, Rev. B.01.11.

## 10.4. Tecan System

- 10.4.1. Method of Use
  - 10.4.1.1. Refer to the appropriate operating instructions for proper handling, use, and troubleshooting.
- 10.4.2. Reagents and Materials
  - 10.4.2.1. Deionized water.
  - 10.4.2.2. 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).
  - 10.4.2.3. 1 M NaOH (sodium hydroxide): Dissolve 40.0 g sodium hydroxide pellets in 1 L deionized water. Store at room temperature (12-month expiration).
- 10.4.3. Maintenance
  - 10.4.3.1. Tecan Freedom EVO 75
    - 10.4.3.1.1. 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-092) or an equivalent method.
    - 10.4.3.1.2. Daily Maintenance Tasks
      - 10.4.3.1.2.1. Fill system fluid containers with deionized water.



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

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

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

10.4.3.1.2.5. Check around the valve for signs of moisture.

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

10.4.3.1.2.7. Empty all waste containers.

10.4.3.1.3. Monthly Maintenance Tasks

10.4.3.1.3.1. Remove racks and waste reservoir from the instrument surface.

Carefully clean the work surface using a disinfectant wipe and a KimWipe.

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

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

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

10.4.3.1.3.5. Perform an Acid/Base Wash

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

10.4.3.2. Tecan HydroFlex Plate Washer

10.4.3.2.1. Documentation of the maintenance performed is on the Tecan Maintenance Log (LAB-092) or an equivalent method.

10.4.3.2.2. 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.

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

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

10.4.4. Verification

10.4.4.1. Verification is performed after significant maintenance, repair, or changes in the method parameters other than data acquisition. A validation will be performed for changes in data acquisition. The verification run must meet acceptance criteria outlined in 10.4.4.3. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification runs can be documented in the maintenance log or equivalent form or a separate PDF file. Data are kept in a retrievable format in the laboratory.

10.4.4.2. Procedure



- 10.4.4.2.1. A major verification consists of three analyses of a curve (two blanks, two negative controls, two cut-off calibrators, and two positive controls), three positive controls, and three negative controls.
  - 10.4.4.2.2. A minor verification consists of one analysis of a curve (two blanks, two negative controls, two cut-off calibrators, and two positive controls), three positive controls, and three negative controls.
  - 10.4.4.2.3. Whether a major or minor verification is performed depends on the extent of the maintenance/repair/changes in the method. Minor maintenance/repair/changes may not warrant a verification.
  - 10.4.4.2.4. Modifications to the aforementioned verification designs and/or additional experiments can be performed depending on the nature and purpose of a verification.
- 10.4.4.3. Acceptance criteria
- 10.4.4.3.1. Positive controls are positive.
  - 10.4.4.3.2. Negative controls are negative.

#### 10.4.5. References

- 10.4.5.1. Tecan. Operating Manual: Freedom EVO 75, Document Part No: 30024538.02, Version 2.2.
- 10.4.5.2. Tecan. Instructions for Use for: HydroFlex, Document Part No: 30026397, 2008-02.
- 10.4.5.3. Tecan. Instructions for Use for: Sunrise Absorbance Reader + Option Touchscreen Color, Document Part No: 30041769, 2008-11.
- 10.4.5.4. Immunalysis. Standard Operating Procedure for EVO 75/2 with NaviTrack-OS V.1.4. Ponama, CA.
- 10.4.5.5. Tecan. Getting Started Guide: Freedom EVOware Standard 2.3 and Freedom EVOware Plus 2.3, Document Part No: 10393318.09, Version 2.3.

### 10.5. GC-MS

#### 10.5.1. Method of Use

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

#### 10.5.2. Performance Check

- 10.5.2.1. Ensure that the tune verification and system suitability have been completed, all acceptance criteria met, and maintenance log filled out.
  - 10.5.2.1.1. SS acceptance criteria include 1) presence of all ions and 2) smooth and symmetrical peak shapes; ion ratios and retention times are visually monitored. As the acceptance limits on the data are from the past calibration, flags associated with the limits are not considered.

#### 10.5.3. Tune Verification and Autotune

- 10.5.3.1. An autotune is used to evaluate the instrument's performance and to check for leaks. An autotune must be performed prior to each day's first





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.

- 10.5.3.1.1. Each day that an autotune is performed, it should be documented on the GC-MS Maintenance Log (LAB-024) or an equivalent form. A copy of the most recent tune file should be recorded in a retrievable format.
- 10.5.3.1.2. 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.
- 10.5.3.1.3. 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.
  - 10.5.3.1.3.1. Tune Specifications
    - 10.5.3.1.3.1.1. The three tuning masses must be within  $\pm 0.2$  amu of 69.00, 219.00, and 502.00 amu.
    - 10.5.3.1.3.1.2. The peak widths of the three tuning masses must be within  $\pm 0.05$  amu of 0.60 amu.
    - 10.5.3.1.3.1.3. The ratio of mass 70 to 69 must be within 0.5 – 1.6%.
    - 10.5.3.1.3.1.4. The ratio of mass 220 to 219 must be within 3.2 – 5.4%.
    - 10.5.3.1.3.1.5. The ratio of mass 503 to 502 must be within 7.9 – 12.3%.
    - 10.5.3.1.3.1.6. The ratio of mass 219 to 69 must be  $>35\%$ .
    - 10.5.3.1.3.1.7. The ratio of mass 502 to 69 must be  $>3\%$ .
    - 10.5.3.1.3.1.8. 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.
  - 10.5.3.1.4. Daily autotune requirement does not apply if the samples in a batch are being injected overnight or some samples in a batch need to be re-injected the next day using the calibration curve initially injected for the batch; this is to ensure that the instrument condition is the same for all samples within the batch. If an entire batch needs to be re-injected next day, an autotune should be performed again that day prior to re-injecting the entire batch.

#### 10.5.4. Maintenance

- 10.5.4.1. A preventive maintenance is performed annually by an external party.
- 10.5.4.2. 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-024) or an equivalent form.

10.5.4.2.1. 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

10.5.4.2.2. Annually:

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

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

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

10.5.5. Methods

10.5.5.1. 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. The analyst initials and dates any updated methods excluding changes in the SIM window parameters.

10.5.6. Sample Preparation and Sequence Set-up

10.5.6.1. Samples are prepared for analysis according to the specific SOP of the analytical method.

10.5.6.2. 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.

10.5.6.3. Retain GC-MS analysis data in the batch file.

10.5.7. Verification

10.5.7.1. Verification is performed after significant changes in the method parameters other than data acquisition. A validation will be performed for changes in data acquisition. The verification run must meet acceptance criteria outlined in 10.5.7.3. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification runs can be documented in



the maintenance log or equivalent form or a separate PDF file. Data are kept in a retrievable format in the laboratory.

#### 10.5.7.2. Procedure

##### 10.5.7.2.1. Quantitative methods

10.5.7.2.1.1. A major verification consists of three analyses of a matrix blank, a negative control, calibrators and at least three replicates of low, mid, and high concentration controls.

10.5.7.2.1.2. A minor verification consists of one analysis of a matrix blank, a negative control, calibrators and at least three replicates of low, mid, and high concentration controls.

10.5.7.2.1.3. Whether a major or minor verification is performed depends on the extent of the changes in the method. Maintenance including annual preventive maintenance, repair, and minor method changes do not warrant a verification.

10.5.7.2.1.4. Modifications to the aforementioned verification designs and/or additional experiments can be performed depending on the nature and purpose of a verification.

##### 10.5.7.2.2. Qualitative methods

10.5.7.2.2.1. A major verification consists of three analyses of a matrix blank, a cut-off calibrator, a negative control, and at least three replicates of positive controls.

10.5.7.2.2.2. A minor verification consists of one analysis of a matrix blank, a cut-off calibrator, a negative control, and at least three replicates of positive controls.

#### 10.5.7.3. Acceptance criteria

##### 10.5.7.3.1. Quantitative methods

10.5.7.3.1.1. Linearity:  $R^2$  value of at least 0.99 and calibrator concentrations within  $\pm 20\%$  of the target.

10.5.7.3.1.2. Bias: %bias  $\leq 20\%$

10.5.7.3.1.3. Within-run precision: %CV  $\leq 20\%$

10.5.7.3.1.4. Between-run precision (for major verifications): %CV  $\leq 20\%$

##### 10.5.7.3.2. Qualitative methods.

10.5.7.3.2.1. Positive controls are positive.

10.5.7.3.2.2. Negative controls are negative.

#### 10.5.8. Reference

10.5.8.1. Agilent Technologies. Agilent G1701EA GC-MSD ChemStation: Getting Started, Part Number G1701-90062, 2007.

10.5.8.2. Dawling S. Gas Chromatography in Clarke's Analysis of Drugs and Poisons, 3rd Edition. Moffat AC; Ossleton MD; Widdop B; (Ed). The Pharmaceutical Press, London, 2004, pp. 425-499.

10.5.8.3. Agilent Technologies. Agilent MSD ChemStation Software: User Information CD, Part Number G1701-60125.



10.5.8.4. Watson D. Mass Spectrometry in Clarke's Analysis of Drugs and Poisons, 3rd Edition. Moffat AC; Ossleton MD; Widdop B; (Ed). The Pharmaceutical Press, London, 2004, pp. 379-391.

## 10.6. LC-MS/MS

### 10.6.1. Method of Use

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

### 10.6.2. Performance Check

10.6.2.1. Needle wash: H<sub>2</sub>O: methanol: isopropanol (1:1:1)

10.6.2.2. Wash solvents for pump: H<sub>2</sub>O: isopropanol (90:10).

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

10.6.2.3.1. **SS acceptance criteria include 1) presence of all ion transitions and 2) smooth and symmetrical peak shapes; ion ratios and retention times are visually monitored. As the acceptance limits on the data are from the past calibration, flags associated with the limits are not considered.**

### 10.6.3. Autotune and Checktune

10.6.3.1. An Autotune must be performed at least monthly, after any maintenance, and at other intervals as deemed necessary by the analyst. During an Autotune, the mass spectrometer 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. A Checktune is used to evaluate the instrument's performance prior to each day's first analytical run per instrument. It determines if the masses of fragment ions generated from the tuning mixture are properly assigned and if the response or sensitivity of these ions is within expectations.

10.6.3.1.1. Each day that an Autotune or Checktune is performed, it should be documented on the LC-MS/MS Maintenance Log (LAB-111) or an equivalent form. A copy of previous tune files should be recorded in a retrievable format.

10.6.3.1.2. Following an Autotune or a Checktune 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 the said criteria. For example, the system may need a refreshed Autotune, a source cleaning, or other maintenance.

10.6.3.1.3. 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.

10.6.3.1.4. Daily checktune requirement does not apply if the samples in a batch are being injected overnight or some samples in a batch are needed to be re-injected next day using the calibration curve initially injected for the batch; this is to



ensure that the instrument condition is the same for all samples within the batch. If an entire batch needs to be re-injected next day, a checktune should be performed again that day prior to re-injecting the entire batch.

10.6.3.2. Instrument Parameters for the positive and negative modes, set by the manufacturer:

Gas Temp	300°C
Gas Flow	8 L/min
Nebulizer	15 psi
Capillary	4000 V
Nozzle Voltage	1500 V
Sheath Gas Temp	250°C
Sheath Gas Flow	7 L/min

10.6.3.3. Autotune Specifications: the following are manufacturer's recommended settings. These will be used as guidelines. Performance of the instrument will be evaluated before each analytical run.

10.6.3.3.1. Target Peak Widths and Tolerances

10.6.3.3.1.1. Unit: Peak width 0.70, tolerance 0.14

10.6.3.3.1.2. Wide: Peak width 1.20, tolerance 0.60

10.6.3.3.1.3. Widest: Peak width 2.50, tolerance 1.25

10.6.3.3.2. Recommended tune abundances in Positive Mode

<i>m/z</i>	<b>MS1</b>	<b>MS2</b>
118	4.E+05	3.E+05
322	2.E+05	1.E+05
622	2.E+05	1.E+05
922	2.E+05	1.E+05
1522	6.E+04	5.E+04
2122	3.E+04	4.E+04

10.6.3.3.3. Recommended tune abundances in Negative Mode

<i>m/z</i>	<b>MS1</b>	<b>MS2</b>
113	7.E+04	4.E+04
302	2.E+05	2.E+05
602	1.E+05	1.E+05
1034	3.E+05	2.E+05
1634	5.E+05	4.E+05
2234	2.E+05	6.E+04

10.6.3.4. Checktune Specifications

10.6.3.4.1. Mass Axis Tolerances

10.6.3.4.1.1. Unit: var

10.6.3.4.1.2. Wide: 0.50



10.6.3.4.1.3. Widest: 0.70

10.6.3.5. The results of the checktune should state "Pass" for the application; performance of the instrument will be evaluated before each analytical run.

#### 10.6.4. Maintenance

10.6.4.1. A preventive maintenance is performed annually by an external party

10.6.4.2. Maintenance should be performed following the manufacturer's guidelines or more frequently as needed. Refer to Agilent 6400 Series Triple Quad LC/MS guide or equivalent. All maintenance and repairs should be documented on the LC-MS/MS Maintenance Log (LAB-111) or an equivalent form.

10.6.4.2.1. Before each analytical run:

- Verify nitrogen tank and generator pressure
- Check the rough pump oil level
- Check/empty waste bottles
- Prepare fresh mobile phase
- Check/replace seal wash and needle wash solvent
- Clean spray chamber
- Perform a Checktune

10.6.4.2.2. Monthly:

- Perform Autotune

10.6.4.2.3. Annually:

- Replace the rough pump oil

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

- Clean the ion source
- Replace column and/or guard column
- Change in-line filters
- Check and replace solvent bottle filters
- Replace nebulizer
- Replace gas cylinders

#### 10.6.5. Methods

10.6.5.1. 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. The analyst initials and dates any updated methods excluding changes in the MRM window parameters.

10.6.5.2. Sample Preparation and Sequence Set-up

10.6.5.3. Samples are prepared for analysis according to the specific SOP of the analytical method.



10.6.5.4. 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.

10.6.5.5. Retain LC-MS/MS analysis data in the batch file.

#### 10.6.6. Verification

10.6.6.1. Verification is performed after significant changes in the method parameters other than data acquisition. A validation will be performed for changes in data acquisition. The verification run must meet acceptance criteria outlined in 10.6.6.3. If acceptance criteria are not met, appropriate measures must be taken to rectify the problem. Verification runs can be documented in the maintenance log or equivalent form or a separate PDF file. Data are kept in a retrievable format in the laboratory.

##### 10.6.6.2. Procedure

###### 10.6.6.2.1. Quantitative methods

10.6.6.2.1.1. A major verification consists of three analyses of a matrix blank, a negative control, calibrators and at least three replicates of low, mid, and high concentration controls.

10.6.6.2.1.2. A minor verification consists of one analysis of a matrix blank, a negative control, calibrators and at least three replicates of low, mid, and high concentration controls.

10.6.6.2.1.3. Whether a major or minor verification is performed depends on the extent of the changes in the method. Maintenance including annual preventive maintenance, repair, and minor changes do not warrant a verification.

10.6.6.2.1.4. Modifications to the aforementioned verification designs and/or additional experiments can be performed depending on the nature and purpose of a verification.

###### 10.6.6.2.2. Qualitative methods

10.6.6.2.2.1. A major verification consists of three analyses of a matrix blank, a cut-off calibrator, a negative control, and at least three replicates of positive controls.

10.6.6.2.2.2. A minor verification consists of one analysis of a matrix blank, a cut-off calibrator, a negative control, and at least three replicates of positive controls.

##### 10.6.6.3. Acceptance criteria

###### 10.6.6.3.1. Quantitative methods

10.6.6.3.1.1. Linearity:  $R^2$  value of at least 0.99 and calibrator concentrations within  $\pm 20\%$  of the target.

10.6.6.3.1.2. Bias: %bias  $\leq 20\%$

10.6.6.3.1.3. Within-run precision: %CV  $\leq 20\%$

10.6.6.3.1.4. Between-run precision (for major verifications): %CV  $\leq 20\%$

###### 10.6.6.3.2. Qualitative methods.



- 10.6.6.3.2.1. Positive controls are positive.
- 10.6.6.3.2.2. Negative controls are negative.

#### 10.6.7. References

- 10.6.7.1. Agilent Technologies. Agilent 6400 Series Triple Quad LC/MS Setup and Verification Guide, Part Number G1960-90125, 2018.
- 10.6.7.2. Agilent Technologies. Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide, Part Number G3335-90228, 2017.

### 10.7. Pipettes

#### 10.7.1. Method of Use

- 10.7.1.1. Refer to appropriate manuals for proper handling and use.

#### 10.7.2. Calibration

- 10.7.2.1. 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.

#### 10.7.3. Performance Check and Maintenance

- 10.7.3.1. Pipette performance checks are performed following maintenance or cleaning.

10.7.3.1.1. External calibration can be performed in lieu of a performance check.

10.7.3.1.2. External calibration of pipettes performed onsite by an approved vendor is not considered maintenance and thus does not require a performance check.

- 10.7.3.2. Performance check procedure:

10.7.3.2.1. Room temperature deionized water should be pipetted into a weighing vessel on an analytical balance.

10.7.3.2.2. 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.

10.7.3.2.3. The mass of the water delivered should be recorded on the Pipette Performance Check Form (LAB-041) or an equivalent form.

10.7.3.2.4. The average value of each pipette should fall within the performance limit set by the external vendor's calibration document ( $\pm 3\%$ ) before it may be used for casework.

- 10.7.3.3. Due to the inability to gravimetrically verify the small volumes ( $2.0 \mu\text{L}$ ) associated with low volume pipettes that dispense volumes  $\leq 25 \mu\text{L}$ , these pipettes will not be checked internally. Instead, these shall be checked via an approved calibration vendor before placing back into service.

- 10.7.3.4. Follow manufacturer's instructions for troubleshooting maintenance if needed.





10.7.3.5. If a pipette fails a performance check or if an analyst has reason to believe that a pipette is not working properly, they must:

10.7.3.5.1. Perform a pipette performance check and if the pipette is not in proper working order:

10.7.3.5.1.1. Clearly mark the pipette "OUT OF SERVICE".

10.7.3.5.1.2. Inform the section manager. No laboratory case work will be performed using the pipette until an external calibration is completed.

10.7.3.5.1.3. Repair or send out the pipette for repairs.

10.7.3.5.1.4. Externally calibrate following repair.

10.7.3.5.1.5. Documentation must be maintained in a retrievable format in the laboratory.

10.7.3.5.2. 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-041) or an equivalent form.

10.7.3.5.3. All pipettes marked as "OUT OF SERVICE" are not subject to routine external calibration unless the pipettes need to be placed back into service.

10.7.3.6. 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).

10.7.3.7. Maintenance of Hamilton® Microlab 600 follows as described in Hamilton Maintenance Log (LAB-085) or an equivalent form.

10.7.3.7.1. 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, if possible. Firmware or software versions may also be updated as provided by Hamilton Company.

#### 10.7.4. References

10.7.4.1. Gilson. Microman Pipette Manual. Middleton, WI, 2006.

10.7.4.2. VWR. VWR Signature Ergonomic High-Performance Pipettor Manual. West Chester, PA.

10.7.4.3. Hamilton. Microlab® 600 Basic Manual: Hardware Installation and Basic Operation. Part Number: 61440-01, Rev. F.

10.7.4.4. Hamilton. Microlab® 600 Advanced Manual: Wizards and Custom Method Operation. Part Number: 61441-01, Rev. D.

10.7.4.5. Thermo Scientific. Finnpiquette F2 Single Channel & Variable Fixed Volume Multichannel: Instructions for use, 2017.

10.7.4.6. Eppendorf. Multipette/Repeater Xstream and Multipette/Repeater stream: Operating Manual, 2013.



- 10.7.4.7. Eppendorf. Multipette Plus and Repeater Plus: Operating Manual, 2007.
- 10.7.4.8. Eppendorf. Multipette M4 and Repeater M4: Operating Manual, 2019.

## 10.8. pH Meter

### 10.8.1. Method of Use

10.8.1.1. Refer to the User's Manual for detailed instructions on proper handling and use.

### 10.8.2. Performance Check and Maintenance

10.8.2.1. A three-point performance check is used for measuring pH of a solution when pH accuracy better than  $\pm 0.1$  is required. This check is performed prior to each use and documented on pH Meter Performance Check (LAB-048) or an equivalent form.

10.8.2.1.1. Measured pH shall be **at the target pH rounded to one decimal place (e.g., pH 4.0, 7.0, and 10.0)** at the reported temperature; if measured temperature falls between the listed temperatures on the pH buffer solution bottle label (e.g., 18 °C), the target pH value closer to the measured temperature (**pH 7.0** at 20 °C) is used. Slope shall be between **95-105%**. **If Mettler-Toledo SevenDirect™ SD50 pH/Ion Meter is used, a verification shall be performed and passed.**

10.8.2.2. Weekly maintenance **includes 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-048) or an equivalent form.

### 10.8.3. References

- 10.8.3.1. **Mettler-Toledo. SevenDirect™ SD50 pH/Ion Meter: Reference Manual, 2022.**
- 10.8.3.2. **Mettler-Toledo. SevenDirect™ SD50 pH/Ion Meter: User Manual, 2021.**

## 10.9. Refrigerators and Freezers

### 10.9.1. Maintenance

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

10.9.2. If a refrigerator or freezer is open for an extended period (e.g., cleaning, inventory), this should be documented on the Temperature Log (LAB-069) or by an equivalent method.

10.9.3. 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.

### 10.9.4. References

- 10.9.4.1. VWR. Chromatography Refrigerators: Owner's Guide, Part Number 20047, Rev. O, 7-30-2013.



- 10.9.4.2. VWR. Pharmacy, Laboratory, and Chromatography Glass Door(s) Refrigerator or Freezer with D-100 Alarm System & Microprocessor Temp Control V5.2: Owner's Guide. All Rev. 7-27-2016.
- 10.9.4.3. VWR. Laboratory Refrigerator or Freezer with Mechanical Temp Control: Owner's Guide. FD Rev. 8-11-2016.
- 10.9.4.4. VWR. Laboratory Refrigerator or Freezer with Mechanical or Digital Temp Control: Owner's Guide. Rev. 3-11-2022.

#### **10.10. NIST Traceable Thermometers**

- 10.10.1. In the event the temperature monitoring system is not working, temperature measurements for refrigerators and freezers must be performed using NIST traceable thermometers weekly at the minimum. Temperature measured with a method other than the temperature monitoring system is documented on the Temperature Log (LAB-069) or an equivalent method.
- 10.10.2. If any thermometer is suspected of not working properly, laboratory management **or designee** should be notified, and a record made. It will then be removed from service and replaced with a working thermometer.

#### **10.11. NIST Traceable Volumetric Flasks**

- 10.11.1. Class A volumetric flasks will be used for the preparation of calibrators and will be dedicated for this purpose. Pre-calibrated flasks will be purchased, and the flasks will be replaced every ten years.
- 10.11.2. References
  - 10.11.2.1. ANSI/ASB Standard 017, First Edition 2018. Standard Practices for Measurement Traceability in Forensic Toxicology.

#### **10.12. Balances**

- 10.12.1. Method of Use
  - 10.12.1.1. Refer to the appropriate operating instructions for proper handling and use.
- 10.12.2. Performance Check and Maintenance
  - 10.12.2.1. Performance check and maintenance should be performed following the manufacturer's guidelines and the schedule described below.
  - 10.12.2.2. Balances
    - 10.12.2.2.1. Balances must be calibrated and certified with a traceable certificate by an external vendor once per year.
    - 10.12.2.2.2. An internal performance check must be conducted weekly using NIST traceable reference weights. This is documented using form Balance Performance Check (LAB-014), or an equivalent method.
    - 10.12.2.2.3. Additional performance checks may be performed as necessary.



- 10.12.2.2.4. Balances should be checked for accuracy each time the balance is moved and after maintenance is performed.
- 10.12.2.3. Weights
- 10.12.2.3.1. Weights used to performance check the balance shall be sent to a vendor for recertification every year.
- 10.12.2.3.2. 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-012), or an equivalent method.
- 10.12.2.3.3. Laboratory weights should be stored, transported, and handled using pre-cautions to protect them from contamination and deterioration.
- 10.12.2.4. General
- 10.12.2.4.1. All records of the performance checks of balances and weight sets, maintenance, and calibration certificates are maintained in a retrievable format.
- 10.12.2.4.2. 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 **or designee** should be notified, and the problem documented on LAB-012, LAB-014, or an equivalent method.
- 10.12.2.4.3. 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

### 10.12.3. References

- 10.12.3.1. Mettler-Toledo. Operating Instructions: B-S/FACT line of balances, 2007.

## 10.13. Heating Block

### 10.13.1. Method of Use

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

### 10.13.2. 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-084).

#### 10.13.3. References

10.13.3.1. VWR. Analog Dry Block Heaters: Instruction Manual, Rev. 2-3/09.

### 10.14. Millipore Water Purification System

#### 10.14.1. Method of Use

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

#### 10.14.2. Performance Check and Maintenance

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

10.14.2.2. 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-083) and the User Manual.

#### 10.14.3. References

10.14.3.1. Millipore. Direct-Q 3 System, Direct-Q 3 UV System: User Manual, Rev. 3-12/08, 2006.

### 10.15. Centrifuge

#### 10.15.1. Method of Use

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

#### 10.15.2. References

10.15.2.1. Eppendorf Centrifuge 5804/5804R/5810/5810R: Instruction Manual.

### 10.16. Evaporator

10.16.1. The Zymark Turbo Vap<sup>®</sup> LV is a microprocessor-controlled evaporator that provides simultaneous and automated concentration of multiple samples, unattended operation, convenience, and speed. The Turbo Vap will be used routinely during extractions in order to concentrate extracts or as needed.

#### 10.16.2. Equipment Parts

10.16.2.1. Zymark Turbo Vap LV Workstation and accessories

10.16.2.2. Compatible test tube rack

10.16.2.3. Inert gas supply (nitrogen)

10.16.2.4. Distilled or deionized water

10.16.2.5. Timer

10.16.2.6. Siphon tube

10.16.2.7. Spectrum Clear Bath

#### 10.16.3. Precautions



- 10.16.3.1. 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.
- 10.16.3.2. Note: Exhaust gases may be hazardous. Consult the Safety Data Sheets for all solvents used.
- 10.16.3.3. The workstation must be used on a flat, level, stable surface.
- 10.16.3.4. The workstation must never be used with hydrogen or other flammable gases which may explode or catch on fire.
- 10.16.3.5. DO NOT move the unit when the bath is full of water. It is a burn hazard.
- 10.16.3.6. DO NOT operate the Turbo Vap LV without water in the bath to avoid the risk of fire or burn injuries.
- 10.16.3.7. To avoid injury, DO NOT EXCEED 100 psi maximum inlet pressure.
- 10.16.4. Procedure
  - 10.16.4.1. Turn on the unit and gas supply.
  - 10.16.4.2. 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.
  - 10.16.4.3. Check the gas supply and pressure.
  - 10.16.4.4. 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.
  - 10.16.4.5. Set the gas pressure and time setting in accordance with the SOP.
  - 10.16.4.6. 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.
  - 10.16.4.7. Note: Leaving empty rows unplugged while in use can cause condensation on the unit lid, which may in turn contaminate the sample.
  - 10.16.4.8. Load sample tubes into the evaporator by opening the cover and placing the sample racks into the water bath.
  - 10.16.4.9. 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.
  - 10.16.4.10. 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.
  - 10.16.4.11. Note: Highly volatile samples can be lost if they are allowed to remain in the unit.



10.16.4.12. 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.

#### 10.16.5. Maintenance

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

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

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

10.16.5.4. Open the cover and remove the rack.

10.16.5.5. Siphon the water out of the bath.

10.16.5.6. 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.

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

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

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

#### 10.16.6. References

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

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

10.16.6.3. Biotage. TurboVap: User Manual. P/N 415390-E, 2019.

### **10.17. Pressure Processor**

10.17.1. During solid phase extraction (SPE), samples require processing and drying which may be carried out using positive pressure. The CEREX<sup>®</sup> 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. 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.

#### 10.17.2. Equipment Parts

10.17.2.1. CEREX System 48 ("System 48") or CEREX System 48-II ("CEREX 48")

10.17.2.2. Collection tube rack, 16x100 mm

10.17.2.3. SPE Rack, 6CC, for 48 Place Processor

10.17.2.4. Waste bin(s) and waste bin rack

10.17.2.5. Nitrogen and Air compressed gas cylinder/supply

#### 10.17.3. Precautions

10.17.3.1. 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.

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





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

#### 10.17.4. Procedure

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

10.17.4.2. 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.

10.17.4.3. Turn on gas flow to the unit.

10.17.4.4. 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.

10.17.4.5. To begin applying pressure to the rack assembly:

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

10.17.4.5.2. 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.

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

10.17.4.6. 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.)

10.17.4.7. 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.

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

10.17.4.7.2. '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 gentlest, limiting flow setting.

10.17.4.7.3. '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.

10.17.4.7.3.1. **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.

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





10.17.4.8.1. 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.

10.17.4.8.2. 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.

#### 10.17.5. Maintenance

10.17.5.1. 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:

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

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

#### 10.17.6. References

10.17.6.1. SPEware Corporation. Cerex 48 Pressure Processor: Operating Instructions, Rev. A.



## **11. Analysis of Alcohol and Other Volatiles by Headspace Gas Chromatography-Flame Ionization Detection**

### **11.1. Purpose**

11.1.1. 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).

### **11.2. Scope**

11.2.1. This procedure is used for the qualitative or quantitative analysis of ethanol, methanol, acetone, and isopropanol in blood and liquid specimens.

### **11.3. Reagents and Solvents**

- 11.3.1. Ethyl Alcohol
- 11.3.2. n-Propanol
- 11.3.3. Isopropanol
- 11.3.4. Acetone
- 11.3.5. Methanol
- 11.3.6. Deionized (DI) Water
- 11.3.7. Blank Blood

### **11.4. Equipment and Materials**

- 11.4.1. Instrumentation using a method that is approved and validated for use in the section
- 11.4.2. Compressed gas cylinders or equivalent (helium, hydrogen, air, and nitrogen)
- 11.4.3. Vials, caps/septa, stoppers, and crimpers
- 11.4.4. Hamilton pipettor-dilutor or equivalent
- 11.4.5. Volumetric flasks
- 11.4.6. Analytical balance
- 11.4.7. Homogenizer
- 11.4.8. Centrifuge
- 11.4.9. Vortex mixer or rocker

### **11.5. Instrumentation**

#### **11.5.1. Parameters**

11.5.1.1. Capillary columns: Restek BAC1 and BAC2 (or equivalent), 30 m x 320  $\mu\text{m}$  id x 1.8  $\mu\text{m}$  (BAC 1) or 0.6  $\mu\text{m}$  (BAC2) film thickness. After a 10:1 split injection, the column flow rates are approximately 7 mL/min with a pressure of 24.011 psi.



11.5.1.2. HS-GC: Agilent 7697A-7890B

GC Oven Temperature:	40 °C hold for 4 minutes
Total Run Time:	4 minutes
HS Oven and Loop Temperatures:	70 °C
Equilibration Time:	7 minutes
HS Transfer Line Temperature:	90 °C
Detector Temperature:	250 °C
Detector Flows:	H <sub>2</sub> 30 mL/min Air 400 mL/min N <sub>2</sub> 25 mL/min

11.5.1.3. ALC.M

11.5.2. Performance Check

11.5.2.1. Ensure gas cylinder pressure is sufficient, test sequence is completed (see 13.5.2.2), all acceptance criteria are met, and maintenance log is filled out.

11.5.2.2. A test sequence is composed of two air blanks and an SS. The results will be documented in the Headspace GC Maintenance Log (LAB-039 or equivalent form). 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.

11.5.2.2.1. The air blanks must be clear of peaks of interest (i.e., no analytes of interest identified by both FIDs).

11.5.2.2.2. Resolution for the reporting analyte(s) in SS must be confirmed.

**11.6. Calibration**

11.6.1. Ethanol Calibration (0.010 – 0.500 g/100 mL)

Volatile Analyte	Weighting Factor (Calibration Model)
Ethanol	1/x (linear)
Methanol	1/x (linear)
Isopropanol	1/x (linear)
Acetone	1/x (linear)

11.6.1.1. 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.

11.6.1.2. External (e.g., Cerilliant, Lipomed, or equivalent) NIST traceable 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

11.6.1.3. 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.

#### 11.6.2. Methanol/Isopropanol/Acetone (M/I/A) Calibration (0.010 – 0.400 g/100 mL)

11.6.2.1. External (e.g., Cerilliant, Lipomed, or equivalent) NIST traceable 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

### 11.7. Internal Standard

11.7.1. 0.01% n-Propanol Internal Standard can be prepared using either Method 1 or Method 2.

#### 11.7.2. 1.0% n-Propanol Internal Standard Stock Solution (%w/v) – Method 1

11.7.2.1. 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.

#### 11.7.3. 0.01% n-Propanol Internal Standard Working Solution (%v/v) – Method 1

11.7.3.1. 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.

#### 11.7.4. 0.01% n-Propanol Internal Standard Working Solution (%v/v) – Method 2



11.7.4.1. 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.

## 11.8. Controls

### 11.8.1. Whole Blood Controls

11.8.1.1. 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.

11.8.1.2. Purchased whole blood mixed volatile controls (MQC) will be used for quantitative methanol/isopropanol/acetone analysis.

**Storage:** Store refrigerated. **Discard:** Manufacturer expiration date; 45 days opened.

### 11.8.2. Low Aqueous Mixed Volatile Control (LMQC) (0.0192 g/100 mL)

11.8.2.1. 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.

### 11.8.3. Aqueous Ethanol Control (EQC) – 0.080 g/100 mL

11.8.3.1. 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.

### 11.8.4. High Aqueous Ethanol Control (HEQC) – 0.400 g/100 mL

11.8.4.1. 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.

### 11.8.5. Mixed Volatile System Suitability Control (SS)

11.8.5.1. 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.

### 11.8.6. Negative Control

11.8.6.1. Negative control is used to monitor any potential carryover from the highest calibrator concentration. It consists of 100 µL of DI water and 1000 µL of internal standard.



#### 11.8.7. Dilution Control (DQC)

11.8.7.1. A dilution of the HEQC or other analyte solvent (e.g., 10% ethanol) with known concentration is analyzed in the batch, if necessary, to verify dilution results of an alcoholic beverage or other case sample(s) employing the same dilution factor used in the case sample.

11.8.7.2. 10% Ethanol (v/v) – 7.89 g/100 mL

11.8.7.2.1. Fortify 500 µL of ethanol (ACS grade, 99.5% purity or better) into a 5 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.

11.8.7.2.2. **Storage:** Store refrigerated. **Discard:** 1 year.

#### 11.8.8. DI Water Control

11.8.8.1. The DI water control is a quality control used to monitor any potential environmental contamination throughout the sample preparation. It consists of 100 µL of DI water added to a headspace vial and left open (uncapped) for the duration of the aliquoting process. Internal standard is added at the end of sampling.

### 11.9. Sample Preparation

11.9.1. Allow calibrators, controls, and case samples to come to room temperature prior to sampling.

11.9.2. Mix all calibrators, controls, and case samples well prior to sampling by gentle inversion or rocking. Avoid shaking.

11.9.3. Prepare and leave open the DI Water Control for the duration of the aliquoting process. When all sampling is completed, add internal standard, cap, and crimp tightly.

11.9.4. Dilutions will be performed with DI water prior to aliquoting.

11.9.4.1. When alcoholic beverages or other case samples requiring dilution are analyzed, fluid from the sample 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.

11.9.4.2. A DQC, using the same dilution as the case sample, must be included in the batch.

11.9.4.3. Commonly used dilutions are as follows:

Specimen Type	Dilution	Specimen (µL)	Deionized Water (µL)
blood	1:2	500	500
beer	1:20	50	950
wine or unknown	1:50	20	980
liquor	1:100	10	990



- 11.9.5. 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  $\mu$ L of the calibrators, controls, or case samples. The pipettor-dilutor will deliver 1000  $\mu$ L of IS working solution along with the 100  $\mu$ L sample aliquot into a headspace vial. Label the headspace vial appropriately, cap, and crimp tightly.
- 11.9.6. Samples must be prepared and labeled appropriately in the same order they will be analyzed (see example sequence below).
- 11.9.7. 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.
- 11.9.8. 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.
- 11.9.9. 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: gray>lavender>pink>tan>royal blue (if it contains anticoagulant). See 3.7.2 and 3.7.3 of the Evidence Handling section for further details.
- 11.9.10. 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 3.7 of the Evidence Handling section for further details.
- 11.9.11. Load headspace vials on the HS-GC autosampler and inject the sequence using the ALC.m method.



**11.10. Volatiles Sequence Example:**

- Calibrators (Low to High)
  - Negative Control (Carryover check)
  - HEQC
  - DQC<sup>2</sup>
  - MQC<sup>1</sup>
  - 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
  - LMQC
  - EQC
  - DI Water Control
- <sup>1</sup> Only necessary if quantifying M/I/A.  
<sup>2</sup> Only necessary if diluted case sample(s) are included in batch.

- 11.10.1. 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.
- 11.10.2. Batch size will accommodate a maximum of 40 case samples unless determined otherwise by the supervisor or manager.

**11.11. Calculation and Acceptance Criteria**

- 11.11.1. 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.
- 11.11.2. Calibrators and Controls (Ethanol and M/I/A)
  - 11.11.2.1. Retention time of reporting analyte(s) must be within  $\pm 1\%$  of the mean retention time of calibrators (i.e., "Default RT Windows" for "Other Peaks" under Calibration Settings was set at 2%).
  - 11.11.2.2. Negative and DI Water controls must be clear of significant (i.e., area counts  $>10\%$  of the LOQ) peaks of interest.
  - 11.11.2.3. For volatile concentrations of reporting analyte(s)  $>0.050$  g/100 mL, average values from FID1 and FID2 must be within  $\pm 5\%$  of target value.
  - 11.11.2.4. For volatile concentrations of reporting analyte(s)  $\leq 0.050$  g/100 mL, average values from FID1 and FID2 must be within  $\pm 10\%$  of target value.





- 11.11.2.5. Calibrator and control results from FID1 and FID2 per aliquot must be within  $\pm 10\%$  of their average for the concentrations  $\leq 0.050$  g/100 mL and  $\pm 5\%$  of their average for the concentrations  $> 0.050$  g/100 mL.
- 11.11.2.6. The calibration curve must yield an  $R^2$  value of 0.99 or greater for reporting analyte(s).
- 11.11.2.7. 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.
  - 11.11.2.7.1. HEQC, MQC, EQC, and LMQC must be within the allowable range for reporting analyte(s) to report quantitative results.
  - 11.11.2.7.2. Negative results may be reported with documented agreement by the technical reviewer.
- 11.11.3. Internal Standard Recovery
  - 11.11.3.1. For each batch, the consistency of internal standard signal must be evaluated.
  - 11.11.3.2. 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 within the batch for reporting quantitative results.
- 11.11.4. Chromatography
  - 11.11.4.1. All chromatography must be symmetric and well resolved from any interfering peaks.
  - 11.11.4.2. 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.
  - 11.11.4.3. Peak resolution will be evaluated for every peak associated with a reporting analyte. The instrument software will generate and report the peak-to-valley ratio for peaks that have an apparent shoulder or interfering peak. A number greater than  $|10|$  indicates acceptable resolution.
  - 11.11.4.4. Any samples with unresolved or asymmetric peaks for reporting analyte(s) will be re-analyzed.
- 11.11.5. Case Samples
  - 11.11.5.1. Case sample results are calculated using values obtained from both FID1 and FID2.
  - 11.11.5.2. Results from FID1 and FID2 per aliquot must be within  $\pm 10\%$  of their average for the concentrations  $\leq 0.050$  g/100 mL and  $\pm 5\%$  of their average for the concentrations  $> 0.050$  g/100 mL.
  - 11.11.5.3. If the average, truncated to three decimals, of aliquot 1 and aliquot 2  $\leq 0.050$  g/100 mL, all four values must be within  $\pm 10\%$  of the average.



$$\begin{aligned} \text{Acceptable Range} \\ = \frac{(Alq\ 1\ FID1 + Alq\ 1\ FID2 + Alq\ 2\ FID1 + Alq\ 2\ FID2)}{4} \times 0.9 \text{ to} \\ \frac{(Alq\ 1\ FID1 + Alq\ 1\ FID2 + Alq\ 2\ FID1 + Alq\ 2\ FID2)}{4} \times 1.1 \end{aligned}$$

11.11.5.4. If the average, truncated to three decimals, of aliquot 1 and aliquot 2 >0.050 g/100 mL, all four values must be within ±5% of the average.

$$\begin{aligned} \text{Acceptable Range} \\ = \frac{(Alq\ 1\ FID1 + Alq\ 1\ FID2 + Alq\ 2\ FID1 + Alq\ 2\ FID2)}{4} \times 0.95 \text{ to} \\ \frac{(Alq\ 1\ FID1 + Alq\ 1\ FID2 + Alq\ 2\ FID1 + Alq\ 2\ FID2)}{4} \times 1.05 \end{aligned}$$

11.11.5.5. Case samples must be immediately bracketed by acceptable controls to report positive results.

11.11.5.5.1. Any positive case samples not bracketed by an acceptable control must be re-analyzed.

11.11.5.6. If a case sample is not within the given range or does not meet other acceptable criteria, that positive case sample must be re-analyzed. Negative results may be reported upon documented review of the data by the analyst and technical reviewer.

11.11.5.7. 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 sample will be reported as unsuitable for the HFSC alcohol analysis. Any further attempts should be investigated and discussed with the section manager, supervisor, and/or Quality Director.

## 11.12. Contamination

11.12.1. See 11.11.2.2 for acceptance criteria for Negative and DI Water Controls.

11.12.2. Air blanks are capped empty headspace vials included in sequences to clean the instrument. Air blanks are not controls.

## 11.13. Interference

11.13.1. If a case sample with a calculated result above the LOQ fails due to resolution, the sample will be re-analyzed at least once to confirm the interference is from the sample rather than from the analysis if there is sufficient volume. The re-analysis may not be possible if the sample volume is not sufficient.

11.13.1.1. If the re-analysis still shows unacceptable interference (e.g., the peak-to-valley ratio or peak symmetry value still fails), the result will be reported as unsuitable for analysis.



#### **11.14. Reinjection**

11.14.1. Due to the nature of headspace analysis, reinjections of unknown samples (i.e., case samples) are not permitted. For instance, if an instrument error causes a stopped run, the previous bracketing control may be reinjected followed by any subsequent unpunctured vials.

11.14.2. Reinjection of known samples (i.e., calibrators or controls) can be performed when required to complete a sequence.

11.14.2.1. All acceptance criteria must be met as described in section 11.11 to accept the reinjected data.

#### **11.15. Carryover**

11.15.1. If a case sample is greater than the ULOQ, it may be necessary to dilute the sample as stated in section 11.9.4.

11.15.2. If a highly concentrated case sample is greater than 1.0 g/100 mL, the case samples immediately following each aliquot in the batch sequence require reanalysis to rule out carryover.

11.15.2.1. The Laboratory has demonstrated in validation experiments that no carryover occurs following samples with concentrations  $\leq 1.0$  g/100 mL

11.15.2.2. If a negative case sample follows a case sample greater than the ULOQ, no reanalysis is necessary for the negative sample.

#### **11.16. References**

11.16.1. Caplan, Yale H., Goldberger, Bruce A., eds. *Garriott's Medicolegal Aspects of Alcohol*, 6<sup>th</sup> ed. Tucson, AZ: Lawyers & Judges Publishing Company, Inc. 2015.



## 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
- Buprenorphine Direct ELISA Kit
- Cocaine Metabolite (Benzoyllecgonine Specific) Direct ELISA Kit
- Cannabinoids (THCA/CTHC) Direct ELISA Kit
- Carisoprodol Direct ELISA Kit
- Fentanyl Direct ELISA Kit
- Methamphetamine Direct ELISA Kit
- Opiates Direct ELISA Kit
- PCP Direct ELISA Kit
- Oxycodone/Oxymorphone 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:



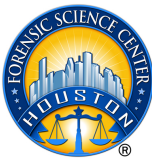
- 12.3.5.1.1. Add 14.0 g of dibasic sodium phosphate to a 1000 mL volumetric flask and bring to volume with deionized water while stirring.
  - 12.3.5.1.2. In a 500 mL volumetric flask, add 6.0 g of monobasic sodium phosphate and bring to volume with deionized water while stirring.
  - 12.3.5.1.3. Adjust the pH of the monobasic sodium phosphate solution by adding dibasic sodium phosphate solution until the pH reaches  $7.0 \pm 0.5$ .
  - 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
  - 12.5.4.1. Cut-off Calibrator
    - 12.5.4.1.1. 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	750	75,000
(-)-11-nor-9-carboxy-THC	0.1	500	5,000
Benzoylcegonine	1	250	25,000
d-Amphetamine	1	100	10,000
d-Methamphetamine	1	100	10,000
Oxazepam	1	100	10,000
Buprenorphine	0.1	50	500
Fentanyl	0.1	50	500
Morphine	1	50	5,000
Phencyclidine	1	50	5,000

\*Cerilliant or equivalent

12.5.4.1.2. Cut-off Calibrator Sub-stock

12.5.4.1.2.1. Transfer 1 mL of the 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 the 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.

12.5.4.1.4. Urine Cut-off Calibrator

12.5.4.1.4.1. Add 250 µL of the Cut-off Calibrator Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

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

Cut-off Calibrator	Final Concentration (ng/mL)
d-Amphetamine	20
Secobarbital	150
Oxazepam	20
Buprenorphine	1
Benzoylcegonine	50
(-)-11-nor-9-carboxy-THC	10
Carisoprodol	500
Fentanyl	1
d-Methamphetamine	20
Morphine	10
Phencyclidine	10
Oxycodone*	10

\*Oxycodone is not a part of the mixed calibrator and is in its own cut-off calibrator solution.



12.5.4.2. Mixed Controls

12.5.4.2.1. Buprenorphine Control Stock – 0.1 mg/mL

12.5.4.2.1.1. Prepare by adding 100 µL of Buprenorphine 1 mg/mL CRM to 900 µL of methanol.

12.5.4.2.2. Control Stock

12.5.4.2.2.1. Prepare by spiking the following amounts of CRMs into a 10 mL class A volumetric flask and QS with methanol. Add 50 µL of Buprenorphine Control Stock 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	750	75,000
(-)-11-nor-9-carboxy-THC	0.1	500	5,000
Benzoylcegonine	1	250	25,000
d-Amphetamine	1	100	10,000
d-Methamphetamine	1	100	10,000
Oxazepam	1	100	10,000
Fentanyl	0.1	50	500
Morphine	1	50	5,000
Phencyclidine	1	50	5,000

\*Lipomed or equivalent

12.5.4.2.3. Negative Control Sub-Stock

12.5.4.2.3.1. Transfer 1 mL of the Control Stock to a 10 mL class A volumetric flask. Add 200 µL of Carisoprodol 1 mg/mL CRM and QS with PBS.

12.5.4.2.4. Blood Negative Control

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

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

12.5.4.2.5. Urine Negative Control

12.5.4.2.5.1. Add 250 µL of the Negative Control Sub-Stock to a 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

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



Negative Control	Final Concentration (ng/mL)
d-Amphetamine	10
Secobarbital	75
Oxazepam	10
Buprenorphine	0.5
Benzoyllecgonine	25
(-)-11-nor-9-carboxy-THC	5
Carisoprodol	200
Fentanyl	0.5
d-Methamphetamine	10
Morphine	5
Phencyclidine	5
Oxycodone*	5

\*Oxycodone is not a part of the mixed negative control.

12.5.4.2.6. Positive Control Sub-Stock

12.5.4.2.6.1. Transfer 1 mL of the Control Stock to a 5 mL class A volumetric flask.

Add 250 µL of Carisoprodol 1 mg/mL CRM and QS with PBS.

12.5.4.2.7. Blood Positive Control

12.5.4.2.7.1. Add 1.5 mL of the Positive Control Sub-Stock to a 50 mL class A volumetric flask, QS with synthetic blood, and thoroughly mix.

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

12.5.4.2.8. Urine Positive Control

12.5.4.2.8.1. Add 1.5 mL of the Positive Control Sub-Stock to a 50 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.

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

Positive Control	Final Concentration (ng/mL)
d-Amphetamine	60
Secobarbital	450
Oxazepam	60
Buprenorphine	3
Benzoyllecgonine	150
(-)-11-nor-9-carboxy-THC	30
Carisoprodol	1,500
Fentanyl	3
d-Methamphetamine	60
Morphine	30
Phencyclidine	30
Oxycodone*	30

\*Oxycodone is not a part of the mixed **positive control**.

12.5.5. Oxycodone Standards for Blood and Urine Analyses

12.5.5.1. Oxycodone Cut-off Calibrators

12.5.5.1.1. Oxycodone Cut-off Calibrator Stock - 5 µg/mL





- 12.5.5.1.1.1. Prepare by spiking 50  $\mu$ L of the Oxycodone 1 mg/mL CRM in methanol into 10 mL class A volumetric flask and QS with PBS.
- 12.5.5.1.2. Oxycodone Blood Cut-off Calibrator - 10 ng/mL
  - 12.5.5.1.2.1. Transfer 50  $\mu$ L 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.
  - 12.5.5.1.2.2. Aliquot out into appropriately labeled tubes and store in the freezer.
- 12.5.5.1.3. Oxycodone Urine Cut-off Calibrator - 10 ng/mL
  - 12.5.5.1.3.1. Transfer 50  $\mu$ L 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.
- 12.5.5.2. Oxycodone Controls
  - 12.5.5.2.1. Oxycodone Control Stock - 5  $\mu$ g/mL
    - 12.5.5.2.1.1. Prepare by spiking 50  $\mu$ L of the Oxycodone 1 mg/mL CRM in methanol into 10 mL class A volumetric flask and QS with PBS.
  - 12.5.5.2.2. Oxycodone Blood Negative Control - 5 ng/mL
    - 12.5.5.2.2.1. Transfer 25  $\mu$ L of the Oxycodone Control Stock to an appropriately labeled 25 mL class A volumetric flask and QS with unfortified blood (synthetic) and thoroughly mix.
    - 12.5.5.2.2.2. Aliquot out into appropriately labeled tubes and store in the freezer.
  - 12.5.5.2.3. Oxycodone Blood Positive Control - 30 ng/mL
    - 12.5.5.2.3.1. Transfer 300  $\mu$ L of the Oxycodone Control Stock to an appropriately labeled 50 mL class A volumetric flask and QS with unfortified blood (synthetic) and thoroughly mix.
    - 12.5.5.2.3.2. Aliquot out into appropriately labeled tubes and store in the freezer.
  - 12.5.5.2.4. Oxycodone Urine Negative Control - 5 ng/mL
    - 12.5.5.2.4.1. Transfer 25  $\mu$ L of the Oxycodone Control Stock to an appropriately labeled 25 mL class A volumetric flask, QS with unfortified urine, and thoroughly mix.
    - 12.5.5.2.4.2. Aliquot out into appropriately labeled tubes and store in the freezer.
  - 12.5.5.2.5. Oxycodone Urine Positive Control - 30 ng/mL
    - 12.5.5.2.5.1. Transfer 300  $\mu$ L of the Oxycodone Control Stock to an appropriately labeled 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.

## 12.6. Procedure

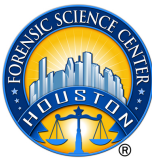
- 12.6.1. Allow all biological specimens and reagents to come to room temperature before starting the procedure.
- 12.6.2. Label 12x75 mm test tubes accordingly for the following calibrators and quality controls:
  - 12.6.2.1. Blank QC (in duplicate) – drug free matrix.



- 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 carisoprodol.
- 12.6.2.4. Positive QC (in duplicate) – fortified with target drugs at three times the concentration found in the calibrator.
- 12.6.3. [Urine only] Have another individual verify the case urine containers against the worklist and document the verification on batch records (e.g., worklist).
- 12.6.4. Pipet 100  $\mu$ L of calibrators, quality controls, and case samples into the appropriately labeled 12x75 mm test tube.
  - 12.6.4.1. Mix all calibrators, quality controls and case samples well prior to sampling by gentle priming of the pipette tip.
  - 12.6.4.2. Aliquoting occurs one case sample at a time.
  - 12.6.4.3. Every 10 case samples must be bracketed by a Positive QC.
- 12.6.5. Pipet 900  $\mu$ L of PBS into each test tube. Vortex.
- 12.6.6. 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.
  - 12.6.6.1. The instrument will pipet the following sample volumes for each assay, followed by 100  $\mu$ L of the appropriate conjugate:

Assay	Volume ( $\mu$ L)
Amphetamines	10
Barbiturates	20
Benzodiazepines	30
Buprenorphine	50
Benzoylcegonine	75
Cannabinoids	50
Carisoprodol	10
Fentanyl	75
Methamphetamine	25
Opiates	10
Phencyclidine	10
Oxycodone	10

- 12.6.6.2. 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.
- 12.6.6.3. Tecan will pipet 100  $\mu$ L of the appropriate TMB substrate into each sample well.
- 12.6.6.4. After TMB incubation, the instrument will pipet 100  $\mu$ L of the stop solution into each sample well.



12.6.6.5. 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.

12.6.6.6. 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	
C	Negative	SAMPLE	Positive	SAMPLE	SAMPLE	
D	Negative	SAMPLE	SAMPLE	SAMPLE	SAMPLE	
E	Calibrator	SAMPLE	SAMPLE	SAMPLE	SAMPLE	
F	Calibrator	SAMPLE	SAMPLE	Positive	SAMPLE	
G	Positive	SAMPLE	SAMPLE	SAMPLE	SAMPLE	
H	Positive	SAMPLE	SAMPLE	SAMPLE	SAMPLE	

12.7.3. Batch size will accommodate a maximum of 30 case samples.

### 12.8. Interpretation of Results

12.8.1. All samples with B/B<sub>0</sub> (%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 =  $(\text{Avg. Abs.}_{\text{calibrator}} / \text{Avg. Abs.}_{\text{blank control}}) \times 100$

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

Example:

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

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. References

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, 5<sup>th</sup> Edition. Levine B and Kerrigan S. (Eds). Springer, Switzerland, 2020, pp. 177-196.

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-068) 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
- Potassium hydroxide
- Dibasic sodium phosphate
- Monobasic sodium phosphate
- Sodium acetate
- Methylene chloride
- Isopropanol
- Methanol
- Ethyl acetate
- Hexane
- 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 Acetic acid

13.3.3.1. 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.

13.3.3.2. Store: Room temperature

13.3.3.3. Expiration: 1 year

13.3.4. 10% Acetic acid

13.3.4.1. In a graduated cylinder add 10 mL glacial acetic acid and 90 mL deionized water. Cap and thoroughly mix.

13.3.4.2. Store: Room temperature

13.3.4.3. Expiration: 1 year

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

13.3.6. 5 M Ammonium formate

13.3.6.1. Add 7.88 g ammonium formate to 25 mL LC/MS grade water. Cap and thoroughly mix.

13.3.6.2. Store: Room temperature

13.3.6.3. Expiration: 6 months

13.3.7. 1 M Ammonium hydroxide

13.3.7.1. Add 17 mL ammonium hydroxide (28-30% concentrated) to deionized water in a 250 mL volumetric flask and bring to volume.

13.3.7.2. Store: Room temperature

13.3.7.3. Expiration: 1 year

13.3.8. 100 mM Phosphate buffer, pH 6.0

13.3.8.1. 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 solution by adding the dibasic sodium phosphate solution until the pH reaches  $6.0 \pm 0.5$ .

13.3.8.2. Alternatively, a commercial pH buffer pouch containing 1.70 g disodium hydrogen phosphate and 12.14 g of sodium dihydrogen phosphate hydrate can be used. Add 600 mL of deionized water to a 1000 mL volumetric flask.



Add in the contents of the buffer pouch and mix/stir. Dilute to the mark with deionized water. Check the pH is  $6.0 \pm 0.5$ .

13.3.8.3. Store: Room temperature

13.3.8.4. Expiration: 6 months

13.3.9. 10 M Potassium hydroxide

13.3.9.1. Weigh approximately 56.1 g of potassium hydroxide pellets and add to a 100 mL volumetric flask containing approximately 50 mL of deionized water. Sit and let the solution completely dissolve and cool before bringing to volume with deionized water.

13.3.9.2. Store: Room temperature

13.3.9.3. Expiration: 1 year

13.3.10. 100 mM Sodium acetate buffer, pH 4.5

13.3.10.1. Weigh approximately 11.72 g of sodium acetate trihydrate and add to a 2000 mL volumetric flask. Add approximately 1800 mL of deionized water. Add 6.5 mL glacial acetic acid. Bring to volume with deionized water. Mix. Adjust the pH to  $4.5 \pm 0.5$ . with sodium acetate or acetic acid if needed.

13.3.10.2. Alternatively, a commercial pH buffer pouch containing 5.86 g sodium acetate trihydrate can be used. Add 300 mL of deionized water to a 500 mL volumetric flask. Mix in the contents of the buffer pouch and shake/stir well. Add 3.24 mL of glacial acetic acid to the volumetric flask and dilute to the mark with deionized water. Check pH is  $4.5 \pm 0.5$ .

13.3.10.3. Store: Room temperature.

13.3.10.4. Expiration: 6 months.

13.3.11. 100 mM Sodium acetate buffer, pH 5.0

13.3.11.1. Weigh approximately 17.16 g of sodium acetate trihydrate and add to a 2000 mL volumetric flask. Add approximately 1800 mL of deionized water. Add 4.16 mL glacial acetic acid. Bring to volume with deionized water. Mix. Adjust the pH to  $5.0 \pm 0.5$ . with sodium acetate or acetic acid if needed.

13.3.11.2. Alternatively, a commercial pH buffer pouch containing 4.29 g sodium acetate trihydrate can be used. Add 300 mL of deionized water to a 500 mL volumetric flask. Mix in the contents of the buffer pouch and shake/stir well. Add 1.04 mL of glacial acetic acid to the volumetric flask and dilute to the mark with deionized water. Check pH is  $5.0 \pm 0.5$ .

13.3.11.3. Store: Room temperature.

13.3.11.4. Expiration: 6 months.



## 14. Evaluation of Results from Gas/Liquid Chromatography-Mass Spectrometry

### 14.1. Purpose

14.1.1. The GC-MS and LC-MS are composed of two major components: the gas or liquid chromatograph (GC or LC) and the mass spectrometer (MS). The LC utilizes liquid mobile phases and the stationary phase of an analytical column to facilitate the separation of complex mixtures while the GC utilizes a capillary column for separation. The dimensions (length, diameter, film thickness (GC)) 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 and LC-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 and LC-MS/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 designated characteristic ions 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 (both analytes and internal standards):

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





- 14.3.4.2. Case mass spectrum must be of comparable quality to a reference spectrum in mass assignment and intensity for applicable methods.
- 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 for applicable methods.
- 14.3.5. Qualitative identification is determined using characteristic retention time and mass spectral characteristics. Validated internal standards in each sample are used to identify the correct retention time of the drug. For SIM/MRM, all designated characteristic ions/ion transitions must be present. Ion intensity and ratios should be taken into consideration. Ion ratios should be within  $\pm 20\%$  of the average ratio from all calibrators used in the calibration response curve to meet acceptance criterion.
- 14.3.6. An analyte needs to have an acceptable internal standard to report qualitative results, both none detected and positive findings.
- 14.3.7. **Negative controls must be negative and positive controls must be positive.**

#### **14.4. Quantitative Analysis**

- 14.4.1. Qualitative identification described in sections 14.3 must be met.
- 14.4.2. Concentration of a drug is determined using linear or quadratic regression analysis. At least 4 calibrators for linear regression or at least 6 calibrators for quadratic regression and a blank blood sample must be used for each quantitation.  $R^2$  values of at least 0.99 should be attained following linear and quadratic regression analyses.
  - 14.4.2.1. A calibrator must have a concentration within  $\pm 20\%$  of the target, acceptable ion ratios, and retention time within  $\pm 2\%$  of the target. If a calibrator needs to be excluded from the curve, the action should be described and justified in the batch file.
  - 14.4.2.2. If at least 6 calibrators are included in the run for linear regression, up to two may be excluded if the response factor is not within  $\pm 20\%$  of the remaining calibrators, the calculated concentration is not within  $\pm 20\%$  of the target, retention time is not within  $\pm 2\%$  of the target, 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. If 7 calibrators are included in the run for quadratic regression, one calibrator may be excluded for the reasons stated above.
  - 14.4.2.3. If the lowest or highest calibrator needs to be excluded, the next lowest or highest calibrator becomes the LOQ as long as there is a control level between the next lowest/highest calibrator and the calibrator that comes immediately after. The revised calibration curve and controls need to meet the linearity and accuracy acceptance criteria as described in this section for the calibration curve to be considered valid.



14.4.2.3.1. If the lowest or highest calibrator needs to be excluded and the LQC or HQC, respectively, is outside of the revised calibration curve, hence not meeting the aforementioned criteria, the new upper limit will be the next control level (e.g., MQC or UTAK).

14.4.2.4. If LOQ changes due to exclusion of the lowest or highest calibrator, any samples meeting all acceptance criteria (e.g., ion ratio, retention time, etc.) other than being within the calibration curve will be re-analyzed (e.g., if the lowest calibrator at 5 ng/mL is excluded, a case sample with 3 ng/mL, 0.5 ng/mL or even 0.0 ng/mL will be re-analyzed if it meets other acceptance criteria). The samples with concentrations between the original LOQ and the new LOQ will be re-analyzed even if the ion ratios are out; however, if one or more ion ratios are out in the re-analysis, the sample will be deemed unsuitable.

14.4.3. Quantitative values above the highest calibrator must be reported as greater than the appropriate calibrator concentration if no dilution has been 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 or dilution factor 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  $\pm 20\%$  of the target concentration.

14.4.5. Quantitative analysis can be converted to qualitative analysis by reprocessing the data using the LOQ as the cut-off calibrator in a one-point curve forced through the zero. The concentration above LOQ will be considered positive. All appropriate positive controls must be positive and negative controls negative.

#### **14.5. Contamination**

14.5.1. Negative controls must be clear of significant peaks of interest (i.e., calculated concentration < 10% of LOQ or quantifier ion response < 10% of LOQ with acceptable ion ratios).

14.5.2. GC/LC methods include wash samples to clean the instrument; the injection frequency of the wash samples varies among the methods. Wash samples are not controls.

#### **14.6. Interference**

14.6.1. If an ion ratio fails for a case sample that has a quantified result above LOQ, the sample will be re-analyzed at least once to confirm that the interference is from the sample rather than from the analysis if there is sufficient sample volume. The re-analysis may not be possible if the sample volume is not sufficient.



- 14.6.1.1. If the re-analysis still shows unacceptable interference (i.e., the ion ratio still fails), the result will be reported as unsuitable for analysis.
- 14.6.2. Re-analysis will not be performed if a sample is likely negative for the analyte of interest (e.g., the quantified result below LOQ, instrument software selecting a wrong peak, one or more ion/ion transition missing). In this case, the result will be considered none detected.
- 14.6.3. Significantly overlapping peaks, even if the ion ratios are acceptable, may trigger re-analysis and subsequent inconclusive results if the peaks are still overlapped in the re-analysis. The criteria for re-analysis may include approximately >20% overlapping of the quantifier (either analyte of interest or internal standard), multiple overlapping peaks for the analyte, or other reasons as stated in case records. Minor interfering peaks are deemed acceptable unless there is a comment indicating otherwise on chromatograms.

#### **14.7. Reinjection**

- 14.7.1. 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.
- 14.7.2. Case samples: If a batch run has been completed, 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.
  - 14.7.2.1. 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.
- 14.7.3. Calibrators or control samples: the samples with known analyte concentrations can be re-injected if the samples have initially mis-injected (i.e., analyte as well as internal standard responses are unusually low).
- 14.7.4. If the instrument needs to be switched in the middle of analysis, the entire batch samples will be reinjected on the other instrument.

#### **14.8. Carryover**

- 14.8.1. If a case sample is greater than the ULOQ, it may be necessary to dilute the sample as stated in section 14.4.3.
- 14.8.2. The sample immediately following the case sample greater than the ULOQ needs to be evaluated for the potential of carryover unless validation demonstrated insignificant carryover with a concentration greater than ULOQ; if so, the carryover limit follows the value listed in the assay specific SOP. 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.

14.8.2.1. 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.

14.8.3. If a wash is injected immediately following the case sample greater than the validated carryover limit, potential carryover is evaluated using the wash sample. Carryover is deemed negligible if the wash sample has an analyte response no greater than 10% of the cut-off calibrator or lower LOQ.

14.8.4. If negative control sample shows carryover from calibrator(s), all calibrators, controls, and positive case samples can be reinjected after an appropriate number of washes between each injection. The reinjected negative control must be clear of significant peaks of interest, demonstrating additional washes were effective and the reinjected calibrators/controls must meet the acceptance criteria in order for the reinjected case results to be reported.

#### **14.9. Manual Integration in Chromatograms**

14.9.1. Manual integration may be necessary in rare occasions where a chromatogram is not properly integrated by the instrument data system (i.e., partial integration of an analyte peak either vertically or horizontally) because of small analyte peak size, fluctuating baseline, or other issues.

14.9.2. Manual integration cannot be used to affect quantitative values of the analyte or to adjust ion ratios when the data system properly integrates the peak.

14.9.3. If manual integration is performed,

14.9.3.1. The original chromatogram integrated by the instrument data system shall be retained and included in the batch file.

14.9.3.2. The manually integrated chromatogram shall include the reason for the manual integration, the person performing the integration, and the date/time of the integration (i.e., the date/time of the last calibration update).



## 15. Drug Screen and Qualitative Confirmation by Gas Chromatography-Mass Spectrometry

### 15.1. Purpose

15.1.1. Analysis is performed in full-scan mode for screen and qualitative confirmation using solid phase extraction (SPE) and gas chromatography-mass spectrometry. Mepivacaine is used as an internal standard.

### 15.2. Scope

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

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

### 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: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix, and then add 78 mL methylene chloride. Cap and mix well. Prepare fresh daily.
- 15.3.4. Acidic methanol (1% HCl in methanol)
- 15.3.5. Methanol
- 15.3.6. Ethyl acetate
- 15.3.7. Deionized (DI) water
- 15.3.8. Blank blood (preserved with potassium oxalate and sodium fluoride)
- 15.3.9. Blank urine

### 15.4. Equipment and Materials

- 15.4.1. Air displacement pipettes (1000-5000  $\mu$ L; 100-1000  $\mu$ L; 20-200  $\mu$ L; 2-20  $\mu$ L)
- 15.4.2. Repeater pipette
- 15.4.3. Cerex<sup>®</sup> 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: 30 m DB-5MS Agilent J&W Column (or equivalent), 0.25 mm id X 0.25  $\mu$ m film thickness. The flow rate is 1.52 mL/min with an injection volume of 1  $\mu$ L in splitless mode. NOTE: This is a retention time locked method to SKF-525a (Proadifen) at 17.14 minutes, so the pressure and flow are subject to change.

15.5.1.1.1. **Retention time locking solution: 1 mg/mL SKF-525a (Proadifen) – Add 10 mg SKF-525a (Proadifen) to methanol in a 10 mL volumetric flask and QS. Expiration: 1 year.**

15.5.1.2. GC-MS: Agilent 7890B-5977B MSD or 8890-5977B MSD

Initial Temperature:	100 °C hold for 1 minute 10 °C/min to 325 °C for 5 minutes
Total Run Time:	28.5 minutes
Injector Temperature:	280 °C
Interface Temperature:	300 °C
MS Quads:	150 °C
MS Source:	230 °C

15.5.1.3. Full-Scan acquisition: BSD.M

### 15.5.2. Wash solvents for the autosampler

15.5.2.1. **Methanol and ethyl acetate are used as the wash solvents. A minimum of 3 pre and 3 post injection rinses are performed. Each rinse cycle consists of 3 methanol rinses followed by 3 ethyl acetate rinses.**

## 15.6. Standards and Solutions

### 15.6.1. Qualitative Working Standards (for blood and urine)

15.6.1.1. This analysis requires preparation of four solutions.

15.6.1.1.1. Preparation of the Cut-off Calibrator Solution (blood): add 100  $\mu$ L of 1 mg/mL CRM of ketamine, diphenhydramine, doxylamine, tramadol, venlafaxine, brompheniramine, methadone, dextromethorphan, and cyclobenzaprine, add 50  $\mu$ L of 1 mg/mL CRM of imipramine and sertraline then add 20  $\mu$ L of 1 mg/mL CRM of chlorpheniramine, amitriptyline, and zolpidem to a 25 mL volumetric flask. Bring to volume with methanol. Refer to the table below for the composition and concentration of the current drug mixture.

15.6.1.1.2. Preparation of the Positive Control Solution (blood): add 100  $\mu$ L of 1 mg/mL CRM of ketamine, diphenhydramine, doxylamine, tramadol, venlafaxine, brompheniramine, methadone, dextromethorphan, and cyclobenzaprine, add 50  $\mu$ L of 1 mg/mL CRM of imipramine and sertraline then add 20  $\mu$ L of 1 mg/mL CRM of chlorpheniramine, amitriptyline, and zolpidem to a 25 mL volumetric flask. Bring to



volume with methanol. Refer to the table below for the composition and concentration of the current drug mixture.

Analyte	Blood Cut-off Calibrator		Positive Control	
	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)
Ketamine	50	4	100	4
Diphenhydramine	50	4	100	4
Doxylamine	50	4	100	4
Tramadol	50	4	100	4
Venlafaxine	50	4	100	4
Brompheniramine	50	4	100	4
Methadone	50	4	100	4
Dextromethorphan	50	4	100	4
Cyclobenzaprine	50	4	100	4
Imipramine	25	2	50	2
Sertraline	25	2	50	2
Chlorpheniramine	10	0.8	20	0.8
Amitriptyline	10	0.8	20	0.8
Zolpidem	10	0.8	20	0.8

15.6.1.1.3. Preparation of the Cut-off Calibrator Solution (urine): add 100 µL of a 1mg/mL CRM of norketamine, venlafaxine, and methadone, add 50 µL of 1 mg/mL CRM of diphenhydramine, doxylamine, dextromethorphan, amitriptyline, and sertraline and add 20 µL of 1 mg/mL CRM of tramadol, chlorpheniramine, brompheniramine, imipramine, cyclobenzaprine, and zolpidem to a 25 mL volumetric flask. Bring to volume with methanol.

15.6.1.1.4. Preparation of the Positive Control Solution (urine): add 100 µL of a 1mg/mL CRM of norketamine, venlafaxine, and methadone, add 50 µL of 1 mg/mL CRM of diphenhydramine, doxylamine, dextromethorphan, amitriptyline, and sertraline and add 20 µL of 1 mg/mL CRM of tramadol, chlorpheniramine, brompheniramine, imipramine, cyclobenzaprine, and zolpidem to a 25 mL volumetric flask. Bring to volume with methanol.



Analyte	Urine Cut-off Calibrator		Positive Control	
	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)
Norketamine	50	4	100	4
Venlafaxine	50	4	100	4
Methadone	50	4	100	4
Diphenhydramine	25	2	50	2
Doxylamine	25	2	50	2
Dextromethorphan	25	2	50	2
Amitriptyline	25	2	50	2
Sertraline	25	2	50	2
Tramadol	10	0.8	20	0.8
Chlorpheniramine	10	0.8	20	0.8
Brompheniramine	10	0.8	20	0.8
Imipramine	10	0.8	20	0.8
Cyclobenzaprine	10	0.8	20	0.8
Zolpidem	10	0.8	20	0.8

15.6.2. Internal Standard for Screen and Qualitative Confirmation

15.6.2.1. This analysis requires the preparation of two solutions

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

15.6.2.1.2. Preparation of the 10 µg/mL Internal Standard: add 500 µL of the 1 mg/mL Internal Standard Stock Solution to a 50 mL volumetric flask and bring to volume with methanol.

**15.7. Fortification Guide**

15.7.1. Calibrator and Control (Drug Screen and Qualitative Confirmation)

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (ng/mL)	Volume Added (µL)	Volume of Methanol Added (µL)
2	Cut-off Calibrator (10-50)	Mixed (0.8-4)	25	25
2	Positive QC (20-100)	Mixed (0.8-4)	50	0

15.7.2. Internal Standard

Volume of Matrix (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)
2	100	10	20





### 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. Pipet 2 mL of drug-free blood/urine for matrix blank, negative control, positive controls, and case samples into the appropriately labeled tubes.
- 15.8.4. Add corresponding drug standard to cut-off calibrator and positive controls.
- 15.8.5. Using a repeater pipette, add 20  $\mu$ L of internal standard (mepivacaine) to each sample to obtain the final concentration of 100 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.
- 15.8.9. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
- 15.8.10. Add 2 mL of deionized water and aspirate.
- 15.8.11. Add 1 mL of 1 M acetic acid and aspirate. Dry columns under full pressure for 5 minutes.
- 15.8.12. Add 2 mL of methanol and aspirate. Dry columns under full pressure for 5 minutes.
- 15.8.13. Elute drugs by adding 2 mL of elution solvent, prepared fresh daily.
- 15.8.14. Add 30  $\mu$ L of acidic methanol to all elution tubes.
- 15.8.15. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi.
- 15.8.16. Reconstitute in 60  $\mu$ L of ethyl acetate using a repeater pipette, vortex, and transfer to appropriately labeled autosampler vials with inserts and cap tightly.
- 15.8.17. Inject 1  $\mu$ L onto the GC-MS using BSD.M method. Data analysis method for screen is BSD\_SCN.M and BSD\_CNF.M for qualitative confirmation.

### 15.9. Sequence Table

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

#### **BSD Screen sequence example (blood and urine):**

Matrix Blank  
Negative Control  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control



**BSD Confirmation sequence example (blood and urine):**

Matrix Blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control

**15.10. Data Analysis**

15.10.1. GC-MS Drug Screen using the drug analysis method (BSD\_SCN.m) – Interpretation of results using the AMDIS Deconvolution Reporting Software (DRS) with the AMDIS library.

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

15.10.1.1.1. The match factor should be within the range of 60 to 100, based on the AMDIS library. 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.

15.10.1.1.2. 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 other means of peak identification (i.e., screener or library searching).

15.10.1.1.3. The following is a list of characteristic ions for each analyte. These characteristic ions must be present for an analyte in its extracted spectrum to be considered as a positive finding. This requirement is for case samples only and does not apply to controls and calibrators.

15.10.1.1.3.1. Note: depending on their abundance, characteristic ion numbers may not be listed in extracted spectra. In such instances, supplemental spectra data showing their presence in the case sample will be printed.

15.10.1.1.3.2. Additionally, monitor that abundant ions approximately match with the library's abundant ions.



Analyte	Molecular Ion	Characteristic Ions
Norketamine*	223	131, 166
Ketamine#	237	152, 180, 209
Diphenhydramine	255	73, 165
Doxylamine	270	71, 167
Tramadol	263	135, 188, 263
Chlorpheniramine	274	167, 203
Mepivacaine	246	70, 98
Venlafaxine	277	134, 179
Brompheniramine	435	167, 247
Methadone	309	72, 165, 294
Dextromethorphan	271	150, 214, 271
Amitriptyline	277	115, 202
Imipramine	280	193, 234, 280
Cyclobenzaprine	275	215
Sertraline	306	159, 274
Zolpidem	307	219, 235, 307

\*Urine only; #blood only

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

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	All characteristic ions as identified in 15.10.1.1.3 are present at the <b>approximately</b> appropriate relative abundance (visually compared with the library).	The characteristic ions are missing, including the ones with a significant relative abundance and/or with the <b>approximately</b> appropriate abundance.
Library Match	60-100%	Less than 60%

15.10.2. GC-MS qualitative confirmation using the drug analysis method (BSD\_CNFB.m/BSDFU.m)

15.10.2.1. The criteria from section 15.10.1.1.1 – 15.10.1.1.4 must be met.

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

15.10.2.2.1. 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 a decision on acceptability.

15.10.2.2.2. Review chromatography of positive controls throughout the batch for unusual peak shape, tailing, fronting, splitting, or interferences. Questionable performance must be reviewed with the supervisor/manager for acceptability and documented in the case record.

15.10.2.2.3. 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.).

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

#### 15.10.2.3. Batch Review of Controls

15.10.2.3.1. For qualitative confirmation analysis, the compounds contained in these controls will be identified using DRS and Chemstation.

15.10.2.3.2. All drugs in positive controls must be present and above the cut-off concentrations based on the one-point curve using the cut-off calibrator and forced through the zero.

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

15.10.2.3.4. All negative controls must be negative for reporting analyte(s) and have acceptable internal standard response. If a negative control is positive for reporting analyte(s), it must be evaluated for potential contamination to ensure the results of case samples are not compromised.

#### 15.10.2.4. Case Sample Review

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

15.10.2.4.2. 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.

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

15.10.2.4.4. Batch outcomes not described in 15.10.2.4 will be evaluated on a case-by-case basis and acceptability will be determined by toxicology management **or designee**.

### 15.10.3. Reinjection

15.10.3.1. Reinjections can be made up to 24 hours or longer after the completion of the initial injection of the particular sample, as indicated in the latest validation study.

## 15.11. References

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: BSD.M



## 16. Amphetamines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 16.1. Purpose

16.1.1. A targeted analysis is performed for confirmatory analysis of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA) by solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Drugs are isolated from the matrix using a basic extraction. Deuterated internal standard and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 16.2. Scope

16.2.1. This procedure describes a confirmatory analysis of amphetamines in biological specimens including blood. Urine confirmations are reported only qualitatively.

### 16.3. Reagents and Solvents

- 16.3.1. 100 mM Acetic acid
- 16.3.2. 100 mM Phosphate buffer, pH 6.0
- 16.3.3. Elution Solvent: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. **In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix and then add 78 mL of methylene chloride. Cap and thoroughly mix. Prepare fresh daily.**
- 16.3.4. Mobile Phase A: H<sub>2</sub>O with 0.1% formic acid. Add 1 mL formic acid to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).
- 16.3.5. Mobile Phase B (organic, may be in another position in method report): LC/MS grade methanol.
- 16.3.6. Acidic methanol (1% HCl in methanol)
- 16.3.7. Methanol
- 16.3.8. Utak **Drugs** of Abuse Level 1
- 16.3.9. Deionized (DI) water
- 16.3.10. Blank blood (preserved with potassium oxalate and sodium fluoride)
- 16.3.11. Blank urine

### 16.4. Equipment and Materials

- 16.4.1. Air displacement pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)
- 16.4.2. Repeater pipette
- 16.4.3. UCT Clean Screen® DAU, 6 mL columns, 200 mg
- 16.4.4. Positive pressure SPE manifold
- 16.4.5. Analytical/Top-loading balance
- 16.4.6. pH meter
- 16.4.7. Evaporator
- 16.4.8. Vortex mixer
- 16.4.9. **Centrifuge**



## 16.5. Instrumentation

### 16.5.1. LC Parameters

16.5.1.1. Column: Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7 μm

16.5.1.2. Agilent 1290 Infinity II LC system

Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 0.1% formic acid	
Mobile Phase B	Methanol	
Flow Rate	0.6 mL/min	
Needle Wash	5 seconds	
Injection Volume	1 μL	
Gradient	Initial	10% B
	0.5 minutes	10% B
	2.5 minutes	50 % B
	2.51 minutes	90% B
	4.0 minutes	90% B
	Post time	2.5 minutes

### 16.5.2. MS/MS Parameters

16.5.2.1. Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	300°C
Gas Flow	6 L/min
Nebulizer Pressure	40 psi
Sheath Gas Heater	400°C
Sheath Gas Flow	12 L/min
Capillary	3,000 V

16.5.2.2. MRM acquisition: AMP.m

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor (Calibration model)
+/-Amphetamine-d11	147.2	<b>130.2</b>	66	8	1.39	1/x <sup>2</sup> (quadratic)
		98.1		24		
		70.1		48		
+/-Amphetamine	136.1	<b>119.1</b>	69	8	1.48	
		91.1		20		
		65.1		44		



+/-Methamphetamine-d14	164.2	<b>130.1</b> 98.1 70.1	91	12 24 52	1.59	1/x (quadratic)
+/-Methamphetamine	150.1	<b>119.1</b> 91.1 65.1	76	12 20 48	1.68	
+/- MDA-d5	185.1	<b>168.1</b> 138.1 110.1	76	8 20 24	1.72	1/x <sup>2</sup> (linear)
+/-MDA	180.1	<b>163.1</b> 133.1 79.1	76	8 16 36	1.74	
+/-MDMA-d5	199.1	<b>165.1</b> 135.1 107.1	85	12 20 28	1.83	1/x <sup>2</sup> (linear)
+/-MDMA	194.1	<b>163.0</b> 105.1 77.1	82	12 28 52	1.84	
+/-MDEA-d6	214.2	<b>166.1</b> 136.1 108.1	94	12 24 32	2.15	1/x <sup>2</sup> (linear)
+/-MDEA	208.1	<b>163.1</b> 105.1 77.1	88	12 28 56	2.16	
*Quantifier ion in bold **Retention times will vary						

## 16.6. Standards and Solutions

### 16.6.1. Amphetamines Working Standards for Calibrators (for blood and urine)

16.6.1.1. This analysis requires preparation of three solutions. The solutions contain (±)-amphetamine, (±)-methamphetamine, (±)- MDA, (±)-MDMA, (±)-MDEA at 10 µg/mL, 2.5 µg/mL, and 0.2 µg/mL.

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

16.6.1.1.2. Preparation of the 2.5 µg/mL Amphetamines Calibrator: add 25 µL of 1 mg/mL CRM of (±)-amphetamine, (±)-methamphetamine, (±)- MDA, (±)-MDMA, (±)-MDEA to a 10 mL volumetric flask and bring to volume with methanol.

16.6.1.1.3. Preparation of the 0.2 µg/mL Amphetamines Calibrator: add 800 µL of 2.5 µg/mL Amphetamines Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

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

16.6.2.1. This analysis requires preparation of three solutions. The three solutions contain (±)-methamphetamine and (±)-MDMA at 10 µg/mL, 5 µg/mL and 0.5



µg/mL and (±)-amphetamine, (±)-MDA, (±)-MDEA at 5 µg/mL, 5 µg/mL and 0.5 µg/mL.

16.6.2.1.1. Preparation of the 5/10 µg/mL Amphetamines Control: add 50 µL of 1 mg/mL CRM of (±)-amphetamine, (±)-MDA, (±)-MDEA and 100 µL of 1 mg/mL CRM of (±)-methamphetamine and (±)-MDMA to a 10 mL volumetric flask and bring to volume with methanol.

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

16.6.2.1.3. Preparation of the 0.5 µg/mL Amphetamines Control: add 1 mL of the 5 µg/mL Amphetamines Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

### 16.6.3. Amphetamines Internal Standard (for blood and urine)

16.6.3.1. This solution contains (±)-amphetamine-d11, (±)-methamphetamine-d14, (±)-MDA-d5, (±)-MDMA-d5, (±)-MDEA-d6 at a concentration of 1 µg/mL.

16.6.3.1.1. Preparation of the 1 µg/mL Amphetamines Internal Standard: add 100 µL of 100 µg/mL CRM of (±)-amphetamine-d11, (±)-methamphetamine-d14, (±)-MDA-d5, (±)-MDMA-d5, and (±)-MDEA-d6 to a 10 mL volumetric flask and bring to volume with methanol.

## 16.7. Fortification Guide

### 16.7.1. 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)
0.5	10	0.2	25	25
0.5	20	0.2	50	0
0.5	50	2.5	10	40
0.5	100	2.5	20	30
0.5	250	2.5	50	0
0.5	500	10	25	25
0.5	1000*	10	50	0

\*(±)-methamphetamine and (±)-MDMA only

### 16.7.2. 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)
0.5	25	0.5	25	25
0.5	400/800	5/10	40	10

(±)-amphetamine, (±)-MDA, and (±)-MDEA/(±)-methamphetamine and (±)-MDMA





### 16.7.3. 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)
0.5	10	0.2 (Calibrator)	25	25
0.5	20	0.5 (QC)	20	30

### 16.7.4. Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	50	1	25	0

## 16.8. Extraction Procedure

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

16.8.2. Label all round bottom glass tubes accordingly.

16.8.3. Pipet 0.5 mL of drug-free blood/urine for matrix blank, negative control, calibrators, and positive controls into the appropriately labeled tubes.

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

16.8.5. Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50 µL of methanol. Vortex.

16.8.6. Using a repeater pipette, add 25 µL of internal standard to each sample (except matrix blank) to obtain the final concentration of 50 ng/mL. Vortex.

16.8.7. Add 1 mL of 100 mM phosphate buffer, pH 6.0 to each tube. Vortex.

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

16.8.8.1. Add 3 mL methanol and aspirate.

16.8.8.2. Add 3 mL deionized water and aspirate.

16.8.8.3. Add 1 mL of 100 mM phosphate buffer, pH 6.0 and aspirate.

16.8.8.4. Pour samples into appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.

16.8.8.5. Add 3 mL of deionized water and aspirate.

16.8.8.6. Add 1 mL of 100 mM acetic acid and aspirate.

16.8.8.7. Add 3 mL of methanol and aspirate. Dry columns under full pressure for 5 minutes.

16.8.8.8. Elute amphetamines by adding 3 mL of elution solvent, prepared fresh daily.

16.8.9. Add 50 µL of acidic methanol to all tubes.

16.8.10. Evaporate eluates to dryness at approximately 40 °C under nitrogen at 20 psi (2.9 L/min). NOTE: avoid over drying.

16.8.11. Reconstitute extracts in 200 µL of 90:10 LC-MS grade water:methanol. Vortex.



- 16.8.12. Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 16.8.13. Load autosampler vials on the LC-MS/MS and inject 1  $\mu$ L using the AMP.m method.

### 16.9. Sequence Table

16.9.1. 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).

16.9.1.1. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.

#### AMP sequence example:

Blood	Urine
Matrix blank	Matrix blank
10 ng/mL Calibrator	Cut-off Calibrator
20 ng/mL Calibrator	Negative Control
50 ng/mL Calibrator	Positive Control
100 ng/mL Calibrator	10 Case Samples
250 ng/mL Calibrator	Positive Control
500 ng/mL Calibrator	10 Case Samples
1000 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	

### 16.10. Data Analysis

#### 16.10.1. Blood

16.10.1.1. The calibration curve for ( $\pm$ )-amphetamine, ( $\pm$ )-MDA, and ( $\pm$ )-MDEA ranges from 10-500 ng/mL, and for ( $\pm$ )-methamphetamine and ( $\pm$ )-MDMA from 10-1000 ng/mL.

16.10.1.1.1. Note: because the amphetamine method consists of 6 calibrators and uses a quadratic calibration model, none of its calibrators can be dropped.

16.10.1.2. The low-quality control (LQC) for amphetamines is 25 ng/mL.



- 16.10.1.2.1. An external Utak quality control is used for the mid quality control (MQC). An in-house MQC of 100 ng/mL may be used if Utak is unavailable or if the concentration cannot be verified.
- 16.10.1.2.2. The high-quality control (HQC) for (±)-amphetamine, (±)-MDA, and (±)-MDEA is 400 ng/mL, and for (±)-methamphetamine and (±)-MDMA 800 ng/mL.
- 16.10.2. Urine
  - 16.10.2.1. The cut-off calibrator for amphetamines is 10 ng/mL forced through zero.
  - 16.10.2.2. The positive control (PQC) for amphetamines is 20 ng/mL.
- 16.10.3. **Reinjection**
  - 16.10.3.1. Reinjections can be made up to 48 hours for blood samples or 72 hours for urine samples after the completion of the initial injection of the particular sample(s).
- 16.10.4. **Carryover**
  - 16.10.4.1. Carryover analysis will be performed if case sample concentration is greater than 2000 ng/mL.
- 16.10.5. **Dilutions**
  - 16.10.5.1. It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

#### **16.11. References**

- 16.11.1. Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 12th ed. Seal Beach, CA: Biomedical Publications, 2020.
- 16.11.2. Hudson, J. et al., Amphetamines, Phentermine, and Designer Stimulant Quantitation Using an Agilent 6430 LC/MS/MS. Agilent Technologies Inc., Application Note 5991-5059EN, 2015.
- 16.11.3. Levine, Barry and Kerrigan, Sarah, eds. "Amphetamines/Sympathomimetic Amines." Principles of Forensic Toxicology, 5th ed. Springer, Switzerland, 2020, 449-466.
- 16.11.4. United Chemical Technologies Inc. Sympathomimetic Amines in Blood, Plasma/Serum, and Urine by LC-MS/MS or GC-MS Clean Screen® Extraction Column. Application Note, 2016, 149-152.
- 16.11.5. Method File: AMP.m



## 17. Benzodiazepines and Zolpidem Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 17.1 Purpose

17.1.1 A targeted analysis is performed for confirmatory analysis of 7-aminoclonazepam, zolpidem,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, temazepam, and diazepam using solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 17.2 Scope

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

### 17.3 Reagents and Solvents

- 17.3.1 100 mM Sodium acetate buffer, pH 4.5
- 17.3.2 100 mM Sodium acetate buffer, pH 5.0
- 17.3.3  $\beta$ -glucuronidase solution from Helix pomatia
- 17.3.4 Wash solvent: 100 mM Sodium acetate buffer, pH 4.5 with 20% acetonitrile. In a 100 mL graduated cylinder add 80 mL 0.1 M Sodium acetate buffer, pH 4.5, and 20 mL acetonitrile. Cap and thoroughly mix. Prepare fresh daily.
- 17.3.5 Elution solvent: Ethyl acetate with 2% ammonium hydroxide. In a 100 mL graduated cylinder add 98 mL ethyl acetate and 2 mL ammonium hydroxide. Cap and thoroughly mix. Prepare fresh daily.
- 17.3.6 Mobile Phase A: H<sub>2</sub>O with 0.1% formic acid. Add 1 mL formic acid to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).
- 17.3.7 Mobile Phase B (organic, may be in another position in method report): LC/MS grade acetonitrile.
- 17.3.8 Acetonitrile
- 17.3.9 Methanol
- 17.3.10 Hexane
- 17.3.11 Deionized (DI) water
- 17.3.12 Utak Benzodiazepines Plus 100 Control
- 17.3.13 Blank blood (preserved with potassium oxalate and sodium fluoride)
- 17.3.14 Blank urine

### 17.4 Equipment and Materials

- 17.4.1 Air displacement pipettes (1000-5000  $\mu$ L; 100-1000  $\mu$ L; 20-200  $\mu$ L; 2-20  $\mu$ L)
- 17.4.2 Repeater pipette



- 17.4.3 Pasteur pipettes
- 17.4.4 Analytical/Top-loading balance
- 17.4.5 pH meter
- 17.4.6 Evaporator
- 17.4.7 Vortex mixer
- 17.4.8 Centrifuge
- 17.4.9 Heating block
- 17.4.10 Cerex® Clin II, 6 mL columns, 50 mg
- 17.4.11 Positive pressure SPE manifold

## 17.5 Instrumentation

### 17.5.1 LC Parameters

17.5.1.1 Column: Agilent Poroshell 120 EC-C18 2.1 x 75 mm, 2.7 µm

17.5.1.2 Agilent 1290 Infinity II LC system

Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 0.1% formic acid	
Mobile Phase B	Acetonitrile	
Flow Rate	0.5 mL/min	
Needle Wash	5 seconds	
Injection Volume	2 µL	
Gradient	Initial	10% B
	4.0 minutes	30% B
	8.0 minutes	40 % B
	8.5 minutes	95% B
	10.5 minutes	95% B
	Stop time	11.0 minutes
	Post time	3.0 minutes

### 17.5.2 MS/MS Parameters

17.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	300°C
Gas Flow	8 L/min
Nebulizer Pressure	40 psi
Sheath Gas Heater	250°C
Sheath Gas Flow	10 L/min
Capillary	3,500 V



17.5.3 MRM acquisition: BNZ.m (Dynamic MRM (dMRM))

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT (min)**	Weighting Factor (Calibration model)
7-Aminoclonazepam-d4	290.1	<b>226.0</b> 121.0 94.0	135	28 36 48	2.14	1/X (linear)
7-Aminoclonazepam	286.1	<b>250.0</b> 121.0 222.0	147	20 36 28	2.15	
Zolpidem-d6	314.2	<b>235.0</b> 236.1 65.1	163	40 32 80	3.75	1/X (quadratic)
Zolpidem	308.2	<b>235.0</b> 236.1 65.1	153	40 32 80	3.78	
α-Hydroxyalprazolam-d5	330.1	<b>302.0</b> 210.0	135	32 56	5.96	1/X (linear)
α-Hydroxyalprazolam	325.1	<b>297.0</b> 216.0 205.0	150	28 44 52	6.00	
Oxazepam-d5	292.1	<b>246.0</b> 274.0 109.0	119	24 16 40	6.17	1/X (linear)
Oxazepam	287.1	<b>241.0</b> 268.9 104.0	119	24 16 40	6.22	
Nordiazepam-d5	276.1	<b>140.0</b> 213.0 165.0	138	32 32 32	6.39	1/X (linear)
Nordiazepam	271.1	<b>140.0</b> 164.9 77.0	150	32 32 72	6.48	
Clonazepam-d4	320.1	<b>274.0</b> 218.0	156	28 48	6.45	1/X <sup>2</sup> (linear)
Clonazepam	316.1	<b>270.0</b> 214.0 241.0 <sup>#</sup>	135	28 44 40	6.50	
Lorazepam-d4	327.0	<b>280.9</b> 308.9 233.0	128	24 16 36	6.52	1/X (linear)
Lorazepam	321.0	<b>228.9</b> 302.9	122	36 16	6.55	
Alprazolam-d5	314.1	<b>286.0</b> 210.1	166	32 52	6.61	1/X (linear)



		279.1		28		
Alprazolam	309.1	<b>205.0</b>	160	52	6.67	
		281.0		28		
		151.0		80		
Temazepam-d5	306.1	<b>260.0</b>	122	24	7.29	1/X (linear)
		288.0		12		
		176.9		48		
Temazepam	301.1	<b>255.0</b>	116	24	7.34	
		283.0		12		
		176.9		48		
Diazepam-d5	290.1	<b>198.0</b>	141	36	8.03	1/X (quadratic)
		154.0		32		
		227.1		32		
Diazepam	285.1	<b>193.0</b>	163	36	8.13	
		154.0		32		
		89.0		80		
*Quantifier ion in bold **Retention times will vary #UTAK controls may not use this transition due to interference						

## 17.6 Standards and Solutions

### 17.6.1 Benzodiazepines/Zolpidem Working Standards for Calibrators (for blood and urine)

17.6.1.1 This analysis requires preparation of three solutions. The solutions contain 7-aminoclonazepam, zolpidem,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, temazepam, and diazepam at 5  $\mu\text{g/mL}$ , 1  $\mu\text{g/mL}$ , and 0.1  $\mu\text{g/mL}$ .

17.6.1.1.1 Preparation of the 5  $\mu\text{g/mL}$  Benzodiazepines/Zolpidem Calibrator: add 50  $\mu\text{L}$  of each 1 mg/mL CRM of 7-aminoclonazepam, zolpidem,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, temazepam, and diazepam to a 10 mL volumetric flask and bring to volume with methanol.

17.6.1.1.2 Preparation of the 1  $\mu\text{g/mL}$  Benzodiazepines/Zolpidem Calibrator: add 2 mL of 5  $\mu\text{g/mL}$  Benzodiazepines Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

17.6.1.1.3 Preparation of the 0.1  $\mu\text{g/mL}$  Benzodiazepines/Zolpidem Calibrator: add 1 mL of 1  $\mu\text{g/mL}$  Benzodiazepines Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

### 17.6.2 Benzodiazepines/Zolpidem Working Standards for Quality Controls (Blood)

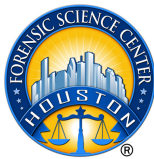
17.6.2.1 This analysis requires preparation of five solutions.

17.6.2.1.1 Preparation of the 10  $\mu\text{g/mL}$  Benzodiazepines/Zolpidem Control Stock Solution: add 100  $\mu\text{L}$  of each 1 mg/mL CRM of 7-aminoclonazepam, zolpidem,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam,



- lorazepam, alprazolam, temazepam, and diazepam to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.2.1.2 Preparation of the 5/2.5  $\mu\text{g}/\text{mL}$  Mixed Benzodiazepines/Zolpidem Control Solution: add 50  $\mu\text{L}$  of each 1 mg/mL CRM of 7-aminoclonazepam,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, temazepam, and diazepam and 25  $\mu\text{L}$  of 1 mg/mL CRM of zolpidem to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.2.1.3 Preparation of the 1  $\mu\text{g}/\text{mL}$  Benzodiazepines/Zolpidem Control Solution: add 1 mL of 10  $\mu\text{g}/\text{mL}$  Benzodiazepines/Zolpidem Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.2.1.4 Preparation of the 10  $\mu\text{g}/\text{mL}$  7-aminoclonazepam Control Stock Solution: add 100  $\mu\text{L}$  of 1 mg/mL CRM of 7-aminoclonazepam to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.2.1.5 Preparation of the 1  $\mu\text{g}/\text{mL}$  7-aminoclonazepam Control Solution: add 1 mL of the 10  $\mu\text{g}/\text{mL}$  7-aminoclonazepam Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.3 Benzodiazepines/Zolpidem Working Standards for Quality Controls (Urine)
- 17.6.3.1.1 Preparation of the 0.1  $\mu\text{g}/\text{mL}$  Benzodiazepines/Zolpidem Control Solution: add 1 mL of 1  $\mu\text{g}/\text{mL}$  Benzodiazepines/Zolpidem Control Solution to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.3.1.2 Preparation of 1  $\mu\text{g}/\text{mL}$  Benzodiazepines Hydrolysis Control Stock: add 100  $\mu\text{L}$  of 100  $\mu\text{g}/\text{mL}$  CRM of oxazepam-glucuronide to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.3.1.3 Preparation of 0.1  $\mu\text{g}/\text{mL}$  Benzodiazepines Hydrolysis Control: add 1 mL of 1  $\mu\text{g}/\text{mL}$  Benzodiazepines Hydrolysis Control to a 10 mL volumetric flask and bring to volume with methanol.
- 17.6.4 Benzodiazepines/Zolpidem Internal Standard
- 17.6.4.1 This solution contains 7-aminoclonazepam-d<sub>4</sub>, zolpidem-d<sub>6</sub>,  $\alpha$ -hydroxyalprazolam-d<sub>5</sub>, oxazepam-d<sub>5</sub>, nordiazepam-d<sub>5</sub>, clonazepam-d<sub>4</sub>, lorazepam-d<sub>4</sub>, alprazolam-d<sub>5</sub>, temazepam-d<sub>5</sub>, and diazepam-d<sub>5</sub> at 1  $\mu\text{g}/\text{mL}$ .
- 17.6.4.1.1 Preparation of the 1  $\mu\text{g}/\text{mL}$  Benzodiazepines/Zolpidem Internal Standard: add 10  $\mu\text{L}$  of each 1 mg/mL or 100  $\mu\text{L}$  of each 100  $\mu\text{g}/\text{mL}$  CRM of 7-aminoclonazepam-d<sub>4</sub>, zolpidem-d<sub>6</sub>,  $\alpha$ -hydroxyalprazolam-d<sub>5</sub>, oxazepam-d<sub>5</sub>, nordiazepam-d<sub>5</sub>, clonazepam-d<sub>4</sub>, lorazepam-d<sub>4</sub>, alprazolam-d<sub>5</sub>, temazepam-d<sub>5</sub>, and diazepam-d<sub>5</sub> 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)
0.5	10	0.1	50	0
0.5	20	1	10	40
0.5	50	1	25	25
0.5	100	1	50	0
0.5	150	5	15	35
0.5	250	5	25	25
0.5	500*	5	50	0

\*Benzodiazepines only

### 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)
0.5	30	1	15	35
0.5	80*	1	40	10
0.5	400/200**	5/2.5	40	10

\*7-aminoclonazepam only; \*\*Zolpidem only

### 17.7.3 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)
0.5	5.0	0.1 (Calibrator)	25	25
0.5	10	0.1 (QC)	50	0
0.5	10	0.1 (Hydrolysis QC)	50	0

### 17.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	50	1	25	0

## 17.8 Extraction Procedure

### 17.8.1 Blood Extraction

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

17.8.1.2 Label all round bottom glass tubes accordingly.

17.8.1.3 Pipet 0.5 mL of drug-free blood for matrix blank, negative control, calibrators, and positive controls into the appropriately labeled tubes.



- 17.8.1.4 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 17.8.1.5 Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50  $\mu$ L of methanol. Vortex.
- 17.8.1.6 Using a repeater pipette, add 25  $\mu$ L of internal standard to each sample (except matrix blank) to obtain the final concentration of 50 ng/mL. Vortex.
- 17.8.1.7 Add 1 mL of cold acetonitrile dropwise while vortexing.
- 17.8.1.8 Centrifuge tubes approximately at 3082 rcf for 10 minutes to achieve separation.
- 17.8.1.9 Transfer the supernatant into an appropriately labeled round bottom screw cap tube.
- 17.8.1.10 Add 1.5 mL of 100 mM sodium acetate buffer pH 4.5. Vortex.
- 17.8.1.11 Place SPE columns in the extraction manifold. Throughout the SPE, do not let the SPE sorbent dry, unless specified.
  - 17.8.1.11.1 Add 3 mL methanol and aspirate.
  - 17.8.1.11.2 Add 3 mL deionized water and aspirate.
  - 17.8.1.11.3 Add 2 mL 100 mM sodium acetate buffer, pH 4.5 and aspirate.
  - 17.8.1.11.4 Pour samples in **appropriate** SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 17.8.1.11.5 Add 3 mL deionized water and aspirate.
  - 17.8.1.11.6 Add 2 mL 100 mM sodium acetate buffer, pH 4.5 with 20% acetonitrile and aspirate. Dry columns under full pressure for 10 minutes.
  - 17.8.1.11.7 Add 1 mL hexane and aspirate. Dry columns under full pressure for 5 minutes.
  - 17.8.1.11.8 Elute benzodiazepines with 3 mL BNZ elution solvent, prepared fresh daily.
- 17.8.1.12 Evaporate eluates to dryness at approximately 50  $^{\circ}$ C under nitrogen at 20 psi (2.9 L/min).
- 17.8.1.13 Reconstitute in 100  $\mu$ L of 90:10 LC-MS grade water: acetonitrile and vortex.
- 17.8.1.14 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 17.8.1.15 Load autosampler vials on the LC-MS/MS and inject 2  $\mu$ L using BNZ.m method.

#### 17.8.2 Urine Extraction

- 17.8.2.1 Pipet 0.5 mL drug-free urine for matrix blank, negative control, calibrators, and positive controls into appropriately labeled tubes.
- 17.8.2.2 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 17.8.2.3 Pipet 0.5 mL case samples into appropriately labeled tubes followed by 50  $\mu$ L of methanol. Vortex.



- 17.8.2.4 Using a repeater pipette, add 25  $\mu\text{L}$  of internal standard to each sample to obtain the final concentration of 50 ng/mL. Vortex.
- 17.8.2.5 Add 500  $\mu\text{L}$  of 100 mM sodium acetate buffer pH 5.0 and 50  $\mu\text{L}$  of  $\beta$ -glucuronidase solution to each tube. Cap and vortex briefly.
- 17.8.2.6 Incubate **on the heating block** for 2 hours at 60°C. Allow tubes to cool to room temperature before proceeding.
- 17.8.2.7 Centrifuge tubes approximately at 4200 rcf for 10 minutes if the samples are cloudy or contain particulates.
- 17.8.2.8 Follow the blood extraction procedure from 17.8.1.11 – 17.8.1.15.

### 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, hydrolysis control, and 10% positive controls that bracket case samples.

#### BNZ sequence example:

##### Blood

Matrix blank  
Calibrator 10 ng/mL  
Calibrator 20 ng/mL  
Calibrator 50 ng/mL  
Calibrator 100 ng/mL  
Calibrator 150 ng/mL  
Calibrator 250 ng/mL  
Calibrator 500 ng/mL  
Negative Control  
LQC  
HQC  
MQC  
Utak Benzodiazepines Plus 100  
10 case samples  
MQC  
Utak Benzodiazepines Plus 100  
10 case samples  
MQC  
Utak Benzodiazepines Plus 100  
10 case samples  
MQC  
Utak Benzodiazepines Plus 100  
10 case samples  
HQC  
LQC

##### Urine

Matrix blank  
Cut-off Calibrator  
Negative Control  
Hydrolysis Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control



## 17.10 Data Analysis

### 17.10.1 Blood

- 17.10.1.1 The calibration curve for 7-aminoclonazepam,  $\alpha$ -hydroxyalprazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, temazepam, and diazepam ranges from 10-500 ng/mL and zolpidem from 10-250 ng/mL
- 17.10.1.2 The low-quality control (LQC) for benzodiazepines/zolpidem is 30 ng/mL.
- 17.10.1.3 The mid quality control (MQC) for 7-aminoclonazepam is 80 ng/mL. An external Utak quality control is used for the MQC for all benzodiazepines and zolpidem. An in-house MQC of 80 ng/mL may be used if Utak is unavailable or if the concentration cannot be verified. Alternatively, Utak control can be used instead of internal MQC for 7-aminoclonazepam if it is verified.
- 17.10.1.4 The high-quality control (HQC) for benzodiazepines is 400 ng/mL and zolpidem 200 ng/mL.
- 17.10.1.5 **Positive alprazolam will be reported only if the analyte elutes after alprazolam-d5 because the compound eluting before alprazolam-d5 could be 4'-chloro-deschloroalprazolam. Any positive alprazolam findings with retention time before alprazolam-d5 will be reported as not suitable for the HFSC alprazolam analysis due to possible interference.**

### 17.10.2 Urine

- 17.10.2.1 The cut-off calibrator for benzodiazepines/zolpidem is 5.0 ng/mL forced through zero.
- 17.10.2.2 The positive control (PQC) for benzodiazepines/zolpidem is 10 ng/mL.  
**Hydrolysis control must be positive.**

### 17.10.3 Reinjection

- 17.10.3.1 Reinjections can be made up to 72 hours for blood samples or 48 hours for urine samples after the completion of the initial injection of the particular sample(s).

### 17.10.4 Carryover

- 17.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 1000 ng/mL or greater than 100 ng/mL in urine.

### 17.10.5 Dilutions

- 17.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

### 17.10.6 Limitations

- 17.10.6.1 **Blood:** In the presence of cyclobenzaprine at concentrations greater than 300 ng/mL, an interference with nordiazepam was observed in validation experiments (i.e., decreased quantitation).
- 17.10.6.2 If a case sample is positive for zolpidem via BNZ method but has not



been previously analyzed by GC-MS drug screen, the sample will be analyzed by qualitative confirmation BSD method unless the case sample needs to be re-analyzed with BNZ method for another analyte in the method.

#### **17.11 References**

- 17.11.1 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 12th ed. Seal Beach, CA: Biomedical Publications, 2020.
- 17.11.2 Levine, Barry and Kerrigan, Sarah, eds. "Benzodiazepines." Principles of Forensic Toxicology, 5th ed. Springer, Switzerland, 2020, 317-331.
- 17.11.3 Moore, C. and Zumwalt, M. Determination of Benzodiazepines in Urine and Blood Using Rapid Resolution Liquid Chromatography/ Triple Quadrupole Mass Spectrometry. Agilent Technologies Inc., Application Note. 2007.
- 17.11.4 Method File: BNZ.m



## 18. Cannabinoids Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 18.1 Purpose

18.1.1 A targeted analysis is performed for confirmatory analysis of 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THC-COOH), cannabidiol (CBD),  $\Delta$ 9-tetrahydrocannabinol ( $\Delta$ 9-THC), and  $\Delta$ 8-tetrahydrocannabinol ( $\Delta$ 8-THC) by liquid-liquid extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 18.2 Scope

18.2.1 This procedure describes a confirmatory analysis of THC, its target metabolites, and other cannabinoids in biological specimens including blood. Urine confirmations are reported only qualitatively.

### 18.3 Reagents and Solvents

18.3.1 10 M Potassium hydroxide

18.3.2 10% acetic acid

18.3.3 9:1 hexane: ethyl acetate solution: In a graduated cylinder add 9 parts hexane and 1 part ethyl acetate. Cap and thoroughly mix. Prepare fresh daily.

18.3.4 Mobile Phase A: H<sub>2</sub>O with 5 mM ammonium formate and 0.1% formic acid. Add 1 mL formic acid and 1 mL 5 M ammonium formate to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).

18.3.5 Mobile Phase B (organic, may be in another position in method report): LC/MS grade methanol

18.3.6 Deionized (DI) water

18.3.7 Methanol

18.3.8 Blank blood (preserved with potassium oxalate and sodium fluoride)

18.3.9 Blank urine

### 18.4 Equipment and Materials

18.4.1 Air displacement pipettes (1000-5000  $\mu$ L; 100-1000  $\mu$ L; 20-200  $\mu$ L; 2-20  $\mu$ L)

18.4.2 Repeater pipette

18.4.3 Analytical/Top-loading balance

18.4.4 Evaporator

18.4.5 Vortex mixer

18.4.6 Centrifuge

18.4.7 Rotator

18.4.8 Heating block



## 18.5 Instrumentation

### 18.5.1 LC Parameters

18.5.1.1 Column: Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7  $\mu$ m

18.5.1.2 Agilent 1290 Infinity II LC system

Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 5 mM ammonium formate and 0.1% formic acid	
Mobile Phase B	Methanol	
Flow Rate	0.4 mL/min	
Needle Wash	5 seconds	
Injection Volume	5 $\mu$ L	
Gradient	Initial	70% B
	2.0 minutes	80% B
	6.0 minutes	98% B
	7.0 minutes	98% B
	7.1 minutes	70%B
	Stop time	7.1 minutes
	Post time	2.5 minutes

### 18.5.2 MS/MS Parameters

18.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	300°C
Gas Flow	8 L/min
Nebulizer Pressure	50 psi
Sheath Gas Heater	400°C
Sheath Gas Flow	12 L/min
Capillary	4,000 V



18.5.3 MRM acquisition: THC.M

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor (Calibration model)
11-OH-THC-d3	334.2	<b>196.1</b> 316.2 201.0	131	28 12 28	2.74	1/x (linear)
11-OH-THC	331.2	<b>193.1</b> 313.1 201.0	122	28 12 28	2.75	
THC-COOH-d3	348.2	<b>302.1</b> 330.1 196.0	152	20 16 32	3.08	1/x (linear)
THC-COOH	345.2	<b>299.2</b> 193.0	124	22 30	3.09	
CBD-d3	318.2	<b>196.0</b> 123.0 262.1	116	24 36 20	3.12	1/x (linear)
CBD	315.2	<b>193.0</b> 123.0 135.1	131	24 40 20	3.13	
Delta9-THC-d3	318.2	<b>196.0</b> 123.0 135.0	131	28 40 24	4.50	1/x (linear)
Delta9-THC	315.2	<b>193.0</b> 123.0 135.0	140	24 36 20	4.51	
Delta8-THC-d3	318.2	<b>196.0</b> 123.0 135.0	131	28 40 24	4.66	1/x (linear)
Delta8-THC	315.2	<b>193.1</b> 123.0 135.0	140	24 36 20	4.67	
*Quantifier ion in bold **Retention times will vary						

**18.6 Standards and Solutions**

18.6.1 Cannabinoids Working Standards for Calibrators (Blood)

18.6.1.1 This analysis requires preparation of four solutions. The four solutions contain 11-OH-THC, CBD, delta9-THC, and delta8-THC at 10, 1, 0.1, 0.02 µg/mL and THC-COOH at 0, 5, 0.5, and 0.1 µg/mL, respectively.

18.6.1.1.1 Preparation of the 10 µg/mL Cannabinoids Calibrator stock solution: add 100 µL of each 1 mg/mL CRM of 11-OH-THC, CBD, delta9-THC, and delta8-THC to a 10 mL volumetric flask and bring to volume with methanol.





- 18.6.1.1.2 Preparation of the 1/5 µg/mL Cannabinoids Calibrator: add 1 mL of the 10 µg/mL Cannabinoids Calibrator stock solution and 50 µL of 1 mg/mL CRM of THC-COOH to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.1.1.3 Preparation of the 0.1/0.5 µg/mL Cannabinoids Calibrator: add 1 mL of the 1/5 µg/mL Cannabinoids Calibrator to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.1.1.4 Preparation of the 0.02/0.1 µg/mL Cannabinoids Calibrator: add 200 µL of the 1/5 µg/mL Cannabinoids Calibrator to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.2 Cannabinoids Working Standards for Calibrators (Urine)
  - 18.6.2.1 This analysis requires preparation of two solutions. The two solutions contain 11-OH-THC, THC-COOH, CBD, delta9-THC, and delta8-THC at 10 µg/mL and 0.1 µg/mL.
    - 18.6.2.1.1 Preparation of the 10 µg/mL Cannabinoids Calibrator stock solution: add 100 µL of each 1 mg/mL CRM of 11-OH-THC, THC-COOH, CBD, delta9-THC, and delta8-THC to a 10 mL volumetric flask and bring to volume with methanol.
    - 18.6.2.1.2 Preparation of the 0.1 µg/mL Cannabinoids Calibrator: add 100 µL of the 10 µg/mL Cannabinoids Calibrator stock solution to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.3 Cannabinoids Working Standards for Quality Controls (Blood)
  - 18.6.3.1 This analysis requires preparation of three solutions. The three solutions contain 11-OH-THC, CBD, delta9-THC, and delta8-THC at 10, 1, and 0.05 µg/mL, and THC-COOH at 0, 2, and 0.25 µg/mL, respectively.
    - 18.6.3.1.1 Preparation of the 10 µg/mL Cannabinoids Control Stock: add 100 µL of each 1 mg/mL CRM of 11-OH-THC, CBD, delta9-THC, and delta8-THC to a 10 mL volumetric flask and bring to volume with methanol.
    - 18.6.3.1.2 Preparation of the 1/2.5 µg/mL Cannabinoids Control: add 1 mL of the 10 µg/mL Cannabinoids Control Stock Solution and 250 µL of 100 µg/mL CRM of THC-COOH to a 10 mL volumetric flask and bring to volume with methanol.
    - 18.6.3.1.3 Preparation of the 0.05/0.25 µg/mL Cannabinoids Control: add 50 µL of the 10 µg/mL Cannabinoids Control Stock Solution and 25 µL of 100 µg/mL CRM of THC-COOH to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.4 Cannabinoids Working Standards for Quality Controls (Urine)
  - 18.6.4.1 This analysis requires preparation of four solutions. Two solutions contain 11-OH-THC, THC-COOH, CBD, delta9-THC, and delta8-THC at 10 µg/mL and 0.1



µg/mL. The other two solutions contain THC-COOH-glucuronide at 1 µg/mL and 0.1 µg/mL.

- 18.6.4.1.1 Preparation of the 10 µg/mL Cannabinoids Control stock solution: add 100 µL of each 1 mg/mL CRM of 11-OH-THC, THC-COOH, CBD, delta9-THC, and delta8-THC to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.4.1.2 Preparation of the 0.1 µg/mL Cannabinoids Control: add 100 µL of the 10 µg/mL Cannabinoids Calibrator stock solution to a 10 mL volumetric flask and bring to volume with methanol.
- 18.6.4.1.3 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.
- 18.6.4.1.4 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.

18.6.5 Cannabinoids Internal Standard Solutions (for blood and urine)

18.6.5.1 This analysis requires preparation of one solution. The solutions contain concentrations of 11-OH-THC-d3, THC-COOH-d3, CBD-d3, delta9-THC-d3, and delta8-THC-d3 at 0.2 µg/mL.

- 18.6.5.1.1 Preparation of the 0.2 µg/mL Internal Standard Stock Solution: add 20 µL of each 100 µg/mL CRM of 11-OH-THC-d3, THC-COOH-d3, CBD-d3, delta9-THC-d3, and delta8-THC-d3 to a 10 mL volumetric flask and bring to volume with methanol.

**18.7 Fortification Guide**

18.7.1 Blood Calibration Curve (delta9-THC, 11-OH-THC, CBD, delta8-THC/THC-COOH)

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	1.0/5.0	0.02/0.1	25	25
0.5	2.0/10	0.02/0.1	50	0
0.5	5.0/25	0.1/0.5	25	25
0.5	10/50	0.1/0.5	50	0
0.5	20/100	1/5	10	40
0.5	50/250	1/5	25	25
0.5	100**	1/5	50	0

\*\* 11-OH-THC, CBD, delta9-THC, and delta8-THC only



18.7.2 Blood Quality Controls (delta9-THC, 11-OH-THC, CBD, delta8-THC/THC-COOH)

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	3.0/15	0.05/0.25	30	20
0.5	30/75	1/2.5	15	35
0.5	80/200	1/2.5	40	10

18.7.3 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)
0.5	5.0	0.1 (Calibrator)	25	25
0.5	10	0.1 (QC)	50	0
0.5	10	0.1 (Hydrolysis QC)	50	0

18.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	10	0.2	25	0

**18.8 Extraction Procedure**

18.8.1 Blood Extraction

- 18.8.1.1 Allow all biological specimens to come to room temperature before starting the procedure.
- 18.8.1.2 Label all round bottom screw cap tubes accordingly.
- 18.8.1.3 Pipet 0.5 mL of drug-free blood for matrix blank, negative control, calibrators, and positive controls into appropriately labeled tubes.
- 18.8.1.4 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 18.8.1.5 Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50 µL of methanol. Vortex.
- 18.8.1.6 Using a repeater pipette, add 25 µL of internal standard to each sample (except matrix blank) to obtain the final concentration of 10 ng/mL. Vortex.
- 18.8.1.7 Add 1 mL of deionized water. Vortex.
- 18.8.1.8 Add 400 µL of 10% acetic acid. Vortex.
- 18.8.1.9 Add 4 mL of 9:1 hexane:ethyl acetate solution. Cap and rotate tubes for 15 min.
- 18.8.1.10 Centrifuge tubes approximately at 3082 rcf for 15 minutes to achieve separation.
- 18.8.1.11 Transfer the organic (upper) layer into an appropriately labeled conical glass tube.



- 18.8.1.12 Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 18.8.1.13 Reconstitute in 100 µL of 70:30 LC-MS grade methanol:water and vortex.
- 18.8.1.14 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 18.8.1.15 Load autosampler vials on the LC-MS/MS and inject 5 µL onto the LC-MS/MS using THC.m method.

#### 18.8.2 Urine Extraction

- 18.8.2.1 Pipet 0.5 mL of drug-free urine for matrix blank, negative control, calibrators, and positive controls into appropriately labeled tubes.
- 18.8.2.2 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 18.8.2.3 Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50 µL of methanol. Vortex.
- 18.8.2.4 Using a repeater pipette, add 25 µL of internal standard to each sample (except matrix blank) to obtain the final concentration of 10 ng/mL. Vortex.
- 18.8.2.5 Add 40 µL of 10 M potassium hydroxide to all tubes. Vortex.
- 18.8.2.6 Incubate **on the heating block** for 20 minutes at 60 °C. Allow tubes to cool to room temperature.
- 18.8.2.7 Centrifuge tubes approximately at 3082 rcf for 15 minutes if the samples are cloudy or contain particulates.
- 18.8.2.8 Follow the blood extraction procedure from 18.8.1.7-18.8.1.15.

#### 18.9 **Sequence Table**

- 18.9.1 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).
- 18.9.2 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**

Matrix blank  
Calibrator 1.0/5.0 ng/mL  
Calibrator 2.0/10 ng/mL  
Calibrator 5.0/25 ng/mL  
Calibrator 10/50 ng/mL  
Calibrator 20/100 ng/mL  
Calibrator 50/250 ng/mL  
Calibrator 100 ng/mL  
Negative Control  
LQC  
HQC  
MQC  
10 case samples  
MQC  
10 case samples  
MQC  
10 case samples  
MQC  
HQC  
LQC

**Urine**

Matrix blank  
Cut-off Calibrator  
Negative Control  
Hydrolysis Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control

**18.10 Data Analysis**

18.10.1 Blood

18.10.1.1 The calibration curve for 11-OH-THC, CBD, and delta9-THC, and delta8-THC ranges from 1.0-100 ng/mL, and for THC-COOH from 5.0-250 ng/mL.

18.10.1.1.1 The low-quality control (LQC) for delta9-THC, 11-OH-THC, CBD, and delta8-THC is 3 ng/mL and for THC-COOH 15 ng/mL.

18.10.1.1.2 The mid quality control (MQC) for delta9-THC, 11-OH-THC, CBD, and delta8-THC is 30 ng/mL and for THC-COOH 75 ng/mL.

18.10.1.1.3 The high-quality control (HQC) for delta9-THC, 11-OH-THC, CBD, and delta8-THC is 80 ng/mL and for THC-COOH 200 ng/mL.

18.10.2 Urine

18.10.2.1 The cut-off calibrator for cannabinoids is 5.0 ng/mL forced through zero. The positive control (PQC) for cannabinoids is 10 ng/mL. **Hydrolysis control must be positive.**

18.10.3 Reinjections can be made up to 72 hours after the completion of the initial injection of the particular sample(s).

18.10.4 **Carryover**



18.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 200 ng/mL for 11-OH-THC, CBD, delta9-THC, and delta8-THC or 1000 ng/mL for THC-COOH in blood, or greater than 200 ng/mL for all cannabinoids in urine.

**18.10.5 Dilutions**

18.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

**18.11 References**

18.11.1 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 12th ed. Seal Beach, CA: Biomedical Publications, 2020.

18.11.2 Hudson, J., et al., Cannabinoid Quantitation Using an Agilent 6430 LC/MS/MS. Agilent Technologies Inc., Application Note 5991-2521 EN, 2013.

18.11.3 Levine, Barry and Kerrigan, Sarah, eds. "Cannabis." Principles of Forensic Toxicology, 5th ed. Springer, Switzerland, 2020, 389-448.

18.11.4 Szczesniewski, A., et al. Analysis of cannabinoids and their metabolites in urine using MassHunter StreamSelect LC/MS system. Agilent Technologies Inc., Application Note 5994-0879 EN, 2019.

18.11.5 Method File: THC.m



## 19. Carisoprodol/Meprobamate Confirmation by Gas Chromatography-Mass Spectrometry

### 19.1 Purpose

19.1.1 A targeted analysis is performed for confirmatory analysis of meprobamate and carisoprodol using liquid-liquid extraction (LLE) and gas chromatography-mass spectrometry (GC-MS). 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 meprobamate and carisoprodol in biological specimens including blood. Urine confirmation results are reported only qualitatively.

### 19.3 Reagents and Solvents

- 19.3.1 1M Ammonium hydroxide
- 19.3.2 Ethyl acetate
- 19.3.3 Methanol
- 19.3.4 Blank blood (preserved with potassium oxalate and sodium fluoride)
- 19.3.5 Blank urine

### 19.4 Equipment and Materials

- 19.4.1 Air displacement pipettes (1000-5000  $\mu$ L; 100-1000  $\mu$ L; 20-200  $\mu$ L; 2-20  $\mu$ L)
- 19.4.2 Repeater pipette
- 19.4.3 Evaporator
- 19.4.4 Rocker
- 19.4.5 Vortex mixer
- 19.4.6 Centrifuge

### 19.5 Instrumentation

- 19.5.1 Parameters
  - 19.5.1.1 Capillary Column: 30 m DB-5MS Agilent J&W GC Column (or equivalent), 0.25 mm id X 0.25  $\mu$ m film thickness. The flow rate is 1.0 mL/min with an injection volume of 1  $\mu$ L in split mode (10:1).
  - 19.5.1.2 GC-MS: Agilent GC 7890B-5977B MSD (GCMS-5)



Initial Temperature:	150 °C hold for 1 minute 50 °C/min to 210 °C hold for 2 minutes 25 °C/min to 300 °C hold for 2 minutes
Total Run Time:	9.8 minutes
Injector Temperature:	250 °C
Interface Temperature:	280 °C
MS Quad:	150 °C
MS Source:	230 °C

#### 19.5.1.3 SIM acquisition: CAR.M

Drug	Quant Ion	Qualifier Ions	RT*	Weighting Factor (Calibration Model)
Meprobamate-d7	151.1	89.0, 121.2	4.7	1/x <sup>2</sup>
Meprobamate	144.0	83.0, 114.0	4.8	(quadratic)
Carisoprodol-d7	165.2	252.2, 191.2	5.2	1/x <sup>2</sup>
Carisoprodol	158.0	245.0, 184.0	5.3	(quadratic)

\*Retention Time (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 total of 3 pre- and 6 post- rinses are performed. Each rinse cycle consists of 2 methanol rinses followed by 2 ethyl acetate rinses.

### 19.6 Standards and Solutions

#### 19.6.1 CAR Working Standards for Calibrators (for blood and urine)

19.6.1.1 This analysis requires preparation of two solutions. The solutions contain concentrations of meprobamate and carisoprodol at 0.10 mg/mL and 0.01 mg/mL.

19.6.1.1.1 Preparation of the 0.10 mg/mL CAR Calibrator: add 1 mL of 1 mg/mL CRM of meprobamate and carisoprodol to a 10 mL volumetric flask and bring to volume with methanol.

19.6.1.1.2 Preparation of the 0.01 mg/mL CAR Calibrator: add 1 mL of the 0.10 mg/mL CAR Calibrator solution to a 10 mL volumetric flask and bring to volume with methanol.

#### 19.6.2 CAR Working Standards for Quality Controls (for blood and urine)





19.6.2.1 This analysis requires preparation of two solutions. The solutions contain concentrations of carisoprodol and meprobamate at 0.10 mg/mL and 0.01 mg/mL.

19.6.2.1.1 Preparation of the 0.10 mg/mL CAR QC Solution: add 1 mL of 1 mg/mL CRM of meprobamate and carisoprodol to a 10 mL volumetric flask and bring to volume with methanol.

19.6.2.1.2 Preparation of the 0.01 mg/mL CAR QC Solution: add 1 mL of the 0.10 mg/mL CAR QC solution to a 10 mL volumetric flask and bring to volume with methanol.

### 19.6.3 CAR Internal Standard (for blood and urine)

19.6.3.1 This solution contains a concentration of 10 µg/mL of meprobamate-d7 and carisoprodol-d7.

19.6.3.1.1 Preparation of 10 µg/mL CAR Internal Standard: add 1 mL of 0.1 mg/mL CRM of meprobamate-d7\* and carisoprodol-d7 to a 10 mL volumetric flask and bring to volume with methanol.

19.6.3.1.2 \*Meprobamate-d7 standard should contain 2-methyl-2-propyl-D7-propane-1,3-diol dicarbamate, or equivalent.

## 19.7 Fortification Guide

### 19.7.1 Blood Calibration

Volume of Blood (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	0.5	0.01	12.5
250	1.0	0.01	25
250	3.0	0.01	75
250	6.0	0.10	15
250	10	0.10	25
250	20	0.10	50
250	30	0.10	75

### 19.7.2 Blood Quality Controls

Volume of Blood (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	1.2	0.01	30
250	16	0.10	40
250	24	0.10	60

### 19.7.3 Blood Internal Standard

Volume of Blood (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	3.0	0.01	75



#### 19.7.4 Urine Calibration

Volume of Urine (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	0.5	0.01	12.5
250	20	0.10	50

#### 19.7.5 Urine Positive Control

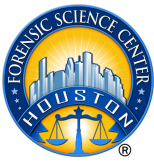
Volume of Urine (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	1.0	0.01	25

#### 19.7.6 Urine Internal Standard

Volume of Urine (µL)	Target Concentration (µg/mL)	Drug Standard Concentration (mg/mL)	Volume of Drug Standard Added (µL)
250	1.0	0.01	25

### 19.8 Extraction Procedure

- 19.8.1 Allow all biological specimens to come to room temperature before starting the procedure.
- 19.8.2 Label all round bottom screw cap tubes accordingly.
- 19.8.3 Add corresponding drug standards to calibrators and controls. Subsequently dry down at approximately 50°C under nitrogen at 20 psi (2.9 L/min) for approximately 30 seconds.
- 19.8.4 Pipet 250 µL of drug-free blood /urine for matrix blank, calibrators, controls. Vortex.
- 19.8.5 Pipet 250 µL case samples into appropriately labeled tubes.
- 19.8.6 Using a repeater pipette, add internal standard indicated below to each sample tube (except matrix blank). Vortex.
  - 19.8.6.1 Blood: Add 75 µL to obtain the final concentration of 3 µg/mL.
  - 19.8.6.2 Urine: Add 25 µL to obtain the final concentration of 1 µg/mL.
- 19.8.7 Add 100 µL of 1M ammonium hydroxide to each tube. Vortex.
- 19.8.8 Add 2 mL ethyl acetate to each tube. Cap and vortex.
- 19.8.9 Place tubes on a rocker for 15 minutes.
- 19.8.10 Centrifuge tubes approximately at 3082 rcf for 10 minutes.
- 19.8.11 Transfer the organic (upper) layer to an appropriately labeled conical tube.
  - 19.8.11.1 Blood: Slowly pour the organic layer off.
  - 19.8.11.2 Urine: Pipet approximately 1.5 mL of the organic layer, careful not to collect the aqueous (bottom) layer.
- 19.8.12 Evaporate samples to dryness at approximately 50 °C under nitrogen at 20 psi.
- 19.8.13 Reconstitute using 100 µL ethyl acetate to each tube using a repeater pipette. Vortex.
- 19.8.14 Transfer extracts to appropriately labeled autosampler vials with inserts and crimp caps tightly.



19.8.15 Inject 1 µL onto the GC-MS using the CAR.M method.

### 19.9 Sequence Table

19.9.1 Every batch must contain a matrix blank, negative control, calibrators, and quality controls. Case samples must be bracketed by 10% positive controls (LQC, MQC, HQC for blood and/or PQC for urine).

#### CAR sequence example:

Blood	Urine
Matrix Blank	Matrix Blank
0.5 µg/mL Calibrator	Low Calibrator
1 µg/mL Calibrator	High Calibrator
3 µg/mL Calibrator	Negative Control
6 µg/mL Calibrator	PQC
10 µg/mL Calibrator	10 Case Samples
20 µg/mL Calibrator	PQC
30 µg/mL Calibrator	10 Case Samples
Negative Control	PQC
LQC	10 Case Samples
HQC	PQC
MQC	
10 Case Samples	
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 meprobamate and carisoprodol ranges from 0.50-30 µg/mL.

19.10.1.2 The low-quality control (LQC) for meprobamate and carisoprodol is 1.2 µg/mL.

19.10.1.3 The mid-quality control (MQC) for meprobamate and carisoprodol is 16 µg/mL.

19.10.1.4 The high-quality control (HQC) for meprobamate and carisoprodol is 24 µg/mL.

#### 19.10.2 Urine

19.10.2.1 The low and high calibrators for meprobamate and carisoprodol are 0.5



and 20 µg/mL, respectively.

19.10.2.2 The positive control (PQC) for meprobamate and carisoprodol is 1 µg/mL.

19.10.3 Carryover

19.10.3.1 No carryover was observed in drug-free samples following samples containing meprobamate or carisoprodol above the ULOQ and up to 120 µg/mL in validation studies. Case samples shown to contain drug at up to 120 µg/mL will not require reinjection to evaluate carryover.

19.10.4 Dilutions

19.10.4.1 Highly concentrated case samples (i.e., analyte concentration >ULOQ) may be diluted with blank matrix at 2x or 5x dilution factors prior to analysis.

19.10.5 Limitations

19.10.5.1 Blood: In the presence of fluoxetine at concentrations greater than 2.5 µg/mL, an interference with carisoprodol-d7 was observed in validation experiments (i.e., ion ratios failed).

19.10.5.1.1 If carisoprodol is indicated during drug screening of a case sample in the presence of fluoxetine greater than 2.5 µg/mL, the sample may be sent to an outside laboratory for confirmation.

19.10.5.2 Urine: In the presence of fluoxetine at concentrations greater than 2.5 µg/mL, an interference with carisoprodol-d7 (i.e., ion ratios failed) was observed in validation experiments. In the presence of fluoxetine at 1 µg/mL, an interference with meprobamate (i.e., ion ratios failed) was also observed in validation experiments.

19.10.5.2.1 If carisoprodol is indicated during drug screening of a case sample in the presence of fluoxetine greater than 2.5 µg/mL, or if meprobamate is indicated during drug screening of a case sample in the presence of fluoxetine, the sample may be sent to an outside laboratory for confirmation.

**19.11 Literature and Supporting Documentation**

19.11.1 Baselt, Randall C. *Disposition of Toxic Drugs and Chemicals in Man*, 12th ed. Seal Beach, CA: Biomedical Publications, 2020.

19.11.2 Logan et al. "Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities—2017 Update." *Journal of Analytical Toxicology*, Volume 42, Issue 2, March 2018, Pages 63–68.

19.11.3 Acquisition method file: CAR.M



## 20. Cocaine Confirmation by Liquid Chromatography-Tandem 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 liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) 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: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. **In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix and then add 78 mL of methylene chloride. Cap and thoroughly mix. Prepare fresh daily.**

20.3.4 Mobile Phase A: H<sub>2</sub>O with 0.1% formic acid. Add 1 mL formic acid to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).

20.3.5 Mobile Phase B (organic, may be in another position in method report): LC/MS grade acetonitrile

20.3.6 Methanol

20.3.7 Deionized (DI) water

20.3.8 Utak **Drugs** of Abuse Level 1

20.3.9 Blank blood (preserved with potassium oxalate and sodium fluoride)

20.3.10 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**



## 20.5 Instrumentation

### 20.5.1 LC Parameters

20.5.1.1 Column: Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7  $\mu$ m

20.5.1.2 Agilent 1290 Infinity II LC system

Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 0.1% formic acid	
Mobile Phase B	Acetonitrile	
Flow Rate	0.5 mL/min	
Needle Wash	15 seconds	
Injection Volume	1 $\mu$ L	
Gradient	Initial	10% B
	0.5 minutes	10% B
	2.5 minutes	70 % B
	2.51 minutes	95% B
	6.0 minutes	95% B
	Post time	2.5 minutes

### 20.5.2 MS/MS Parameters

20.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	300°C
Gas Flow	8 L/min
Nebulizer Pressure	30 psi
Sheath Gas Heater	400°C
Sheath Gas Flow	12 L/min
Capillary	3,000 V

20.5.2.2 MRM acquisition: COC.m

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor (Calibration model)
Benzoylcognine-d3	293.2	<b>171.1</b>	125	20	1.89	1/x <sup>2</sup> (linear)
		77.1		68		
		105.0		36		
Benzoylecgonine	290.1	<b>168.1</b>	119	20	1.89	
		77.1		72		
		105.0		36		
Cocaine-d3	307.2	<b>185.1</b>	113	20	2.32	1/x <sup>2</sup> (linear)
		85.1		36		
		105.0		40		



Cocaine	304.2	<b>182.1</b> 82.1 105.0	125	20 36 40	2.31	1/x (linear)
Cocaethylene-d3	321.2	<b>199.1</b> 85.1 77.1	135	20 36 72	2.49	
Cocaethylene	318.2	<b>196.1</b> 82.1 77.1	125	20 32 76	2.48	
*Quantifier ion in bold **Retention times will vary						

## 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 benzoylecgonine, cocaine and cocaethylene at 10 µg/mL, 2.5 µg/mL, and 0.2 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 benzoylecgonine, cocaine and cocaethylene to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.1.1.2 Preparation of the 2.5 µg/mL Cocaine Calibrator: add 25 µL of each 1 mg/mL CRM of benzoylecgonine, cocaine and cocaethylene to a 10 mL volumetric flask and bring to volume with acetonitrile.

20.6.1.1.3 Preparation of the 0.2 µg/mL Cocaine Calibrator: add 800 µL of the 2.5 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 two solutions. The two solutions contain benzoylecgonine, cocaine and cocaethylene at 10 µg/mL and 0.5 µg/mL.

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

20.6.2.1.2 Preparation of the 0.5 µg/mL Cocaine Control Solution: add 500 µL of the 10 µg/mL Cocaine Control 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 2 µg/mL.

20.6.3.1.1 Preparation of the 2 µg/mL Internal Standard: add 200 µL of each 100 µg/mL CRM of benzoylecgonine-d3, cocaine-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 ( $\mu\text{g/mL}$ )	Volume of Drug Standard Added ( $\mu\text{L}$ )	Volume of Methanol Added ( $\mu\text{L}$ )
0.5	10	0.2	25	25
0.5	20	0.2	50	0
0.5	50	2.5	10	40
0.5	100	2.5	20	30
0.5	250	2.5	50	0
0.5	500	10	25	25
0.5	1000	10	50	0

### 20.7.2 Blood Quality Controls

Volume of Blood (mL)	Target Concentration (ng/mL)	Drug Standard Concentration ( $\mu\text{g/mL}$ )	Volume of Drug Standard Added ( $\mu\text{L}$ )	Volume of Methanol Added ( $\mu\text{L}$ )
0.5	25	0.5	25	25
0.5	800	10	40	10

### 20.7.3 Urine Calibrator and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration ( $\mu\text{g/mL}$ )	Volume of Drug Standard Added ( $\mu\text{L}$ )	Volume of Methanol Added ( $\mu\text{L}$ )
0.5	10	0.2 (Calibrator)	25	25
0.5	20	0.5 (QC)	20	30

### 20.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration ( $\mu\text{g/mL}$ )	Volume of Drug Standard Added ( $\mu\text{L}$ )	Volume of Methanol Added ( $\mu\text{L}$ )
0.5	100	2	25	0

## 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 glass tubes accordingly.

20.8.3 Pipet 0.5 mL of drug-free blood/urine for matrix blank, negative, calibrators, and positive controls into the appropriately labeled tubes.





- 20.8.4 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 20.8.5 Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50  $\mu$ L of methanol. Vortex.
- 20.8.6 Using a repeater pipette, add 25  $\mu$ L of internal standard to each sample (except matrix blank) to obtain the final concentration of 100 ng/mL. Vortex.
- 20.8.7 Add 1 mL of 100 mM phosphate buffer, pH 6.0 to each tube. Vortex.
- 20.8.8 Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent dry, unless specified.
  - 20.8.8.1 Add 3 mL of methanol and aspirate.
  - 20.8.8.2 Add 3 mL of deionized water and aspirate.
  - 20.8.8.3 Add 1 mL of 100 mM phosphate buffer, pH 6.0 and aspirate.
  - 20.8.8.4 Pour samples into **appropriate** SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 20.8.8.5 Add 3 mL of deionized water and aspirate.
  - 20.8.8.6 Add 1 mL of 1 M acetic acid and aspirate.
  - 20.8.8.7 Add 3 mL methanol and aspirate. Dry columns under full pressure for 5 minutes.
  - 20.8.8.8 Elute drugs by adding 3 mL of elution solvent, prepared fresh daily.
- 20.8.9 Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 20.8.10 Reconstitute in 200  $\mu$ L of LC-MS grade 90:10 water: acetonitrile. Vortex.
- 20.8.11 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 20.8.12 Load autosampler vials on the LC-MS/MS and inject 1  $\mu$ L using the COC.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**

Matrix blank  
Calibrator 10 ng/mL  
Calibrator 20 ng/mL  
Calibrator 50 ng/mL  
Calibrator 100 ng/mL  
Calibrator 250 ng/mL  
Calibrator 500 ng/mL  
Calibrator 1000 ng/mL  
Negative Control  
LQC  
HQC  
Utak  
10 case samples  
Utak  
10 case samples  
Utak  
10 case samples  
Utak  
HQC  
LQC

**Urine**

Matrix blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control

**20.10 Data Analysis**

20.10.1 Blood

- 20.10.1.1 The calibration curve for benzoylecgonine, cocaine and cocaethylene ranges from 10-1000 ng/mL.
- 20.10.1.2 The low-quality control (LQC) for benzoylecgonine, cocaine and cocaethylene is 25 ng/mL.
- 20.10.1.3 An external Utak quality control is used for the mid quality control (MQC). An in-house MQC of 100 ng/mL may be used if Utak is unavailable or if the concentration cannot be verified.
- 20.10.1.4 The high-quality control (HQC) for benzoylecgonine, cocaine and cocaethylene is 800 ng/mL.

20.10.2 Urine

- 20.10.2.1 The cut-off calibrator for benzoylecgonine, cocaine and cocaethylene is 10 ng/mL forced through zero.
- 20.10.2.2 The positive control (PQC) for benzoylecgonine, cocaine and cocaethylene is 20 ng/mL.

20.10.3 **Reinjection**

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



**20.10.4 Carryover**

20.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 2000 ng/mL.

**20.10.5 Dilutions**

20.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

**20.11 References**

- 20.11.1 Dioumaeva, I. SAMSHA-Compliant LC/MS/MS Analysis of Benzoyllecgonine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120. Agilent Technologies, Inc. Application Note, 2013.
- 20.11.2 Johansen, S. S. and Bhatia, H. M., Quantitative Analysis of Cocaine and its Metabolites in Whole Blood and Urine by High-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry. *J Chromatography B*, 2007; 852:338-44.
- 20.11.3 United Chemical Technologies, Inc. Cocaine and Benzoyllecgonine in Blood, Plasma/Serum, Urine, Tissue by LC-MS/MS or GC-MS Clean Screen DAU Extraction Column. *Clinical and Forensic Applications Manual*, 2016, pg. 88-90.
- 20.11.4 Method File: COC.m



## 21. Opioids 1 Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 21.1 Purpose

21.1.1 A targeted analysis is performed for confirmatory analysis of morphine, oxycodone, hydromorphone, codeine, O-desmethyltramadol, 6-acetylmorphine, oxycodone, hydrocodone, tramadol, norbuprenorphine, and buprenorphine using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 21.2 Scope

21.2.1 This procedure describes a confirmatory analysis of opioids in biological specimens including blood. Urine confirmations are reported only qualitatively.

### 21.3 Reagents and Solvents

- 21.3.1 100 mM Phosphate buffer, pH 6.0
- 21.3.2 1 M acetic acid
- 21.3.3 Elution solvent: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. **In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix and then add 78 mL of methylene chloride. Cap and thoroughly mix. Prepare fresh daily.**
- 21.3.4 Mobile Phase A: H<sub>2</sub>O with 5 mM ammonium formate and 0.1% formic acid. Add 1 mL formic acid and 1 mL 5 M ammonium formate to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).
- 21.3.5 Mobile Phase B/C: (organic, may be in another position in method report):
  - 21.3.5.1 Methanol with 0.1% formic acid: Add 1 mL formic acid to 1 LC/MS grade methanol. Prepare fresh daily (may be used up to 1 day after preparation).
  - 21.3.5.2 Acetonitrile with 0.1% formic acid: Add 1 mL formic acid to 1 LC/MS grade acetonitrile. Prepare fresh daily (may be used up to 1 day after preparation).
- 21.3.6 Deionized (DI) water
- 21.3.7 Methanol
- 21.3.8 Blank blood (preserved with potassium oxalate and sodium fluoride)
- 21.3.9 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

## 21.5 Instrumentation

### 21.5.1 LC Parameters

#### 21.5.1.1 Column:

21.5.1.1.1 Restek Raptor Biphenyl 2.1 x 100 mm, 2.7  $\mu$ m

#### 21.5.1.2 Agilent 1290 Infinity II LC system

##### 21.5.1.2.1 OPI1.M

Column Temperature	40°C		
Mobile Phase A	H <sub>2</sub> O with 5 mM ammonium formate and 0.1% formic acid		
Mobile Phase B	Methanol with 0.1 % formic acid		
Mobile Phase C	Acetonitrile with 0.1 % formic acid		
Flow Rate	0.5 mL/min		
Needle Wash	30 seconds		
Injection Volume	4 $\mu$ L		
Gradient	Initial	2% B	0% C
	0.60 minutes	2% B	0% C
	2.00 minutes	15% B	0% C
	2.50 minutes	22% B	0% C
	2.51 minutes	30% B	0% C
	5.00 minutes	40% B	0% C
	6.00 minutes	40% B	0% C
	6.50 minutes	0% B	98% C
	Stop time	10.00 minutes	
	Post time	3.00 minutes	

### 21.5.2 MS/MS Parameters

#### 21.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	350°C
Gas Flow	12 L/min
Nebulizer Pressure	50 psi
Sheath Gas Heater	400°C
Sheath Gas Flow	12 L/min
Capillary	3000 V



21.5.2.2 MRM acquisition: OPI1.M

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor (calibration model)
Morphine-d3	289.2	<b>165.0</b> 128.1 152.0	164	56 70 70	3.72	1/x (linear)
Morphine	286.2	<b>165.0</b> 128.1 152.0	161	24 70 70	3.74	
Oxymorphone-d3	305.2	<b>287.1</b> 230.1 201.1	140	20 36 52	3.83	1/x <sup>2</sup> (linear)
Oxymorphone	302.1	<b>284.1</b> 227.1 198.1	146	8 36 52	3.85	
Hydromorphone-d3	289.2	<b>185.0</b> 128.1 157.0	170	36 70 48	4.01	1/x <sup>2</sup> (linear)
Hydromorphone	286.2	<b>185.0</b> 128.1 157.1	167	15 70 52	4.02	
Codeine-d3	303.2	<b>165.0</b> 115.0 152.0	152	48 70 70	4.66	1/x (quadratic)
Codeine	300.2	<b>165.1</b> 115.0 152.1	146	26 70 70	4.66	
O-Desmethyltramadol-d6	256.2	<b>64.2</b> 77.0 61.0	107	20 70 70	4.67	1/x (linear)
O-Desmethyltramadol	250.2	<b>58.1</b> 77.0 <sup>#</sup> 56.1	101	8 70 70	4.69	
6-Acetylmorphine-d3	331.2	<b>211.0</b> 165.0 61.2	155	28 48 36	4.70	1/x <sup>2</sup> (linear)
6-Acetylmorphine	328.2	<b>211.0</b> 165.0 58.1	164	28 44 32	4.72	
Oxycodone-d3	319.2	<b>301.1</b> 259.1 244.1	134	20 28 32	4.87	1/x (linear)
Oxycodone	316.2	<b>298.1</b> 256.1 241.1	137	8 28 32	4.89	



Hydrocodone-d3	303.2	<b>199.1</b> 171.1 128.1	170	36 48 70	5.00	1/x <sup>2</sup> (linear)
Hydrocodone	300.2	<b>199.1</b> 171.0 128.0	170	15 44 70	5.02	
Tramadol-d3	269.2	<b>58.1</b> 56.1	107	20 70	6.14	1/x (quadratic)
Tramadol	264.2	<b>58.1</b> 77.1 56.1	107	7 70 70	6.19	
Norbuprenorphine-d3	417.3	<b>101.1</b> 55.1 83.1	182	44 70 60	7.49	1/x <sup>2</sup> (linear)
Norbuprenorphine	414.3	<b>101.1</b> 83.1	173	44 56	7.49	
Buprenorphine-d4	472.3	<b>59.1</b> 88.1 101.1	182	70 48 48	7.59	1/x <sup>2</sup> (linear)
Buprenorphine	468.3	<b>84.1</b> 101.1	182	48 48	7.59	
*Quantifier ion in bold **Retention times will vary #Qualifier used in blood data analysis only						

## 21.6 Standards and Solutions

### 21.6.1 Opioids 1 Working Standards for Calibrators (Blood)

#### 21.6.1.1 This analysis requires preparation of five solutions.

21.6.1.1.1 Preparation of 10 µg/mL OPI1 Low Calibrator Stock: Add 1 mL of 100 µg/mL CRM of norbuprenorphine and buprenorphine to a 10 mL volumetric flask and bring to volume with methanol.

21.6.1.1.2 Preparation of 100 µg/mL OPI1 High Calibrator Stock: Add 1 mL of 1 mg/mL CRM of morphine, oxycodone, hydromorphone, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, hydrocodone, and tramadol to a 10 mL volumetric flask and bring to volume with methanol.

21.6.1.1.3 Preparation of 1/5 µg/mL OPI1 Calibrator C: Add 1 mL of OPI1 Low Calibrator Stock and 0.5 mL of OPI1 High Calibrator Stock to a 10 mL volumetric flask and bring to volume with methanol.

21.6.1.1.4 Preparation of 0.1/0.5 µg/mL OPI1 Calibrator B: Add 100 µL of OPI1 Low Calibrator Stock and 50 µL of OPI1 High Calibrator Stock to a 10 mL volumetric flask and bring to volume with methanol.



- 21.6.1.1.5 Preparation of 0.01/0.1 µg/mL OPI1 Calibrator A: Add 25 µL each of OPI1 Low Calibrator Stock and OPI1 High Calibrator stock to a 25 mL volumetric flask and bring to volume with methanol.
- 21.6.2 Opioids 1 Working Standards for Quality Controls (Blood)
  - 21.6.2.1 This analysis requires preparation of six solutions.
    - 21.6.2.1.1 Preparation of 10 µg/mL Buprenorphine and Norbuprenorphine Control Stock: add 100 µL of a 1 mg/mL CRM of buprenorphine and norbuprenorphine to a 10 mL volumetric flask and bring to volume with methanol.
    - 21.6.2.1.2 Preparation of 5 µg/mL OPI1 Control Stock: add 50 µL of a 1 mg/mL CRM of hydrocodone, hydromorphone, and oxymorphone to a 10 mL volumetric flask and bring to volume with methanol.
    - 21.6.2.1.3 Preparation of 10 µg/mL OPI1 Control Stock: add 100 µL of a 1 mg/mL CRM of oxycodone, codeine, morphine, 6-acetylmorphine, tramadol, and o-desmethyltramadol to a 10 mL volumetric flask and bring to volume with methanol.
    - 21.6.2.1.4 Preparation of OPI1 0.05/0.5 µg/mL Low QC Working Solution: add 50 µL of 10 µg/mL Buprenorphine and Norbuprenorphine Control Stock, 500 µL of 10 µg/mL OPI1 Control Stock, and 1 mL of 5 µg/mL OPI1 Control Stock to a 10 mL volumetric flask and bring to volume with methanol.
    - 21.6.2.1.5 Preparation of OPI1 0.2/0.6/2 µg/mL Mid QC Working Solution: add 200 µL of 10 µg/mL Buprenorphine and Norbuprenorphine Control Stock, 600 µL of 10 µg/mL OPI1 Control Stock, and 4 mL of 5 µg/mL OPI1 Control Stock to a 10 mL volumetric flask and bring to volume with methanol.
    - 21.6.2.1.6 Preparation of OPI1 2/2.5/10 µg/mL High QC Working Solution: add 2 mL of 10 µg/mL Buprenorphine and Norbuprenorphine Control, 2.5 mL of 10 µg/mL OPI1 Control Stock, 100 µL of a 1 mg/mL CRM of hydrocodone, hydromorphone, and oxymorphone to a 10 mL volumetric flask and bring to volume with methanol.
- 21.6.3 Opioids 1 Working Standards for Calibrators (Urine)
  - 21.6.3.1 This analysis requires the preparation of one solution.
    - 21.6.3.1.1 Preparation of 0.1 µg/mL OPI1 Urine Calibrator: Add 250 µL of OPI1 Low Calibrator Stock and 25 µL of OPI1 High Calibrator Stock to a 25 mL volumetric flask and bring to volume with methanol.
- 21.6.4 Opioids 1 Working Standards for Quality Controls (Urine)
  - 21.6.4.1 This analysis requires preparation of two solutions.
    - 21.6.4.1.1 Preparation of 5 µg/mL OPI1 Urine Control Stock Solution: add 50 µL of a 1 mg/mL CRM of morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, o-desmethyltramadol, buprenorphine, and norbuprenorphine to a 10 mL volumetric flask and bring to volume with methanol.





21.6.4.1.2 Preparation of 0.1 µg/mL OPI1 Urine Control Solution: add 200 µL of 5 µg/mL OPI1 Urine Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

21.6.5 Opioids 1 Internal Standard (for blood and urine)

21.6.5.1 This solution contains concentrations of hydrocodone-d3, hydromorphone-d3, oxymorphone-d3, and 6-acetylmorphine-d3 at 2 µg/mL, morphine-d3, codeine-d3, oxycodone-d3, tramadol-d3, and O-desmethyltramadol-d6 at 1 µg/mL, and buprenorphine-d4 and norbuprenorphine-d3 at 0.4 µg/mL.

21.6.5.1.1 Preparation of 0.4/1/2 µg/mL OPI1 Internal Standard: add 50 µL each of 1 mg/mL CRM of hydrocodone-d3, hydromorphone-d3, oxymorphone-d3, and 6-acetylmorphine-d3, 25 µL of each 1 mg/mL CRM of codeine-d3, oxycodone-d3, and morphine-d3, 250 µL each of 0.1 mg/mL CRM of tramadol-d3 and O-desmethyltramadol-d6, and 100 µL each of 0.1 mg/mL CRM of buprenorphine-d4 and norbuprenorphine-d3 into a 25 mL volumetric flask and bring to volume with methanol.

**21.7 Fortification Guide**

21.7.1 Blood Calibration Curve (norbuprenorphine, buprenorphine/morphine, oxymorphone, hydromorphone, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, hydrocodone, tramadol)

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	0.5/5.0	0.01/0.1 (A)	50	50
1	1.0/10	0.01/0.1 (A)	100	0
1	5.0/25	0.1/0.5 (B)	50	50
1	10/50	0.1/0.5 (B)	100	0
1	15/75	1/5 (C)	15	85
1	25/125	1/5 (C)	25	75
1	50/250*	1/5 (C)	50	50
1	100/500*	1/5 (C)	100	0

\*norbuprenorphine, buprenorphine/oxymorphone, hydromorphone, and hydrocodone only



21.7.2 Blood Quality Controls (norbuprenorphine, buprenorphine/morphine, oxycodone, hydromorphone, codeine, o-desmethyltramadol, 6-acetylmorphine, tramadol)

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	1.5/15	0.05/0.5	30	70
1	10/30/100*	0.2/0.6/2	50	50
1	80/100/400*	2/2.5/10	40	60

\*MQC at 30 and HQC at 100 ng/mL for morphine, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, and tramadol

21.7.3 Urine Calibrators and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)	Volume of Methanol Added (µL)
1	5.0	0.1 (Calibrator)	50	50
1	10	0.1 (Control)	100	0

21.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	10/25/50*	0.4/1/2	25	0

\*IS at 10 ng/mL for buprenorphine-d4 and norbuprenorphine-d3; 25 ng/mL for codeine-d3, oxycodone-d3, morphine-d3, tramadol-d3 and O-desmethyltramadol-d6; and 50 ng/mL for hydrocodone-d3, hydromorphone-d3, oxycodone-d3, and 6-acetylmorphine-d3

**21.8 Extraction Procedure**

21.8.1 Blood and Urine Extraction

- 21.8.1.1 Allow all biological specimens to come to room temperature before starting the procedure.
- 21.8.1.2 Label all round bottom screw cap tubes accordingly.
- 21.8.1.3 Pipet 1 mL of drug-free blood/case samples into the appropriately labeled tubes.
- 21.8.1.4 Add 2 mL of 100 mM sodium phosphate buffer, pH 6.0 to each tube. Vortex.
- 21.8.1.5 Pipet calibrators/negative control/positive controls into the appropriately labeled tubes. Vortex.
- 21.8.1.6 Pipet the appropriate amount of methanol to applicable calibrators and QCs. Add 100 µL methanol to all case samples.



- 21.8.1.7 Using a repeater pipette, add 25  $\mu$ L of internal standard to each sample (except matrix blank) to obtain the final concentration of 10/25/50 ng/mL. Vortex.
- 21.8.1.8 Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent to dry, unless specified.
  - 21.8.1.8.1 Add 3 mL methanol and aspirate.
  - 21.8.1.8.2 Add 3 mL deionized water and aspirate.
  - 21.8.1.8.3 Add 2 mL 100 mM sodium phosphate buffer, pH 6.0 and aspirate.
  - 21.8.1.8.4 Pour samples in appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 21.8.1.8.5 Add 3 mL deionized water and aspirate.
  - 21.8.1.8.6 Add 1 mL 1 M acetic acid and aspirate.
  - 21.8.1.8.7 Add 3 mL methanol and aspirate. Dry columns under full pressure for 5 minutes.
  - 21.8.1.8.8 Elute opioids with 3 mL elution solvent, prepared fresh daily.
- 21.8.1.9 Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 21.8.1.10 Reconstitute in 50  $\mu$ L 90:10 LC-MS grade water: acetonitrile. Vortex.
- 21.8.1.11 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 21.8.1.12 Load autosampler vials on the LC-MS/MS and run OPI1.M method.

## 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, and 10% positive controls that bracket case samples.



**Opioids sequence example:**

**Blood**

Matrix blank  
0.5/5.0 ng/mL Calibrator  
1.0/10 ng/mL Calibrator  
5.0/25 ng/mL Calibrator  
10/50 ng/mL Calibrator  
15/75 ng/mL Calibrator  
25/125 ng/mL Calibrator  
50/250 ng/mL Calibrator  
100/500 ng/mL Calibrator  
Negative Control  
LQC  
HQC  
MQC  
10 case samples  
MQC  
10 case samples  
MQC  
HQC  
LQC

**Urine**

Matrix Blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control

**21.10 Data Analysis**

21.10.1 Blood

21.10.1.1 The calibration curves for norbuprenorphine and buprenorphine range from 0.5-100 ng/mL; for morphine, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, and tramadol from 5.0-125 ng/mL; and for oxymorphone, hydromorphone, and hydrocodone from 5.0-500 ng/mL.

21.10.1.2 The low-quality control (LQC) is 1.5 ng/mL for norbuprenorphine and buprenorphine and 15 ng/mL for morphine, oxymorphone, hydromorphone, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, hydrocodone, and tramadol.

21.10.1.3 The mid-quality control (MQC) is 10 ng/mL for norbuprenorphine and buprenorphine; 30 ng/mL for morphine, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, and tramadol; and 100 ng/mL for oxymorphone, hydromorphone, and hydrocodone.

21.10.1.4 The high-quality control (HQC) is 80 ng/mL for norbuprenorphine and buprenorphine; 100 ng/mL for morphine, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, and tramadol; and 400 ng/mL for oxymorphone, hydromorphone, and hydrocodone.

21.10.2 Urine

21.10.2.1 The cut-off calibrator for opioids 1 is 5.0 ng/mL for morphine, oxymorphone, hydromorphone, codeine, o-desmethyltramadol, 6-



acetylmorphine, oxycodone, hydrocodone, tramadol, norbuprenorphine, and buprenorphine, forced through zero.

21.10.2.2 The positive control (PQC) for opioids 1 is 10 ng/mL for morphine, oxymorphone, hydromorphone, codeine, o-desmethyltramadol, 6-acetylmorphine, oxycodone, hydrocodone, tramadol, norbuprenorphine, and buprenorphine.

#### 21.10.3 Reinjection

21.10.3.1 Reinjections can be made up to 72 hours after the completion of the initial injection of the particular sample(s).

#### 21.10.4 Carryover

21.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 100/500 ng/mL in blood or 200/1000 ng/mL in urine.

#### 21.10.5 Dilutions

21.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

#### 21.10.6 Limitations

##### 21.10.6.1 Blood

21.10.6.1.1 In the presence of pseudoephedrine at concentrations greater than 250 ng/mL, an interference with oxymorphone was observed in validation experiments (i.e., decreased quantitation).

21.10.6.1.2 In the presence of MDMA at concentrations greater than 100 ng/mL, an interference with oxycodone was observed in validation experiments (i.e., increased quantitation).

21.10.6.1.3 In the presence of both dextromethorphan and diphenhydramine at concentrations greater than 200 ng/mL, an interference with buprenorphine was observed in validation experiments (i.e., ion ratio failure).

##### 21.10.6.2 Urine

21.10.6.2.1 In the presence of pseudoephedrine at concentrations greater than 250 ng/mL, an interference with oxymorphone was observed in validation experiments (i.e., decreased quantitation).

21.10.6.2.2 In the presence of MDMA at concentrations greater than 50 ng/mL, an interference with oxycodone was observed in validation experiments (i.e., increased quantitation).

21.10.6.2.3 In the presence of both dextromethorphan and diphenhydramine at concentrations greater than 100 ng/mL, an interference with buprenorphine was observed in validation experiments (i.e., ion ratio failure).

21.10.6.2.4 In the presence of PCP at concentrations greater than 250 ng/mL, an interference with buprenorphine was observed in validation experiments (i.e., poor chromatography).

21.10.6.3 If a case sample is positive for tramadol via OPI1 method but has not been previously analyzed by GC-MS drug screen, the sample will be analyzed by



qualitative confirmation BSD method unless the case sample needs to be re-analyzed with OPI1 method for another analyte in the method.

21.10.6.4 If a case sample is positive for o-desmethyltramadol via OPI1 method, the sample will be re-analyzed by OPI1 method for the confirmation of the result.

21.10.6.5 If a case sample is positive for both tramadol and o-desmethyltramadol, the sample will be re-analyzed by OPI1 method for the confirmation of **o-desmethyltramadol and of tramadol if GC-MS drug screen has not been performed for tramadol.**

### **21.11 References**

- 21.11.1 Coles, R., et al. Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC-MS-MS. *Journal of Analytical Toxicology*, 2007; 31:1-14
- 21.11.2 Levine, Barry and Kerrigan, Sarah, eds. "Opioids." *Principles of Forensic Toxicology*, 5th ed. Springer, Switzerland, 2020, 347-369.
- 21.11.3 United Chemical Technologies Inc. Free Opiates and Glucuronides in Urine Extracted by Clean Screen® DAU and Analyzed by LC-MS/MS. Application Note, 2014.
- 21.11.4 Method file: OPI1.m



## 22. Opioids 2 Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 22.1 Purpose

22.1.1 A targeted analysis is performed for confirmatory analysis of norfentanyl, fentanyl, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and methadone using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 22.2 Scope

22.2.1 This procedure describes a confirmatory analysis of opioids in biological specimens including blood. Urine confirmations are reported only qualitatively.

### 22.3 Reagents and Solvents

- 22.3.1 100 mM Phosphate buffer, pH 6.0
- 22.3.2 1 M acetic acid
- 22.3.3 Elution solvent: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix and then add 78 mL of methylene chloride. Cap and thoroughly mix. Prepare fresh daily.
- 22.3.4 Mobile Phase A: H<sub>2</sub>O with 5 mM ammonium formate and 0.1% formic acid. Add 1 mL formic acid and 1 mL 5 M ammonium formate to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).
- 22.3.5 Mobile Phase B: (organic, may be in another position in method report): Methanol with 0.1% formic acid: Add 1 mL formic acid to 1 LC/MS grade methanol. Prepare fresh daily (may be used up to 1 day after preparation).
- 22.3.6 Deionized (DI) water
- 22.3.7 Methanol
- 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 UCT Clean Screen® DAU, 6 mL columns, 200 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 LC Parameters

##### 22.5.1.1 Column:

22.5.1.1.1 Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7  $\mu$ m

##### 22.5.1.2 Agilent 1290 Infinity II LC system

##### 22.5.1.2.1 OPI2.M

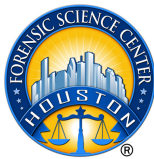
Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 5 mM ammonium formate and 0.1% formic acid	
Mobile Phase B	Acetonitrile with 0.1 % formic acid	
Flow Rate	0.6 mL/min	
Needle Wash	30 seconds	
Injection Volume	1 $\mu$ L	
Gradient	Initial	10% B
	2.00 minutes	80% B
	2.10 minutes	95% B
	Stop time	7.10 minutes
	Post time	2.50 minutes

#### 22.5.2 MS/MS Parameters

##### 22.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	350°C
Gas Flow	12 L/min
Nebulizer Pressure	50 psi
Sheath Gas Heater	400°C
Sheath Gas Flow	12 L/min
Capillary	3000 V





22.5.2.2 MRM acquisition: OPI2.M

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor
Norfentanyl-d5	238.2	<b>84.1</b> 55.1 155.1	101	20 48 20	1.33	1/x (linear)
Norfentanyl	233.2	<b>84.1</b> 55.1 150.1	95	20 44 20	1.34	
Fentanyl-d5	342.3	<b>105.0</b> 188.1 79.1	143	48 24 70	1.81	1/x <sup>2</sup> (linear)
Fentanyl	337.2	<b>105.0</b> 188.1 77.1 <sup>#</sup>	143	48 24 70	1.82	
EDDP-d3	281.2	<b>234.1</b> 249.1 189.1	161	36 28 40	1.95	1/x <sup>2</sup> (linear)
EDDP	278.2	<b>234.1</b> 249.1 186.1	167	36 28 40	1.96	
Methadone-d3	313.2	<b>268.1</b> 105.0 77.1	119	16 32 68	2.08	1/x <sup>2</sup> (linear)
Methadone	310.2	<b>265.1</b> 105.0 77.1	116	16 32 68	2.09	
*Quantifier ion in bold **Retention times will vary <sup>#</sup> Qualifier used in blood data analysis only						

**22.6 Standards and Solutions**

22.6.1 Opioids 2 Working Standards for Calibrators (Blood and Urine)

22.6.1.1 This analysis requires preparation of five solutions.

22.6.1.1.1 Preparation of 10 µg/mL OPI2 Low Calibrator Stock: Add 1 mL of 100 µg/mL CRM of fentanyl and 100 µL of 1 mg/mL CRM of norfentanyl to a 10 mL volumetric flask and bring to volume with methanol.

22.6.1.1.2 Preparation of 100 µg/mL OPI2 High Calibrator Stock: Add 1 mL of 1 mg/mL CRM of EDDP and methadone to a 10 mL volumetric flask and bring to volume with methanol.

22.6.1.1.3 Preparation of 1/5 µg/mL OPI2 Calibrator C: Add 1 mL of OPI2 Low Calibrator Stock and 0.5 mL of OPI2 High Calibrator Stock to a 10 mL volumetric flask and bring to volume with methanol.



- 22.6.1.1.4 Preparation of 0.1/0.5 µg/mL OPI2 Calibrator B: Add 100 µL of OPI2 Low Calibrator Stock and 50 µL of OPI2 High Calibrator Stock to a 10 mL volumetric flask and bring to volume with methanol.
- 22.6.1.1.5 Preparation of 0.01/0.1 µg/mL OPI2 Calibrator A: Add 25 µL each of OPI2 Low Calibrator Stock and OPI2 High Calibrator stock to a 25 mL volumetric flask and bring to volume with methanol.
- 22.6.2 Opioids 2 Working Standards for Quality Controls (Blood)
  - 22.6.2.1 This analysis requires preparation of five solutions.
    - 22.6.2.1.1 Preparation of 10 µg/mL Fentanyl and Norfentanyl Control Stock: add 100 µL of a 1 mg/mL CRM of norfentanyl and 1 mL of a 0.1 mg/mL CRM of fentanyl to a 10 mL volumetric flask and bring to volume with methanol.
    - 22.6.2.1.2 Preparation of 10 µg/mL Methadone and EDDP Control Stock: add 100 µL of a 1 mg/mL CRM of methadone and EDDP to a 10 mL volumetric flask and bring to volume with methanol.
    - 22.6.2.1.3 Preparation of OPI2 0.05/0.5 µg/mL Low QC Working Solution: add 50 µL of 10 µg/mL Fentanyl and Norfentanyl Control Stock and 500 µL of 10 µg/mL Methadone and EDDP Control Stock to a 10 mL volumetric flask and bring to volume with methanol.
    - 22.6.2.1.4 Preparation of OPI2 0.2/2 µg/mL Mid QC Working Solution: add 200 µL of 10 µg/mL Fentanyl and Norfentanyl Control Stock and 2 mL of 10 µg/mL Methadone and EDDP Control Stock to a 10 mL volumetric flask and bring to volume with methanol.
    - 22.6.2.1.5 Preparation of OPI2 2/10 µg/mL High QC Working Solution: add 2 mL of 10 µg/mL Fentanyl and Norfentanyl Control Stock and 100 µL of a 1 mg/mL CRM of methadone and EDDP to a 10 mL volumetric flask and bring to volume with methanol.
- 22.6.3 Opioids 2 Working Standards for Quality Controls (Urine)
  - 22.6.3.1 This analysis requires preparation of three solutions.
    - 22.6.3.1.1 Preparation of norfentanyl 10 µg/mL stock solution: add 100 µL of 1 mg/mL CRM of norfentanyl into a 10 mL volumetric flask bring to volume with methanol.
    - 22.6.3.1.2 Preparation of 0.5/5 µg/mL OPI2 Urine Control Stock Solution: add 500 µL of 10 µg/mL norfentanyl stock solution and 50 µL of 0.1 mg/mL CRM of fentanyl and 50 µL of a 1 mg/mL CRM of methadone and EDDP to a 10 mL volumetric flask and bring to volume with methanol.
    - 22.6.3.1.3 Preparation of 0.01/0.1 µg/mL OPI2 Urine Control Solution: add 200 µL of 0.5/5 µg/mL OPI2 Urine Control Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.



22.6.4 Opioids 2 Internal Standard (for blood and urine)

22.6.4.1 This solution contains concentrations of methadone-d3 and EDDP-d3 at 2 µg/mL and fentanyl-d5 and norfentanyl-d5 at 0.4 µg/mL.

22.6.4.1.1 Preparation of 0.4/2 µg/mL OPI2 Internal Standard: add 200 µL each of 0.1 mg/mL CRM of methadone-d3 and EDDP-d3 and 40 µL of each 0.1 mg/mL CRM of fentanyl-d5 and norfentanyl-d5 into a 10 mL volumetric flask and bring to volume with methanol.

**22.7 Fortification Guide**

22.7.1 Blood Calibration Curve (norfentanyl, fentanyl/EDDP, methadone)

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	0.5/5.0	0.01/0.1 (A)	50	50
1	1.0/10	0.01/0.1 (A)	100	0
1	5.0/25	0.1/0.5 (B)	50	50
1	10/50	0.1/0.5 (B)	100	0
1	25/125	1/5 (C)	25	75
1	50/250	1/5 (C)	50	50
1	100/500	1/5 (C)	100	0

22.7.2 Blood Quality Controls (norfentanyl, fentanyl/EDDP, methadone)

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	1.5/15	0.05/0.5	30	70
1	10/100	0.2/2	50	50
1	80/400	2/10	40	60

22.7.3 Urine Calibrators and Positive Control

Volume of Urine (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume Added (µL)	Volume of Methanol Added (µL)
1	0.5*/5.0	0.01*/0.1 (Calibrator)	50	50
1	1.0*/10	0.01*/0.1 (Control)	100	0
*Norfentanyl and fentanyl only				

22.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
1	10/50	0.4/2	25	0



## 22.8 Extraction Procedure

### 22.8.1 Blood and Urine Extraction

- 22.8.1.1 Allow all biological specimens to come to room temperature before starting the procedure.
- 22.8.1.2 Label all round bottom screw cap tubes accordingly.
- 22.8.1.3 Pipet 1 mL of drug-free blood/case samples into the appropriately labeled tubes.
- 22.8.1.4 Add 2 mL of 100 mM sodium phosphate buffer, pH 6.0 to each tube. Vortex.
- 22.8.1.5 Pipet calibrators/negative control/positive controls into the appropriately labeled tubes. Vortex.
- 22.8.1.6 Pipet the appropriate amount of methanol to applicable calibrators and QCs. Add 100 µL methanol to all case samples.
- 22.8.1.7 Using a repeater pipette, add 25 µL of internal standard to each sample (except matrix blank) to obtain the final concentration of 10/50 ng/mL. Vortex.
- 22.8.1.8 Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent to dry, unless specified.
  - 22.8.1.8.1 Add 3 mL methanol and aspirate.
  - 22.8.1.8.2 Add 3 mL deionized water and aspirate.
  - 22.8.1.8.3 Add 2 mL 100 mM sodium phosphate buffer, pH 6.0 and aspirate.
  - 22.8.1.8.4 Pour samples in **appropriate** SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 22.8.1.8.5 Add 3 mL deionized water and aspirate.
  - 22.8.1.8.6 Add 1 mL 1 M acetic acid and aspirate.
  - 22.8.1.8.7 Add 3 mL methanol and aspirate. Dry columns under full pressure for 5 minutes.
  - 22.8.1.8.8 Elute opioids with 3 mL elution solvent, prepared fresh daily.
- 22.8.1.9 Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 22.8.1.10 Reconstitute:
  - 22.8.1.10.1 Reconstitute in 150 µL 90:10 LC-MS grade water: acetonitrile. Vortex.
- 22.8.1.11 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 22.8.1.12 Load autosampler vials on the LC-MS/MS and run OPI2.M method.

## 22.9 Sequence Table

- 22.9.1 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).
- 22.9.2 Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.



**Opioids sequence example:**

**Blood**

Matrix blank  
0.5/5.0 ng/mL Calibrator  
1.0/10 ng/mL Calibrator  
5.0/25 ng/mL Calibrator  
10/50 ng/mL Calibrator  
25/125 ng/mL Calibrator  
50/250 ng/mL Calibrator  
100/500 ng/mL Calibrator  
Negative Control  
LQC  
HQC  
MQC  
10 case samples  
MQC  
10 case samples  
MQC  
HQC  
LQC

**Urine**

Matrix Blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control

**22.10 Data Analysis**

22.10.1 Blood

- 22.10.1.1 The calibration curves for norfentanyl and fentanyl range from 0.5-100 ng/mL, and for EDDP and methadone from 5.0-500 ng/mL.
- 22.10.1.2 The low-quality control (LQC) is 1.5 ng/mL for norfentanyl and fentanyl and 15 ng/mL for EDDP and methadone.
- 22.10.1.3 The mid-quality control (MQC) is 10 ng/mL for fentanyl and norfentanyl and 100 ng/mL for EDDP and methadone.
- 22.10.1.4 The high-quality control (HQC) is 80 ng/mL for norfentanyl and fentanyl and 400 ng/mL for EDDP and methadone.

22.10.2 Urine

- 22.10.2.1 The cut-off calibrator for opioids 2 is 0.5 ng/mL for norfentanyl and fentanyl and 5.0 ng/mL for EDDP and methadone forced through zero.
- 22.10.2.2 The positive control (PQC) for opioids 2 is 1.0 ng/mL for norfentanyl and fentanyl and 10 ng/mL for EDDP and methadone.

22.10.3 **Reinjection**

- 22.10.3.1 Reinjections can be made up to 72 hours after the completion of the initial injection of the particular sample(s).

22.10.4 **Carryover**

- 22.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 100/500 ng/mL in blood or 200/1000 ng/mL in urine.



#### 22.10.5 Dilutions

22.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

#### 22.10.6 Limitations

##### 22.10.6.1 Blood

22.10.6.1.1 In the presence of ketamine at concentrations greater than 10 ng/mL, an interference with norfentanyl was observed in validation experiments (i.e., ion ratio failure).

22.10.6.1.2 In the presence of alprazolam at concentrations greater than 100 ng/mL, an interference with methadone was observed in validation experiments (i.e., ion ratio failure).

##### 22.10.6.2 Urine

22.10.6.2.1 In the presence of ketamine at concentrations greater than 10 ng/mL, an interference with norfentanyl was observed in validation experiments (i.e., ion ratio failure).

22.10.6.2.2 In the presence of alprazolam at concentrations greater than 10 ng/mL, an interference with methadone was observed in validation experiments (i.e., ion ratio failure).

22.10.6.3 If a case sample is positive for methadone via OPI2 method but has not been previously analyzed by GC-MS drug screen, the sample will be analyzed by qualitative confirmation BSD method unless the case sample needs to be re-analyzed with OPI2 method for another analyte in the method.

22.10.6.4 If a case sample is positive for EDDP via OPI2 method, the sample will be re-analyzed by OPI2 method for the confirmation of the result.

22.10.6.5 If a case sample is positive for both methadone and EDDP, the sample will be re-analyzed by OPI2 method for the confirmation of **EDDP and of methadone if GC-MS drug screen has not been performed for methadone.**

#### 22.11 References

- 22.11.1 Coles, R., et al. Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC-MS-MS. *Journal of Analytical Toxicology*, 2007; 31:1-14
- 22.11.2 Levine, Barry and Kerrigan, Sarah, eds. "Opioids." *Principles of Forensic Toxicology*, 5th ed. Springer, Switzerland, 2020, 347-369.
- 22.11.3 United Chemical Technologies Inc. Free Opiates and Glucuronides in Urine Extracted by Clean Screen® DAU and Analyzed by LC-MS/MS. Application Note, 2014.
- 22.11.4 Method file: OPI2.m



## 23. Phencyclidine Confirmation by Liquid Chromatography-Tandem Mass Spectrometry

### 23.1 Purpose

23.1.1 A targeted analysis is performed for confirmatory analysis of phencyclidine (PCP) by solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Drugs are isolated from the matrix using a basic extraction. Deuterated internal standard and multiple reaction monitoring (MRM) are used in positive electrospray ionization (ESI) mode.

### 23.2 Scope

23.2.1 This procedure describes a confirmatory analysis of PCP in biological specimens including blood. Urine confirmations are reported only qualitatively.

### 23.3 Reagents and Solvents

23.3.1 100 mM Acetic acid

23.3.2 100 mM Phosphate buffer, pH 6.0

23.3.3 Elution Solvent: (78:20:2) methylene chloride: isopropanol: ammonium hydroxide. **In a 100 mL graduated cylinder add 20 mL isopropanol and 2 mL ammonium hydroxide, mix and then add 78 mL of methylene chloride. Cap and thoroughly mix. Prepare fresh daily.**

23.3.4 Mobile Phase A: H<sub>2</sub>O with 0.1% formic acid. Add 1 mL formic acid to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to one day after preparation).

23.3.5 Mobile Phase B (organic, may be in another position in method report): LC/MS grade methanol

23.3.6 Deionized (DI) water

23.3.7 Methanol

23.3.8 Blank blood (preserved with potassium oxalate and sodium fluoride)

23.3.9 Blank urine

### 23.4 Equipment and Materials

23.4.1 Air Displacement Pipettes (1000-5000 µL; 100-1000 µL; 20-200 µL; 2-20 µL)

23.4.2 Repeater pipette

23.4.3 UCT Clean Screen® DAU, 6 mL columns, 200 mg

23.4.4 Positive pressure SPE manifold

23.4.5 Analytical/Top-loading balance

23.4.6 pH Meter

23.4.7 Evaporator

23.4.8 Vortex mixer

23.4.9 Centrifuge



## 23.5 Instrumentation

### 23.5.1 LC Parameters

23.5.1.1 Column: Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7 μm

23.5.1.2 Agilent 1290 Infinity II LC system

Column Temperature	40°C	
Mobile Phase A	H <sub>2</sub> O with 0.1% formic acid	
Mobile Phase B	Methanol	
Flow Rate	0.6 mL/min	
Needle Wash	20 seconds	
Injection Volume	2 μL	
Gradient	Initial	10% B
	0.5 minutes	10% B
	2.5 minutes	75 % B
	2.51 minutes	90% B
	4.0 minutes	90% B
	4.01 minutes	95% B
	6.50 minutes	95% B
	Post time	2.0 minutes

### 23.5.2 MS/MS Parameters

23.5.2.1 Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	300°C
Gas Flow	6 L/min
Nebulizer Pressure	40 psi
Sheath Gas Heater	300°C
Sheath Gas Flow	11 L/min
Capillary	3500 V

23.5.2.2 MRM acquisition: PCP.M

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT**	Weighting Factor (Calibration model)
PCP-d5	249.2	164.1	79	12	2.68	1/x (linear)
		86.1		12		
		96.1		48		
PCP	244.2	159.1	79	12	2.69	
		86.1		12		
		91.1		40		





\*Quantifier ion in bold  
\*\*Retention times will vary

## 23.6 Standards and Solutions

### 23.6.1 PCP Working Standards for Calibrators (for blood and urine)

23.6.1.1 This analysis requires preparation of four solutions. The solutions contain concentrations of PCP at 10 µg/mL, 2 µg/mL, 1 µg/mL, and 0.1 µg/mL.

23.6.1.1.1 Preparation of the 10 µg/mL PCP calibrator stock: add 100 µL of 1 mg/mL CRM of PCP to a 10 mL volumetric flask and bring to volume with methanol.

23.6.1.1.2 Preparation of the 2 µg/mL PCP Calibrator: add 2 mL of 10 µg/mL PCP calibrator stock to a 10 mL volumetric flask and bring to volume with methanol.

23.6.1.1.3 Preparation of the 1 µg/mL PCP Calibrator: add 1 mL of the 10 µg/mL PCP calibrator stock to a 10 mL volumetric flask and bring to volume with methanol.

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

### 23.6.2 PCP Working Standards for Quality Controls (for blood and urine)

23.6.2.1 This analysis requires preparation of three solutions. The solutions contain concentrations of PCP at 10 µg/mL, 2 µg/mL and 0.25 µg/mL.

23.6.2.1.1 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.

23.6.2.1.2 Preparation of the 2 µg/mL PCP QC Solution: add 20 µL of 1 mg/mL CRM of PCP to a 10 mL volumetric flask and bring to volume with methanol.

23.6.2.1.3 Preparation of the 0.25 µg/mL PCP QC Solution: add 250 µL of 10 µg/mL PCP QC Stock Solution to a 10 mL volumetric flask and bring to volume with methanol.

### 23.6.3 PCP Internal Standard (for blood and urine)

23.6.3.1 This solution contains a concentration of 1 µg/mL of phencyclidine-d5.

23.6.3.1.1 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.



### 23.7 Fortification Guide

#### 23.7.1 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)
0.5	5.0	0.1	25	25
0.5	10	0.1	50	0
0.5	20	1	10	40
0.5	50	1	25	25
0.5	100	2	25	25
0.5	200	2	50	0

#### 23.7.2 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)
0.5	15	0.25	30	20
0.5	80	2	20	30
0.5	180	2	45	5

#### 23.7.3 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)
0.5	5.0	0.1 (Calibrator)	25	0
0.5	10	0.25 (QC)	20	5

#### 23.7.4 Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	50	1	25	0

### 23.8 Extraction Procedure

- 23.8.1 Allow all biological specimens to come to room temperature before starting the procedure.
- 23.8.2 Label all round bottom glass tubes accordingly.
- 23.8.3 Pipet 0.5 mL of drug-free blood/urine for matrix blank, negative control, calibrators, and positive controls into the appropriately labeled tubes.
- 23.8.4 Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 23.8.5 Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50 µL methanol. Vortex.



- 23.8.6 Using a repeater pipette, add 25  $\mu\text{L}$  of internal standard to each sample (except matrix blank) to obtain the final concentration of 50 ng/mL. Vortex.
- 23.8.7 Add 1 mL of 100 mM, sodium phosphate buffer, pH 6.0 to each tube. Vortex.
- 23.8.8 Place labeled SPE cartridges in the extraction manifold. Throughout the SPE, do not let SPE sorbent to dry, unless specified.
  - 23.8.8.1 Add 3 mL methanol and aspirate.
  - 23.8.8.2 Add 3 mL deionized water and aspirate.
  - 23.8.8.3 Add 1 mL 100 mM sodium phosphate buffer, pH 6.0 and aspirate.
  - 23.8.8.4 Pour samples in **appropriate** SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 23.8.8.5 Add 3 mL deionized water and aspirate.
  - 23.8.8.6 Add 1 mL 100 mM acetic acid and aspirate.
  - 23.8.8.7 Add 3 mL methanol and aspirate. Dry columns under full pressure for 5 minutes.
  - 23.8.8.8 Elute PCP with 3 mL elution solvent, prepared fresh daily.
- 23.8.9 Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 23.8.10 Reconstitute in **500  $\mu\text{L}$**  of 90:10 LC-MS grade water: methanol. Vortex.
- 23.8.11 Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 23.8.12 Load autosampler vials on the LC-MS/MS and inject 2  $\mu\text{L}$  using the PCP.m method.

### **23.9 Sequence Table**

- 23.9.1 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).
- 23.9.2 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**

Matrix blank  
5.0 ng/mL Calibrator  
10 ng/mL Calibrator  
25 ng/mL Calibrator  
50 ng/mL Calibrator  
100 ng/mL Calibrator  
200 ng/mL Calibrator  
Negative Control  
LQC  
HQC  
MQC  
10 Case Samples  
MQC  
10 Case Samples  
MQC  
10 Case Samples  
MQC  
HQC  
LQC

**Urine**

Matrix blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control  
10 Case Samples  
Positive Control

**23.10 Data Analysis**

23.10.1 Blood

- 23.10.1.1 The calibration curve for phencyclidine ranges from 5.0-200 ng/mL.
- 23.10.1.2 The low-quality control (LQC) for phencyclidine is 15 ng/mL.
- 23.10.1.3 The mid quality control (MQC) for phencyclidine is 80 ng/mL.
- 23.10.1.4 The high-quality control (HQC) for phencyclidine is 180 ng/mL.

23.10.2 Urine

- 23.10.2.1 The cut-off calibrator for phencyclidine is 5.0 ng/mL forced through zero.
- 23.10.2.2 The positive control (PQC) for phencyclidine is 10 ng/mL.

23.10.3 **Reinjection**

- 23.10.3.1 Reinjections can be made up to 72 hours after the completion of the initial injection of the particular sample(s).

23.10.4 **Carryover**

- 23.10.4.1 Carryover analysis will be performed if case sample concentration is greater than 400 ng/mL.

23.10.5 **Dilutions**



23.10.5.1 It is acceptable to dilute blood case samples x2, x5, or x10 prior to analysis.

### **23.11 References**

- 23.11.1 Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man, 12th ed. Seal Beach, CA: Biomedical Publications, 2020.
- 23.11.2 Dioumaeva, I. SAMSHA-Compliant LC/MS/MS Analysis of Phencyclidine in Urine with Agilent Bond Elut Plexa PCX and Agilent Poroshell 120. Agilent Technologies, Inc. Application Note, 2013.
- 23.11.3 Grieshaber, A., et al. Stability of Phencyclidine in Stored Blood Samples. *Journal of Analytical Toxicology*. 1998;22:515-519.
- 23.11.4 Kunsman, G.W., et al. Phencyclidine Blood Concentrations in DRE Cases. *Journal of Analytical Toxicology*. 1997;21:498-502.
- 23.11.5 Pestaner, J.P., et al. Sudden Death During Arrest and Phencyclidine Intoxication. *American Journal of Forensic Medicine and Pathology*. 2003;24:119-122.
- 23.11.6 United Chemical Technologies Inc. Phencyclidine in Whole Blood, Serum/Plasma and Urine Using: 200 mg Clean Screen® Extraction Column. *Clinical and Forensic Applications Manual*, 2009, pg. 129-130.
- 23.11.7 Method File: PCP.m



## 24. Novel Benzodiazepines Confirmation by Liquid Chromatography-Tandem Mass Spectrometry\*

\*Note: a new SOP

### 24.1. Purpose

- 24.1.1. A targeted analysis is performed for confirmatory analysis of 8-aminoclonazepam, clonazepam, flualprazolam, flubromazolam, bromazolam, etizolam, and flubromazepam using solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Deuterated internal standards and dynamic multiple reaction monitoring (dMRM) are used in positive electrospray ionization (ESI) mode.
- 24.1.2. This analysis is an add-on confirmatory analysis to the BNZ analysis.

### 24.2. Scope

- 24.2.1. This procedure describes a confirmatory analysis of selected novel benzodiazepines (NBZ) (8-aminoclonazepam, clonazepam, flualprazolam, flubromazolam, bromazolam, etizolam, and flubromazepam) in biological specimens including blood and urine. Blood confirmations are reported quantitatively. Urine confirmations are reported only qualitatively.

### 24.3. Reagents and Solvents

- 24.3.1. 100 mM Sodium acetate buffer, pH 4.5
- 24.3.2. Wash solvent: 100 mM Sodium acetate buffer, pH 4.5 with 2% acetonitrile. In a 100 mL graduated cylinder add 98 mL 100 mM Sodium acetate buffer, pH 4.5, and 2 mL acetonitrile. Cap and thoroughly mix. Prepare fresh daily.
- 24.3.3. Elution solvent: Ethyl acetate with 2% ammonium hydroxide. In a 100 mL graduated cylinder add 98 mL ethyl acetate and 2 mL ammonium hydroxide. Cap and thoroughly mix. Prepare fresh daily.
- 24.3.4. Mobile Phase A (Aqueous, H<sub>2</sub>O with 0.1% formic acid and 5 mM ammonium formate): Add 1 mL formic acid and 1 mL 5 mM ammonium formate aqueous solution to 1 L LC/MS grade H<sub>2</sub>O. Prepare fresh daily (may be used up to 1 day after preparation).
- 24.3.5. Mobile Phase B (Organic, acetonitrile with 0.1% formic acid): Add 1 mL formic acid to 1 L LC/MS grade acetonitrile. Prepare fresh daily (may be used up to 1 day after preparation).
- 24.3.6. Methanol
- 24.3.7. Acetonitrile
- 24.3.8. *n*-Hexane
- 24.3.9. Deionized (DI) water
- 24.3.10. LC/MS grade water
- 24.3.11. LC/MS grade acetonitrile
- 24.3.12. Blank blood (preserved with potassium oxalate and sodium fluoride)
- 24.3.13. Blank urine

### 24.4. Equipment and Materials

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



- 24.4.3. Analytical/Top-loading balance
- 24.4.4. pH meter
- 24.4.5. Evaporator
- 24.4.6. Vortex mixer
- 24.4.7. Centrifuge
- 24.4.8. Cerex® Clin II, 6 mL column, 50 mg
- 24.4.9. Positive pressure SPE manifold

## 24.5. Instrumentation

### 24.5.1. LC Parameters

- 24.5.1.1. Column: Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7 µm
- 24.5.1.2. Agilent 1290 Infinity II LC system

Column Temperature	50°C	
Mobile Phase A	H <sub>2</sub> O with 0.1% formic acid and 5 mM ammonium formate	
Mobile Phase B	Acetonitrile with 0.1% formic acid	
Flow Rate	0.5 mL/min	
Needle Wash	10 seconds	
Injection Volume	2 µL	
Gradient	Initial	15% B
	3.5 minutes	70% B
	4.5 minutes	95% B
	Stop time	5.50 minutes
	Post time	2.00 minutes

### 24.5.2. MS/MS Parameters

- 24.5.2.1. Agilent 6470 triple quadrupole LC/MS system

Ionization	ESI
Polarity	Positive
Gas Temperature	350 °C
Gas Flow	10 L/min
Nebulizer Pressure	30 psi
Sheath Gas Heater	400 °C
Sheath Gas Flow	12 L/min
Capillary	4,000 V



24.5.3. MRM acquisition: NBZ.m (Dynamic MRM (dMRM))

Drug	Precursor Ion	Product Ions*	Fragment (V)	Collision Energy (V)	RT (min)**	Weighting Factor (Calibration model)
8-Aminoclonazepam	324.1	<b>220.0</b> 296.1 205.0	170	48 28 52	1.76	1/X (Linear)
8-Aminoclonazepam-d4	328.1	<b>224.1</b> 300.1 222.9	168	48 28 40	1.70	
Clonazepam	354.1	<b>308.1</b> 280.0 177.0	161	32 40 72	2.66	1/X (Linear)
Clonazepam-d4	358.1	<b>312.1</b> 284.1 181.0	170	32 44 76	2.65	
Flualprazolam	327.1	<b>223.0</b> 299.1 292.1	176	52 32 28	2.82	1/X (Linear)
Flualprazolam-d4	331.1	<b>227.1</b> 303.1 296.1	170	52 32 32	2.81	
Flubromazolam	371.0	<b>223.0</b> 343.0 292.1	173	56 36 32	2.89	1/X (Linear)
Flubromazolam-d4	375.1	<b>227.1</b> 347.0 296.1	170	56 36 32	2.88	
Bromazolam‡	353.0	<b>205.0</b> 325.0 151.0	165	52 32 84	2.97	1/X (Linear)
Etizolam	343.1	<b>314.0</b> 259.0 224.0	158	16 28 36	3.09	1/X <sup>2</sup> (Linear)
Etizolam-d3	346.1	<b>317.0</b> 292.1 224.0	167	28 28 52	3.08	
Flubromazepam	333.0	<b>226.0</b> 183.9 104.1***	158	32 36 72	3.09	1/X (Linear)
Flubromazepam- <sup>13</sup> C <sub>2</sub> <sup>15</sup> N	336.0	<b>228.1</b> 184.9 105.1	170	32 40 76	3.09	

\*Quantifier Ion in bold  
 \*\*Retention times may vary  
 \*\*\*Not used for urine samples





‡Bromazolam shares internal standard (flubromazolam-d4) with flubromazolam

## 24.6. Standards and Solutions

### 24.6.1. Novel Benzodiazepines Working Standards for Calibrators (for blood and urine)

24.6.1.1. This analysis requires preparation of five solutions. The solutions contain 8-aminoclonazolam, clonazolam, flualprazolam, flubromazolam, bromazolam, etizolam, and flubromazepam at 1 µg/mL, 0.1 µg/mL, and 0.01 µg/mL.

24.6.1.1.1. Preparation of 1 mg/mL 8-Aminoclonazolam Calibrator Stock: Add 1 mL of acetonitrile into the manufacturer's vial of 1 mg 8-aminoclonazolam. Once 8-aminoclonazolam solid dissolves completely, transfer the resulting solution into an amber colored glass vial with screw-on cap.

24.6.1.1.2. Preparation of 1 mg/mL Bromazolam Calibrator Stock: Add 1 mL of methanol into the manufacturer's vial of 1 mg bromazolam. Once bromazolam solid dissolves completely, transfer the resulting solution into an amber colored glass vial with screw-on cap.

24.6.1.1.3. Preparation of 1 µg/mL Novel Benzodiazepines Calibrator: add 10 µL each of 1 mg/mL CRM of clonazolam, flualprazolam, flubromazolam, etizolam, and flubromazepam, and 10 µL each of 1 mg/mL calibrator stock solutions of 8-aminoclonazolam and bromazolam to a 10 mL volumetric flask and bring to volume with methanol.

24.6.1.1.4. Preparation of 0.1 µg/mL Novel Benzodiazepines Calibrator: add 1 mL of 1 µg/mL Novel Benzodiazepines Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

24.6.1.1.5. Preparation of 0.01 µg/mL Novel Benzodiazepines Calibrator: add 1 mL of 0.1 µg/mL Novel Benzodiazepines Calibrator to a 10 mL volumetric flask and bring to volume with methanol.

### 24.6.2. Novel Benzodiazepines Working Standards for Quality Controls (for blood and urine)

24.6.2.1. This analysis requires preparation of four solutions. The quality control solutions contain 8-aminoclonazolam, clonazolam, flualprazolam, flubromazolam, bromazolam, etizolam, and flubromazepam at 1 µg/mL and 0.1 µg/mL.

24.6.2.1.1. Preparation of 1 mg/mL 8-Aminoclonazolam Control Stock: follow the procedure in 24.6.1.1.1.

24.6.2.1.2. Preparation of 1 mg/mL Bromazolam Control Stock: follow the procedure in 24.6.1.1.2.

24.6.2.1.3. Preparation of 1 µg/mL Novel Benzodiazepines Control: add 10 µL each of 1 mg/mL CRM of clonazolam, flualprazolam, flubromazolam, etizolam, and flubromazepam and 10 µL each of 1 mg/mL control stock solutions of 8-aminoclonazolam and bromazolam to a 10 mL volumetric flask and bring to volume with methanol.

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

### 24.6.3. Novel Benzodiazepines Internal Standard



- 24.6.3.1. This analysis requires the preparation of three solutions. The solutions contain 8-aminoclonazepam-d4, clonazepam-d4, flualprazolam-d4, flubromazolam-d4, etizolam-d3, and flubromazepam-<sup>13</sup>C<sub>2</sub><sup>15</sup>N each at 1 µg/mL.
- 24.6.3.1.1. Preparation of 100 µg/mL 8-Aminoclonazepam-d4 Stock: Add approximately 1 mL of acetonitrile into the manufacturer's vial of 1 mg solid. Once the crystalline solid dissolves completely, transfer the resulting solution into a 10 mL volumetric flask. Rinse the manufacturer's vial with approximately 1 mL of acetonitrile and transfer the rinse to the 10 mL volumetric flask. Repeat the rinsing operation two additional times. Add acetonitrile to the volumetric flask and bring the total solution to 10 mL.
- 24.6.3.1.2. Preparation of 100 µg/mL Flubromazolam-d4 Stock: follow the procedure in 24.6.3.1.1 using methanol for reconstitution.
- 24.6.3.1.3. Preparation of 1 µg/mL Novel Benzodiazepines Internal Standard: add 100 µL each of 100 µg/mL CRM of clonazepam-d4, flubromazolam-d4, etizolam-d3, and flubromazepam-<sup>13</sup>C<sub>2</sub><sup>15</sup>N and 100 µL of flubromazolam-d4 and 8-aminoclonazepam-d4 stock solution to a 10 mL volumetric flask and bring to volume with methanol.
- 24.6.3.1.4. NOTE: Flubromazolam-d4 is used as the internal standard for flubromazolam and bromazolam.

## 24.7. Fortification Guide

### 24.7.1. 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)
0.5	1	0.01	50	0
0.5	2	0.1	10	40
0.5	5	0.1	25	25
0.5	10	0.1	50	0
0.5	20	1	10	40
0.5	50	1	25	25
0.5	70	1	35	15

### 24.7.2. 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)
0.5	3	0.1	15	35
0.5	30	1	15	35
0.5	60	1	30	20



24.7.3. 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)
0.5	1.0	0.01 (Calibrator)	50	0
0.5	2.0	0.1 (QC)	10	40

24.7.4. Blood and Urine Internal Standard

Volume of Sample (mL)	Target Concentration (ng/mL)	Drug Standard Concentration (µg/mL)	Volume of Drug Standard Added (µL)	Volume of Methanol Added (µL)
0.5	50	1	25	0

**24.8. Extraction Procedure**

24.8.1. Blood Extraction

- 24.8.1.1. Allow all biological specimens to come to room temperature before starting the extraction procedure.
- 24.8.1.2. Label all round bottom glass tubes accordingly.
- 24.8.1.3. Pipet 0.5 mL of drug-free blood for matrix blank, negative control, calibrators, and positive controls into the appropriately labeled tubes.
- 24.8.1.4. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 24.8.1.5. Pipet 0.5 mL of case samples into appropriately labeled tubes followed by 50 µL of methanol. Vortex.
- 24.8.1.6. Using a repeater pipette, add 25 µL of internal standard to each sample tube (except matrix blank) to obtain the final concentration of 50 ng/mL. Vortex.
- 24.8.1.7. Add 1.5 mL of cold acetonitrile dropwise while vortexing. Then vortex the resulting mixture for approximately 30 seconds.
- 24.8.1.8. Centrifuge sample tubes at approximately 3082 rcf for 10 minutes to achieve separation.
- 24.8.1.9. Transfer the supernatant into an appropriately labeled round bottom screw cap glass tube.
- 24.8.1.10. Add 3.5 mL of 100 mM sodium acetate buffer, pH 4.5. Vortex.
- 24.8.1.11. Place SPE columns in the extraction manifold. Throughout the SPE, do not let the SPE sorbent dry, unless specified.
  - 24.8.1.11.1. Add 3 mL methanol and aspirate.
  - 24.8.1.11.2. Add 3 mL deionized water and aspirate.
  - 24.8.1.11.3. Add 2 mL 100 mM sodium acetate buffer, pH 4.5 and aspirate.
  - 24.8.1.11.4. Pour samples in appropriate SPE columns. Aspirate slowly so that the sample takes at least 2 minutes to pass through the column.
  - 24.8.1.11.5. Add 3 mL deionized water and aspirate.
  - 24.8.1.11.6. Add 2 mL 100 mM sodium acetate buffer, pH 4.5 with 2% acetonitrile and aspirate. Dry columns under full pressure for 10 minutes.



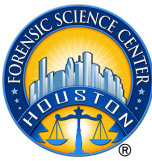
- 24.8.1.11.7. Add 2.5 mL hexane and aspirate. Dry columns under full pressure for 5 minutes.
- 24.8.1.11.8. Elute novel benzodiazepines with 3 mL of the NBZ elution solvent, prepared fresh daily.
- 24.8.1.12. Evaporate eluates to dryness at approximately 50 °C under nitrogen at 20 psi (2.9 L/min).
- 24.8.1.13. Reconstitute in 100 µL of 85:15 LC-MS grade water: acetonitrile and vortex.
- 24.8.1.14. Centrifuge sample tubes for approximately 10 seconds.
- 24.8.1.15. Transfer extracts to appropriately labeled autosampler vials with inserts and cap tightly.
- 24.8.1.16. Load autosampler vials on the LC-MS/MS and inject 2 µL using NBZ.m method.

#### 24.8.2. Urine Extraction

- 24.8.2.1. Pipet 0.5 mL drug-free urine for matrix blank, negative control, calibrators, and positive controls into appropriately labeled tubes.
- 24.8.2.2. Add corresponding drug standards to calibrators and positive controls, followed by the appropriate amount of methanol. Vortex.
- 24.8.2.3. Pipet 0.5 mL case samples into appropriately labeled tubes followed by 50 µL of methanol. Vortex.
- 24.8.2.4. Using a repeater pipette, add 25 µL of internal standard solution to each sample tube (except matrix blank) to obtain the final internal standard concentration of 50 ng/mL. Vortex.
- 24.8.2.5. Follow the blood extraction procedure from 24.8.1.10 – 24.8.1.16.

#### 24.9. **Sequence Table**

- 24.9.1. 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).
- 24.9.2. Every urine batch must contain a matrix blank, negative control, cut-off calibrator, and 10% positive controls that bracket case samples.



**NBZ sequence example:**

**Blood**

Matrix blank  
Calibrator 1.0 ng/mL  
Calibrator 2.0 ng/mL  
Calibrator 5.0 ng/mL  
Calibrator 10 ng/mL  
Calibrator 20 ng/mL  
Calibrator 50 ng/mL  
Calibrator 70 ng/mL  
Negative Control  
LQC  
HQC  
MQC  
10 case samples  
MQC  
10 case samples  
HQC  
MQC  
LQC

**Urine**

Matrix blank  
Cut-off Calibrator  
Negative Control  
Positive Control  
10 case samples  
Positive Control  
10 case samples  
Positive Control

**24.10. Data Analysis**

24.10.1. Blood

- 24.10.1.1. The calibration curve for Novel Benzodiazepines ranges from 1.0 to 70 ng/mL.
- 24.10.1.2. The low-quality control (LQC) for Novel Benzodiazepines is 3.0 ng/mL.
- 24.10.1.3. The mid-quality control (MQC) for Novel Benzodiazepines is 30 ng/mL.
- 24.10.1.4. The high-quality control (HQC) for Novel Benzodiazepines is 60 ng/mL.

24.10.2. Urine

- 24.10.2.1. The cut-off calibrator for Novel Benzodiazepines is 1.0 ng/mL forced through zero.
- 24.10.2.2. The positive control (PQC) for Novel Benzodiazepines is 2.0 ng/mL.

24.10.3. **Reinjection**

- 24.10.3.1. Reinjections can be made up to 72 hours for blood and/or urine samples after the completion of the initial injection of the sample(s).

24.10.4. **Carryover**

- 24.10.4.1. Carryover analysis will be performed if case sample concentration is greater than 150 ng/mL in blood or 100 ng/mL in urine.

24.10.5. **Dilutions**

- 24.10.5.1. It is acceptable to dilute blood case samples for quantitative analysis x2, x5, or x10 prior to analysis.



#### **24.11. References**

- 24.11.1. Brunetti, P., et al. Designer Benzodiazepines: A Review of Toxicology and Public Health Risks. *Pharmaceuticals*, Volume 14, Issue 6, June 2021, Pages 560-605.
- 24.11.2. Mastrovito, R. A., et al. The Development and Validation of a Novel Designer Benzodiazepines Panel by LC-MS-MS. *Journal of Analytical Toxicology*, Volume 45, Issue 5, June 2021, Pages 423–428.
- 24.11.3. Sofalvi, S., et al. Development and Validation of an LC-MS-MS Method for the Detection of 40 Benzodiazepines and Three Z-Drugs in Blood and Urine by Solid-Phase Extraction. *Journal of Analytical Toxicology*, Volume 44, Issue 7, September 2020, Pages 708–717.
- 24.11.4. Garcia, L., et al. Novel and Nonroutine Benzodiazepines and Suvorexant by LC-MS-MS. *Journal of Analytical Toxicology*, Volume 45, Issue 5, June 2021, Pages 462–474.
- 24.11.5. Acquisition method file: NBZ.m
- 24.11.6. Quantitative data analysis method file: NBZ\_Quantitative\_Blood
- 24.11.7. Qualitative data analysis method file: NBZ\_Qualitative\_Urine



## **25. Validation of Quantitative Methods**

### **25.1 Purpose**

25.1.1 This procedure is intended to define the minimum parameters and sets of experiments to validate a quantitative method.

### **25.2 Scope**

25.2.1 This procedure applies to all quantitative bio-analytical methods.

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. The decision regarding which performance characteristics require additional validation shall be based on consideration of the specific parameters likely to be affected by the change(s). These changes may include, but are not limited to a) analytical conditions, b) instrumentation, c) sample processing, and d) data software.

### **25.3 Validation Parameters**

25.3.1 The following validation parameters are adapted from the ASB Standard 036, First Edition 2019, Standard Practices for Method Validation in Forensic Toxicology. Refer to the document for more 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.

- Bias and precision
- Calibration model
- Carryover
- Interference studies
- Ionization suppression/enhancement (for applicable techniques, such as LC-MS)
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Dilution integrity (if applicable)
- Processed sample stability (if applicable)

### **25.4 Validation Plan**

25.4.1 Before starting any validation experiments, a validation plan will be established, which includes the instrument method, sample preparation technique, validation



parameters, evaluation of additional validation parameters, and acceptance criteria for a specific analysis.

- 25.4.2 Information for the plan can be in multiple locations (e.g., instrument method in batch records; sample preparation technique in the SOP draft for the analysis; and validation parameters and acceptance criteria in the validation template file).
- 25.4.3 The validation plan can be adjusted during the validation as needed. However, appropriate experiments will be performed to evaluate the change and demonstrate the method is fit for purpose.

## **25.5 Validation Experiments**

### **25.5.1 General**

- 25.5.1.1 All validation experiments shall be conducted using fortified samples for each matrix type for which the method is intended.
- 25.5.1.2 Validation studies shall be conducted in a manner similar to casework.
- 25.5.1.3 Fortified matrix samples should be prepared from reference materials that are from a different source (e.g., supplier or lot number) than used to prepare calibration samples. In instances where the same source shall be utilized, separate weighing factors or solutions shall be used to prepare these samples.
- 25.5.1.4 Some validation experiments may be conducted concurrently with the same fortified samples.

### **25.5.2 Bias and precision**

#### **25.5.2.1 Procedure**

- 25.5.2.1.1 Prepare pooled fortified matrix samples using a minimum of three separate samples per concentration at three different concentration pools (low, medium and high) over five different runs (n=15 per concentration).
- 25.5.2.1.2 Low concentrations are no more than approximately 3 times the lower LOQ and the high concentrations are within approximately  $\geq 80\%$  of the upper LOQ, unless otherwise noted. Medium concentrations are near the midpoint of the low and high concentrations.
- 25.5.2.1.3 Bias studies can be conducted concurrently with precision studies.
- 25.5.2.1.4 The different runs used to evaluate precision may be performed within the same day, provided a different calibration curve is used for each run.

#### **25.5.2.2 Data analysis**

- 25.5.2.2.1 Use the established calibration curve to calculate the concentration of the analyte of interest.
- 25.5.2.2.2 Calculate bias, within-run precision and between-run precision. These parameters are calculated for each concentration.





$$\text{Bias} = \frac{\text{Grand mean of calculated concentrations} - \text{Nominal concentration}}{\text{Nominal Concentration}} \times 100$$

$$\text{Within - Run Precision} = \frac{\text{Standard deviation of a single run}}{\text{Average of a single run}} \times 100$$

$$\text{Between - Run Precision} = \frac{\text{Standard deviation of all runs}}{\text{Average of all runs}} \times 100$$

25.5.2.2.3 The largest calculated within-run and between-run %CV for each concentration shall be used to assess precision acceptability.

25.5.2.2.4 Both within-run and between-run precisions may be calculated using the one-way analysis of variance (ANOVA) approach with the varied factor (run number) as the grouping variable.

#### 25.5.2.3 Acceptance criteria

25.5.2.3.1 Bias: %bias  $\leq$ 20%

25.5.2.3.2 Within-run precision: %CV  $\leq$ 20%

25.5.2.3.3 Between-run precision: %CV  $\leq$ 20%

25.5.2.3.4 Certain analytical methods (e.g., blood alcohol analysis) should require a much lower %bias and %CV ( $\leq$ 10% or better).

#### 25.5.3 Calibration model

25.5.3.1 The most appropriate calibration model should be determined during method development. Once the working range is determined, signal response (e.g., peak area ratio of analyte and internal standard) is correlated with analyte concentration in the sample. The calibration model is the mathematical model describing this correlation.

25.5.3.2 The use of matrix-matched calibrator samples is encouraged, but not required.

25.5.3.3 The calibrator samples span the range of concentrations expected in day-to-day operations.

#### 25.5.3.4 Procedure

25.5.3.4.1 Prepare calibrator samples spanning the range of concentrations expected in day-to-day operations. At least six (non-zero) concentration levels shall be used.

25.5.3.4.2 A minimum of five replicates per concentration shall be used. The replicates to establish the calibration model may be in the same or in separate runs. All data points from the five replicates shall be plotted



together to establish the calibration model. The origin shall not be included as a calibration point.

#### 25.5.3.5 Data analysis

25.5.3.5.1 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.

25.5.3.5.1.1 These combined curves will be used to verify the weighting scheme based on the response ratios for each calibration point. The response ratio is defined as the peak area of the analyte divided by the peak area of the internal standard.

25.5.3.5.2 Using the data from the appropriate weighting scheme, perform an unweighted linear regression of  $C_{\text{nominal}}$  v.  $C_{\text{calculated}}$  where  $C_{\text{nominal}}$  is nominal (labeled or established) concentration of calibrator and  $C_{\text{calculated}}$  is the concentration obtained from calibration curve.

25.5.3.5.2.1 These data will be used to evaluate the linearity of the analytical measurement range.

25.5.3.5.3 Process each individual run using the selected weighting scheme.

25.5.3.5.3.1 These data will be used to validate the calibration range.

25.5.3.5.4 If neither un-weighted nor weighted linear regression achieves acceptable correlation, other appropriate non-linear model should be applied. Ultimately, the simplest calibration model that best fits the concentration-response relationship should be used.

25.5.3.5.5 If a linear calibration model has been established, fewer calibration samples (i.e., fewer levels or single/fewer replicates) may be used for routine analysis. However, if fewer calibration samples are chosen, the same calibrators (e.g., number, replicates, and concentration levels) shall be used to construct the calibration curves for the bias and precision studies. Further the calibration data shall include the lowest and highest calibration levels used to establish the model, as well as include no fewer than four non-zero calibration points.

25.5.3.5.6 Once the calibration model is established for a validated method, it shall not be arbitrarily changed to achieve acceptable quality control results during a given analytical run.

#### 25.5.3.6 Acceptance criteria

25.5.3.6.1 Verification of weighting scheme

25.5.3.6.1.1 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.



$$\%RE = \frac{C_{\text{calculated}} - C_{\text{nominal}}}{C_{\text{nominal}}} \times 100$$

#### 25.5.3.6.2 Validation of linearity

25.5.3.6.2.1A calibration model shall not be evaluated simply via its correlation coefficient (r).

25.5.3.6.2.2 Visual examination using residual plots of combined calibration curve with appropriate weighting must verify linearity across analytical range.

25.5.3.6.2.2.1 The linear regression of  $C_{\text{nominal}}$  vs  $C_{\text{calculated}}$  should meet the following requirements, unless otherwise noted:

- 95% CI of slope should include 1.
- 95% CI of intercept should include 0.

25.5.3.6.2.2.2 Check for outliers that may be eliminated if found to be statistically significant (e.g., outside of  $\pm 3$  standard deviations).

25.5.3.6.2.2.3 Random distribution of individual residuals around the zero line suggests that a linear model is appropriate.

#### 25.5.3.6.3 Validation of calibration

25.5.3.6.3.1 Visual examination of calibration curve must verify linearity across analytical range.

25.5.3.6.3.1.1 %RE of each calibrator, as determined by the weighted linear regression of the individual run, must be  $\leq \pm 20\%$ .

#### 25.5.4 Carryover

##### 25.5.4.1 Procedure

25.5.4.1.1 Analyze blank matrix samples immediately after a highly concentrated sample or reference material. Perform the analysis three times.

25.5.4.1.2 The high concentration used in the carryover study can be limited to the upper LOQ.

##### 25.5.4.2 Data analysis

25.5.4.2.1 Compare the responses of the matrix blank samples to the method's LOD response for the analyte of interest.

##### 25.5.4.3 Acceptance criteria

25.5.4.3.1 No analyte carryover is observed in the matrix blank samples; the response in the blank samples is  $\leq 20\%$  (10% for the alcohol analysis) of the average response of LOQ.

#### 25.5.5 Interference studies



#### 25.5.5.1 Procedure

- 25.5.5.1.1 Matrix interference – Analyze a minimum of ten different sources of blank matrix (without IS) when possible.
- 25.5.5.1.2 Interference from stable-isotope internal standards – (a) Analyze a blank matrix fortified with IS but no analyte of interest. Additionally, (b) analyze a blank matrix sample fortified with the analyte(s) at a concentration near the upper LOQ without IS.
- 25.5.5.1.3 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. Previously analyzed case samples or neat reference materials of the potential interference(s) may be used instead of fortified matrix samples.

#### 25.5.5.2 Data analysis

- 25.5.5.2.1 Matrix interference – Evaluate response of any peak at the retention time of the analyte of interest.
- 25.5.5.2.2 Interference from stable-isotope internal standards – Evaluate response of any peak at the retention time of the analyte of interest (a) and at the retention time of the internal standard (b).
- 25.5.5.2.3 Interference from commonly encountered exogenous analytes – Use the established calibration curve to calculate the concentration of the analyte of interest.

#### 25.5.5.3 Acceptance criteria

- 25.5.5.3.1 Matrix interference – Response of blank matrix must be <20% of the average response of LOQ.
- 25.5.5.3.2 Interference from stable-isotope internal standards – Response of blank matrix must be <20% of the average response of LOQ.
- 25.5.5.3.3 Interference from commonly encountered exogenous analytes - Concentration of analytes of interest must be within  $\pm 20\%$  of the average concentration obtained in the Bias and Precision studies.

#### 25.5.6 Ionization suppression/enhancement (if applicable)

##### 25.5.6.1 Procedure

- 25.5.6.1.1 Prepare two sets of samples fortified with analyte at low and high concentrations spanning the calibration curve.
- 25.5.6.1.2 Prepare neat standards in reconstitution solvents at one low and one high concentrations (neat samples, Set 1). Inject each of these neat standards a minimum of six times to establish a mean peak area for each concentration.



25.5.6.1.3 Extract blank matrix samples from a minimum of ten different sources, when possible, in duplicate. Fortify each extracted blank matrix sample with either low or high concentration solutions (Set 2).

25.5.6.1.4 Ionization suppression/enhancement can alternatively be evaluated using the post-column infusion method.

#### 25.5.6.2 Data Analysis

25.5.6.2.1 Determine a mean analyte area of samples in each group at each concentration.

25.5.6.2.2 Calculate the ionization suppression/enhancement effect at each concentration:

$$\text{Ionization suppression or enhancement (\%)} = \left( \frac{\text{Mean peak area of Set 2}}{\text{Mean peak area of Set 1}} - 1 \right) \times 100$$

#### 25.5.6.3 Acceptance Criteria

25.5.6.3.1 The average suppression/enhancement of the analyte's target ion (or ion transition)  $\leq \pm 25\%$  or the %CV of the suppression/enhancement  $\leq 20\%$ .

25.5.6.3.2 If the average suppression/enhancement exceeds  $\pm 25\%$ , the influence on these parameters will be assessed by at least tripling the number of different sources of blank matrices as applicable.

#### 25.5.7 Limit of detection (LOD)

25.5.7.1 There are multiple ways to estimate LOD. The LOD shall be determined by one of the following approaches.

##### 25.5.7.2 Procedure

25.5.7.2.1 Define the lowest non-zero calibrator or the decision point concentration as the LOD; analyze a minimum of three LOD samples per run over three runs; use a minimum of three different sources of blank matrix. Or

25.5.7.2.2 Analyze a minimum of three different sources of blank matrix fortified at decreasing concentrations in duplicate for three days to experimentally determine the LOD. The LOD is the lowest concentration that yields reproducible response  $\geq 3.3$  times the noise level of the background signal in an area around the analyte peak and achieves acceptable detection/identification criteria. Or

25.5.7.2.3 Analyze a minimum of three calibration curves using a minimum of three different sources of blank matrix. The LOD can be estimated from the standard deviation of the y intercept ( $S_y$ ) multiplied by 3.3 and divided by the average slope ( $Avg_m$ ).

##### 25.5.7.3 Data analysis



- 25.5.7.3.1 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.
- 25.5.7.3.2 Use the instrument software to evaluate the signal-to-noise ratio in the blanks and the LOD samples as applicable. The signal-to-noise ratio can be manually calculated. If manually calculated, the signal is defined as the height response of the analyte peak and the noise is defined as the amplitude between the highest and lowest point of the baseline in an area around the analyte peak.

$$\text{Signal-to noise} = \frac{\text{Height of analyte}}{\text{amplitude of noise}}$$

#### 25.5.7.4 Acceptance criteria

25.5.7.4.1 The LOD is the lowest concentration that:

25.5.7.4.1.1 Yields a reproducible instrument response greater than or equal to 3.3 times the noise level of the background signal in an area around the analyte peak.

25.5.7.4.1.2 Achieves acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).

#### 25.5.8 Limit of quantitation (LOQ)

##### 25.5.8.1 Procedure

25.5.8.1.1 Analyze a minimum of three samples per run over three runs to demonstrate all detection, identification, bias and precision criteria are met.

25.5.8.1.2 If the lowest non-zero calibrator is used as the LOD, perform the LOQ experiment using at least three different sources of blank matrix.

##### 25.5.8.2 Data analysis

25.5.8.2.1 Use the established calibration curve to quantify the analyte of interest.

25.5.8.2.2 Calculate the bias, within-run precision, and between-run precision.

##### 25.5.8.3 Acceptance criteria

25.5.8.3.1 Bias: %bias  $\leq$ 20%

25.5.8.3.2 Within-run precision: %CV  $\leq$ 20%

25.5.8.3.3 Between-run precision: %CV  $\leq$ 20%

#### 25.5.9 Dilution integrity (if applicable)

##### 25.5.9.1 Procedure

25.5.9.1.1 Dilute 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).

25.5.9.1.2 Analyze a minimum of three replicates of each dilution per run over five runs.

25.5.9.2 Data analysis

25.5.9.2.1 Calculate bias and within-run precision with at least one concentration pool.

25.5.9.3 Acceptance criteria

25.5.9.3.1 The bias and within-run precision values of diluted samples must be within the acceptance criteria (typically 20%) of the bias and within-run precision studies.

25.5.10 Processed sample stability: general (if applicable)

25.5.10.1 Procedure

25.5.10.1.1 If processed samples cannot be analyzed within a reasonable amount of time due to an atypical event (e.g., instrument failure or power loss), the stability of the analyte in the processed samples over that time period should be evaluated.

25.5.10.1.1.1 Determine the point in the procedure at which a break may occur and how long the break may be.

25.5.10.1.1.2 Prepare blank matrix samples fortified at low and high concentrations in a large enough volume to complete the studies.

25.5.10.1.1.3 A single source of blank matrix may be used to evaluate processed sample stability.

25.5.10.1.1.4 Extract multiple aliquots from each concentration set using the method under validation.

25.5.10.1.1.5 Combine, mix, and divide the processed samples for a given concentration pool into different autosampler vials for instrument analysis.

25.5.10.1.1.6 Analyze the first vials of each concentration in triplicate to establish the time zero responses.

25.5.10.1.1.7 Analyze the remaining vials in triplicate at different time intervals that represent the typical time range expected for processed samples to wait before being injected into the instrument.

25.5.10.2 Data analysis

25.5.10.2.1 Monitor the responses of the processed samples stored at different time intervals and compare them to the time zero responses.

25.5.10.3 Acceptance criteria



25.5.10.3.1 The average signal (peak area or ratios of peak area of analyte to internal standard) compared to the time zero average signal within the method's acceptable bias.

25.5.11 Processed sample stability: autosampler stability (if applicable)

25.5.11.1 Procedure

25.5.11.1.1 If there is a possibility that autosampler vials need to be re-injected (e.g., instrument failure or power loss), the stability of the extracts in the autosampler vials should be evaluated.

25.5.11.1.1.1 Determine the maximum residence time of the extracts in the autosampler vials to be tested, possibly including the time a run may sit un-injected over a weekend in the autosampler vials.

25.5.11.1.1.2 Autosampler stability of calibrators: prepare calibrators in a large enough volume to complete the studies; multiple sets of calibrators can alternatively be prepared. Extract them using the method under validation. Inject the calibrators to establish the time zero responses. Re-inject the autosampler vials at different time intervals (e.g., 24, 48, and 72 h).

25.5.11.1.1.3 Autosampler stability of controls: prepare blank matrix samples fortified at low and high concentrations in a large enough volume to complete the studies. At the time zero, prepare two sets of low and high control samples in triplicate each. Extract them using the method under validation. Inject the first set and leave the second set unpunctured. (Re)-inject both sets at different time intervals. Analysis of each time interval should include injection of calibrators so that ion ratios and calculated concentrations of the re-injected control samples can be evaluated.

25.5.11.1.2 Data analysis

25.5.11.1.2.1 Monitor the responses of the autosampler samples stored at different time intervals and compare them to the time zero responses.

25.5.11.1.2.2 **Verify specified time interval calibrators' ion ratios and concentrations calculated against themselves AND against the Time Zero calibrators are acceptable based on the method's criteria.**

25.5.11.1.2.3 Verify controls' ion ratios and concentrations calculated against the Time Zero calibrators and the specified time interval calibrators (e.g., 48 h) are acceptable based on the method's criteria.

25.5.11.1.3 Acceptance criteria





25.5.11.1.3.1 The average signal (peak area or ratios of peak area of analyte to internal standard) compared to the time zero average signal within the method's acceptable bias.

Autosampler stability experiment example:

Time Zero	24 h	48 h	72 h
Inject Calibrators, Set 1 Low Controls x3 and Set 1 High Controls x3	Re-inject Calibrators and Set 1 Controls	Re-inject Calibrators and Set 1 Controls	Re-inject Calibrators and Set 1 Controls
Prepare but do not inject Set 2 Low Controls x3 and Set 2 High Controls x3	Inject Set 2 Controls	Inject Set 2 Controls (need to prepare another set of Set 2 at Time Zero)	Inject Set 2 Controls (need to prepare another set of Set 2 at Time Zero)

## 25.6 Documentation requirements

25.6.1 The method validation records shall include a summary of the validation studies and their results. The summary shall include the following:

25.6.1.1 Scope

25.6.1.2 Validation plan

25.6.1.3 Description of all the parameters evaluated; if any of the parameters were not evaluated, then the reason shall be stated or justified

25.6.1.4 Sample preparation steps to include concentrations and matrices

25.6.1.5 Raw data or reference to where the raw data are stored

25.6.1.6 Results and calculations

25.6.1.7 Conclusions

25.6.1.8 References

25.6.1.9 Documentation of management review and approval

25.6.1.10 Individuals involved in the method validation

25.6.1.11 Specific instrumentation

25.6.1.12 Dates

25.6.2 Method validation documentation shall also include a copy of the newly developed analytical method or a reference to its location. Further, validation documentation should be permanently retained according to the HFSC Record Retention Policy.

## 25.7 References

25.7.1 ASB Standard O36, First Edition, 2019. Standard Practices for Method Validation in Forensic Toxicology.



## **26. Validation of Reportable Qualitative Methods**

### **26.1. Purpose**

26.1.1. This procedure is intended to define the minimum parameters and sets of experiments to validate a reportable qualitative method.

### **26.2. Scope**

26.2.1. This procedure applies to all qualitative bio-analytical methods which produce reportable results.

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:

26.2.3. Development of a new method.

26.2.3.1. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

26.2.3.2. Transfer of a validated method to a new instrument.

26.2.4. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. The decision regarding which performance characteristics require additional validation shall be based on consideration of the specific parameters likely to be affected by the change(s). These changes may include, but are not limited to a) analytical conditions, b) instrumentation, c) sample processing, and d) data software.

### **26.3. Validation Parameters**

26.3.1. The following validation parameters are adapted from the ASB Standard 036, First Edition 2019, Standard Practices for Method Validation in Forensic Toxicology. Refer to the document for more 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.

26.3.2. Carryover

26.3.3. Interference studies

26.3.4. Ionization suppression/enhancement (for applicable techniques, such as LC-MS)

26.3.5. Limit of detection

26.3.6. Processed sample stability (if applicable)

26.3.7. Hydrolysis recovery (if applicable; not included in the ASB standard)

### **26.4. Validation Plan**

26.4.1. Before starting any validation experiments, a validation plan will be established, which includes the instrument method, sample preparation technique, validation parameters, evaluation of additional validation parameters and acceptance criteria for a specific analysis.



- 26.4.2. Information for the plan can be in multiple locations (e.g., instrument method in batch records; sample preparation technique in the SOP draft for the analysis; and validation parameters and acceptance criteria in the validation template file).
- 26.4.3. The validation plan can be adjusted during the validation as needed. However, appropriate experiments will be performed to evaluate the change and demonstrate the method is fit for purpose.

## **26.5. Validation Experiments**

### 26.5.1. General

- 26.5.1.1. All validation experiments shall be conducted using fortified samples for each matrix type for which the method is intended.
- 26.5.1.2. Validation studies shall be conducted in a manner similar to casework.
- 26.5.1.3. Fortified matrix samples should be prepared from reference materials that are from a different source (e.g., supplier or lot number) than used to prepare calibration samples. In instances where the same source shall be utilized, separate weighing factors or solutions shall be used to prepare these samples.
- 26.5.1.4. Some validation experiments may be conducted concurrently with the same fortified samples.

### 26.5.2. Carryover

#### 26.5.2.1. Procedure

- 26.5.2.1.1. Analyze blank matrix samples immediately after a highly concentrated sample or reference material. Perform the analysis three times.

#### 26.5.2.2. Data analysis

- 26.5.2.2.1. Compare the responses of the matrix blank samples to the method's LOD response for the analyte of interest.

#### 26.5.2.3. Acceptance criteria

- 26.5.2.3.1. No analyte carryover is observed in the matrix blank samples; the response in the blank samples is <20% of the average response of LOD.
  - 26.5.2.3.1.1. The criterion is being negative for the GC-MS full scan method due to its spectra not available for library matching <60%.

### 26.5.3. Interference studies

#### 26.5.3.1. Procedure

- 26.5.3.1.1. Matrix interference – Analyze a minimum of ten different sources of blank matrix (without IS) when possible.
- 26.5.3.1.2. Interference from stable-isotope internal standards – (a) Analyze a blank matrix fortified with IS but no analyte of interest. Additionally, (b) analyze a blank matrix sample fortified with the analyte(s) at a high concentration without IS.



26.5.3.1.3. Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the cutoff calibrator concentration and potential interferences at high therapeutic or lethal concentrations. Previously analyzed case samples or neat reference materials of the potential interference(s) may be used instead of fortified matrix samples.

#### 26.5.3.2. Data analysis

26.5.3.2.1. Matrix interference – Evaluate response of any peak at the retention time of the analyte of interest.

26.5.3.2.2. Interference from stable-isotope internal standards – Evaluate response of any peak at the retention time of the analyte of interest (a) and at the retention time of the internal standard (b).

26.5.3.2.3. Interference from commonly encountered exogenous analytes – Use the established calibration curve to calculate the concentration of the analyte of interest.

#### 26.5.3.3. Acceptance criteria

26.5.3.3.1. Matrix interference – Response of blank matrix must be <20% of the average response of LOD.

26.5.3.3.1.1. The criterion is being negative for the GC-MS full scan method due to its spectra not available for library matching <60%.

26.5.3.3.2. Interference from stable-isotope internal standards – Response of blank matrix must be <20% of the average response of LOD.

26.5.3.3.3. Interference from commonly encountered exogenous analytes - Concentration of analytes of interest must be  $\pm 20\%$  of the average response of LOD.

26.5.3.3.3.1. The criterion is  $\pm 50\%$  of the average LOD response for the GC-MS full scan method due to its inherent variability in analyte responses.

#### 26.5.4. Ionization suppression/enhancement (if applicable)

##### 26.5.4.1. Procedure

26.5.4.1.1. Prepare two sets of samples fortified with analyte at the cutoff and at selected high concentrations.

26.5.4.1.2. Fortify **reconstitution** solvent with the cutoff and high concentration solutions (neat samples, Set 1). Inject each of these neat standards a minimum of six times to establish a mean peak area for each concentration.

26.5.4.1.3. Extract blank matrix samples from a minimum of ten different sources, when possible, in duplicate. Fortify each extracted blank matrix sample with the cutoff and high concentration solutions (Set 2).

26.5.4.1.4. Ionization suppression/enhancement can alternatively be evaluated using the post-column infusion method.

##### 26.5.4.2. Data Analysis



26.5.4.2.1. Determine a mean analyte area of samples in each group at each concentration.

26.5.4.2.2. Calculate the ionization suppression/enhancement effect at each concentration:

$$\text{Ionization suppression or enhancement (\%)} = \left( \frac{\text{Mean peak area of Set 2}}{\text{Mean peak area of Set 1}} - 1 \right) \times 100$$

#### 26.5.4.3. Acceptance Criteria

26.5.4.3.1. The average suppression/enhancement of the analyte's target ion (or ion transition)  $\leq \pm 25\%$  or the %CV of the suppression/enhancement  $\leq 20\%$ .

26.5.4.3.2. If the average suppression/enhancement exceeds  $\pm 25\%$ , the influence on these parameters will be assessed by at least tripling the number of different sources of blank matrices as applicable.

#### 26.5.5. Limit of detection (LOD)

26.5.5.1. There are multiple ways to estimate LOD. The LOD shall be determined by one of the following approaches.

##### 26.5.5.2. Procedure

26.5.5.2.1. Define the decision point concentration as the LOD; analyze a minimum of three LOD samples per run over three runs; use a minimum of three different sources of blank matrix. Or

26.5.5.2.2. Analyze a minimum of three different sources of blank matrix fortified at decreasing concentrations in duplicate for three days to experimentally determine the LOD. The LOD is the lowest concentration that yields reproducible response  $\geq 3.3$  times the noise level of the background signal in an area around the analyte peak and achieves acceptable detection/identification criteria. Or

26.5.5.2.3. Analyze a minimum of three calibration curves. The LOD can be estimated from the standard deviation of the y intercept ( $S_y$ ) multiplied by 3.3 and divided by the average slope ( $Avg_m$ ).

##### 26.5.5.3. Data analysis

26.5.5.3.1. 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.

26.5.5.3.2. Use the instrument software to evaluate the signal-to-noise ratio in the blanks and the LOD samples as applicable. The signal-to-noise ratio can be manually calculated. If manually calculated, the signal is defined as the height response of the analyte peak and the noise is defined as the amplitude between the highest and lowest point of the baseline in an area around the analyte peak.



$$\text{Signal-to noise} = \frac{\text{Height of analyte}}{\text{amplitude of noise}}$$

#### 26.5.5.4. Acceptance criteria

26.5.5.4.1. The LOD is the lowest concentration that:

26.5.5.4.1.1. Yields a reproducible instrument response greater than or equal to 3.3 times the noise level of the background signal in an area around the analyte peak.

26.5.5.4.1.2. Achieves acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).

#### 26.5.6. Processed sample stability (if applicable)

##### 26.5.6.1. Procedure

26.5.6.1.1. If processed samples cannot be analyzed within a reasonable amount of time due to atypical event (e.g., instrument failure or power loss), the stability of the analyte in the processed samples over that time period should be evaluated.

26.5.6.1.1.1. Determine the point in the procedure at which a break may occur and how long the break may be.

26.5.6.1.1.2. Prepare blank matrix samples fortified at the cutoff calibrator and positive control concentrations in a large enough volume to complete the studies.

26.5.6.1.1.3. A single source of blank matrix may be used to evaluate processed sample stability.

26.5.6.1.1.4. Extract multiple aliquots from each concentration set using the method under validation.

26.5.6.1.1.5. Combine, mix, and divide the processed samples for a given concentration pool into different autosampler vials for instrument analysis.

26.5.6.1.1.6. Analyze the first vials of each concentration in triplicate to establish the time zero responses.

26.5.6.1.1.7. Analyze the remaining vials in triplicate at different time intervals that represent the typical time range expected for processed samples to wait before being injected into the instrument.

##### 26.5.6.2. Data analysis

26.5.6.2.1. Monitor the responses of the processed samples stored at different time intervals and compare them to the time zero responses.

##### 26.5.6.3. Acceptance criteria

26.5.6.3.1. The average signal (peak area or ratios of peak area of analyte to internal standard) must be  $\pm 20\%$  of the time zero average signal.

#### 26.5.7. Hydrolysis recovery (if applicable)

##### 26.5.7.1. Procedure



- 26.5.7.1.1. Analyze a minimum of three positive controls and two glucuronide positive controls (hydrolysis controls) per run over a minimum of three runs.
- 26.5.7.2. Data analysis
  - 26.5.7.2.1. 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.
- 26.5.7.3. Acceptance Criteria
  - 26.5.7.3.1. If a method satisfies the requirement of hydrolysis controls being positive, its hydrolysis recovery is considered acceptable.

## **26.6. Documentation requirements**

- 26.6.1. The method validation records shall include a summary of the validation studies and their results. The summary shall include the following:
  - 26.6.1.1. Scope
  - 26.6.1.2. Validation plan
  - 26.6.1.3. Description of all the parameters evaluated; if any of the parameters were not evaluated, then the reason shall be stated or justified
  - 26.6.1.4. Sample preparation steps to include concentrations and matrices
  - 26.6.1.5. Raw data or reference to where the raw data are stored
  - 26.6.1.6. Results and calculations
  - 26.6.1.7. Conclusions
  - 26.6.1.8. References
  - 26.6.1.9. Documentation of management review and approval
  - 26.6.1.10. Individuals involved in the method validation
  - 26.6.1.11. Specific instrumentation
  - 26.6.1.12. Dates
- 26.6.2. Method validation documentation shall also include a copy of the newly developed analytical method or a reference to its location. Further, validation documentation should be permanently retained according to the HFSC Record Retention Policy.

## **26.7. References**

- 26.7.1. ASB Standard 036, First Edition, 2019. Standard Practices for Method Validation in Forensic Toxicology.



## **27. Validation of Immunoassay Using Commercial Kits**

### **27.1. Purpose**

27.1.1. This procedure is intended to define a series of experiments that are relevant elements of a qualitative immunoassay method validation.

### **27.2. Scope**

27.2.1. This procedure applies to immunoassay-based methods using commercially available kits.

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:

27.2.2.1. Development of a new method.

27.2.2.2. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

27.2.2.3. Transfer of a validated method to a new instrument.

27.2.3. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. The decision regarding which performance characteristics require additional validation shall be based on consideration of the specific parameters likely to be affected by the change(s). These changes may include, but are not limited to a) analytical conditions, b) instrumentation, c) sample processing, and d) data software.

### **27.3. Validation Parameters**

27.3.1. The following validation parameters are adapted from the ASB Standard 036, First Edition 2019, Standard Practices for Method Validation in Forensic Toxicology. Refer to the document for more 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.

27.3.2. Limit of detection (LOD)

27.3.2.1. 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 regardless of whether the laboratory uses the manufacturer's recommended LOD or not.

27.3.3. Precision at the decision point

27.3.4. Processed sample 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.





## 27.4. Validation Plan

- 27.4.1. Before starting any validation experiments, a validation plan will be established, which includes the instrument method, sample preparation technique, validation parameters, evaluation of additional validation parameters and acceptance criteria for a specific analysis.
- 27.4.2. Information for the plan can be in multiple locations (e.g., instrument method in batch records; sample preparation technique in the SOP draft for the analysis; and validation parameters and acceptance criteria in the validation template file).
- 27.4.3. The validation plan can be adjusted during the validation as needed. However, appropriate experiments will be performed to evaluate the change and demonstrate the method is fit for purpose.

## 27.5. Validation Experiments

### 27.5.1. General

- 27.5.1.1. All validation experiments shall be conducted using fortified samples for each matrix type for which the method is intended.
- 27.5.1.2. Validation studies shall be conducted in a manner similar to casework.
- 27.5.1.3. Fortified matrix samples should be prepared from reference materials that are from a different source (e.g., supplier or lot number) than used to prepare calibration samples. In instances where the same source shall be utilized, separate weighing factors or solutions shall be used to prepare these samples.
- 27.5.1.4. Some validation experiments may be conducted concurrently with the same fortified samples.

### 27.5.2. Limit of detection (LOD)

- 27.5.2.1. The laboratory may assign the decision point (i.e., cutoff concentration) as the LOD for immunoassay. Most of these assays are known to cross-react with numerous drugs and metabolites. When a laboratory declares to their customers that they are able to detect specific analytes demonstrating low cross-reactivity (less than or equal to the target analyte) using the immunoassay, they shall verify their ability to reliably detect these compounds.
  - 27.5.2.1.1. The Section provides the cross-reactivity information of other compounds from the manufacturer in the scope of analysis in the report.

### 27.5.3. Precision at the decision point

#### 27.5.3.1. Procedure

- 27.5.3.1.1. Analyze three separate samples from three different concentration pools over five different runs, including negative controls (generally no more than 50% below decision point; low), controls at the decision point, and positive controls (generally no more than 100% above decision point; high).



- 27.5.3.1.2. For each level, 15 data points are generated. The same lots of reagents and cut-off material are used during the test period.
- 27.5.3.1.3. For Enzyme Linked Immunosorbent Assays (ELISA), the difference between the absorbance of a negative sample ( $B_0$ ) and the absorbance of a specimen (B) should be used as a percentage:  $[B/B_0] \times 100$  (% binding) and not an absolute value.
- 27.5.3.1.4. At least one  $B_0$  is analyzed to establish  $B_0$  for each run.
- 27.5.3.2. Data analysis
  - 27.5.3.2.1. Calculate the grand mean ( $n=15$ ) and related grand standard deviation for each concentration pool.
- 27.5.3.3. Acceptance criteria
  - 27.5.3.3.1.  $\%CV \leq 20$  % binding at each concentration using all 15 sample results per concentration.
  - 27.5.3.3.2. The grand mean  $\pm$  two standard deviations of the low and high concentration pools shall not overlap the mean of the decision point.
- 27.5.3.4. Monitored data
  - 27.5.3.4.1. Overall  $\%CV$  and separation of the calibrator and controls of the absorbance 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.
    - 27.5.3.4.1.1. Overall  $\%CV$  of the absorbance  $<20\%$  at each of the three levels.
    - 27.5.3.4.1.2. The absorbance grand mean  $\pm$  two standard deviations of the low and high concentration pools should not overlap the mean of the decision point.

## 27.6. Documentation requirements

- 27.6.1. The method validation records shall include a summary of the validation studies and their results. The summary shall include the following:
  - 27.6.1.1. Scope
  - 27.6.1.2. Validation plan
  - 27.6.1.3. Description of all the parameters evaluated, if any of the parameters were not evaluated, then the reason shall be stated or justified
  - 27.6.1.4. Sample preparation steps to include concentrations and matrices
  - 27.6.1.5. Raw data or reference to where the raw data are stored
  - 27.6.1.6. Results and calculations
  - 27.6.1.7. Conclusions
  - 27.6.1.8. References
  - 27.6.1.9. Documentation of management review and approval
  - 27.6.1.10. Individuals involved in the method validation
  - 27.6.1.11. Specific instrumentation
  - 27.6.1.12. Dates



27.6.2. Method validation documentation shall also include a copy of the newly developed analytical method or a reference to its location. Further, validation documentation should be permanently retained according to the HFSC Record Retention Policy.

### **27.7. References**

27.7.1. ASB Standard 036, First Edition, 2019. Standard Practices for Method Validation in Forensic Toxicology.



## **28. Validation of Screening Methods**

### **28.1. Purpose**

28.1.1. This procedure is intended to define the minimum parameters and sets of experiments to validate a screening method.

### **28.2. Scope**

28.2.1. This procedure applies to all qualitative bio-analytical methods whose results need to be confirmed by a confirmatory method.

28.2.2. Method validation is required to verify the performance parameters of a method are fit for purpose. Validation is required after the following events occur:

28.2.3. Development of a new method.

28.2.3.1. Modification of a validated method to improve its performance or extend its use beyond that for which it was originally validated.

28.2.3.2. Transfer of a validated method to a new instrument.

28.2.4. Modifications to existing methods or transfer of a validated method to new instruments may not require re-validation of all parameters. The decision regarding which performance characteristics require additional validation shall be based on consideration of the specific parameters likely to be affected by the change(s). These changes may include, but are not limited to a) analytical conditions, b) instrumentation, c) sample processing, and d) data software.

### **28.3. Validation Parameters**

28.3.1. The following validation parameters are adapted from the ASB Standard 036, First Edition 2019, Standard Practices for Method Validation in Forensic Toxicology. Refer to the document for more 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.

28.3.2. Interference studies

28.3.3. Limit of detection

28.3.4. Ionization suppression/enhancement (for applicable techniques, such as LC-MS)

28.3.5. Processed sample stability (if applicable)

### **28.4. Validation Plan**

28.4.1. Before starting any validation experiments, a validation plan will be established, which includes the instrument method, sample preparation technique, validation parameters, evaluation of additional validation parameters and acceptance criteria for a specific analysis.



- 28.4.2. Information for the plan can be in multiple locations (e.g., instrument method in batch records; sample preparation technique in the SOP draft for the analysis; and validation parameters and acceptance criteria in the validation template file).
- 28.4.3. The validation plan can be adjusted during the validation as needed. However, appropriate experiments will be performed to evaluate the change and demonstrate the method is fit for purpose.

## **28.5. Validation Experiments**

### 28.5.1. General

- 28.5.1.1. All validation experiments shall be conducted using fortified samples for each matrix type for which the method is intended.
- 28.5.1.2. Validation studies shall be conducted in a manner similar to casework.
- 28.5.1.3. Fortified matrix samples should be prepared from reference materials that are from a different source (e.g., supplier or lot number) than used to prepare calibration samples. In instances where the same source shall be utilized, separate weighing factors or solutions shall be used to prepare these samples.
- 28.5.1.4. Some validation experiments may be conducted concurrently with the same fortified samples.

### 28.5.2. Interference studies

#### 28.5.2.1. Procedure

- 28.5.2.1.1. Matrix interference – Analyze a minimum of ten different sources of blank matrix (without IS) when possible.
- 28.5.2.1.2. Interference from stable-isotope internal standards – (a) Analyze a blank matrix fortified with IS but no analyte of interest. Additionally, (b) analyze a blank matrix sample fortified with the analyte(s) at a concentration near the upper LOQ without IS.
- 28.5.2.1.3. Interference from commonly encountered exogenous analytes – Analyze blank matrix fortified with analytes of interest at the cutoff calibrator concentration and potential interferences at high therapeutic or lethal concentrations. Previously analyzed case samples or neat reference materials of the potential interference(s) may be used instead of fortified matrix samples.

#### 28.5.2.2. Data analysis

- 28.5.2.2.1. Matrix interference – Evaluate response of any peak at the retention time of the analyte of interest.
- 28.5.2.2.2. Interference from stable-isotope internal standards – Evaluate response of any peak at the retention time of the analyte of interest (a) and at the retention time of the internal standard (b).



28.5.2.2.3. Interference from commonly encountered exogenous analytes – Use the established calibration curve to calculate the concentration of the analyte of interest.

28.5.2.3. Acceptance criteria

28.5.2.3.1. Matrix interference – Response of blank matrix must be <20% of the average response of LOD.

28.5.2.3.1.1. The criterion is being negative for the GC-MS full scan method due to its spectra not available for library matching <60%.

28.5.2.3.2. Interference from stable-isotope internal standards – Response of blank matrix must be <20% of the average response of LOD.

28.5.2.3.3. Interference from commonly encountered exogenous analytes - Concentration of analytes of interest must be  $\pm 20\%$  of the average response of LOD.

28.5.2.3.3.1. The criterion is  $\pm 50\%$  of the average LOD response for the GC-MS full scan method due to its inherent variability in analyte responses.

28.5.3. Limit of detection (LOD)

28.5.3.1. There are multiple ways to estimate LOD. The LOD shall be determined by one of the following approaches.

28.5.3.2. Procedure

28.5.3.2.1. Define the decision point concentration as the LOD; analyze a minimum of three LOD samples per run over three runs; use a minimum of three different sources of blank matrix. Or

28.5.3.2.2. Analyze a minimum of three different sources of blank matrix fortified at decreasing concentrations in duplicate for three days to experimentally determine the LOD. The LOD is the lowest concentration that yields reproducible response  $\geq 3.3$  times the noise level of the background signal in an area around the analyte peak and achieves acceptable detection/identification criteria. Or

28.5.3.2.3. Analyze a minimum of three calibration curves. The LOD can be estimated from the standard deviation of the y intercept ( $S_y$ ) multiplied by 3.3 and divided by the average slope ( $Avg_m$ ).

28.5.3.3. Data analysis

28.5.3.3.1. 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.

28.5.3.3.2. Use the instrument software to evaluate the signal-to-noise ratio in the blanks and the LOD samples as applicable. The signal-to-noise ratio can be manually calculated. If manually calculated, the signal is defined as the height response of the analyte peak and the noise is defined as the amplitude between the highest and lowest point of the baseline in an area around the analyte peak.



$$\text{Signal-to noise} = \frac{\text{Height of analyte}}{\text{amplitude of noise}}$$

#### 28.5.3.4. Acceptance criteria

28.5.3.4.1. The LOD is the lowest concentration that:

28.5.3.4.1.1. Yields a reproducible instrument response greater than or equal to 3.3 times the noise level of the background signal in an area around the analyte peak.

28.5.3.4.1.2. Achieves acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).

#### 28.5.4. Ionization suppression/enhancement (if applicable)

##### 28.5.4.1. Procedure

28.5.4.1.1. Prepare two sets of samples fortified with analyte at low and high concentrations spanning the calibration curve.

28.5.4.1.2. Fortify **reconstitution** solvent with one low and one high concentration solutions (neat samples, Set 1). Inject each of these neat standards a minimum of six times to establish a mean peak area for each concentration.

28.5.4.1.3. Extract blank matrix samples from a minimum of ten different sources, when possible, in duplicate. Fortify each extracted blank matrix sample with low and high concentration solutions (Set 2).

28.5.4.1.4. Ionization suppression/enhancement can alternatively be evaluated using the post-column infusion method.

##### 28.5.4.2. Data Analysis

28.5.4.2.1. Determine a mean analyte area of samples in each group at each concentration.

28.5.4.2.2. Calculate the ionization suppression/enhancement effect at each concentration:

$$\text{Ionization suppression or enhancement (\%)} = \left( \frac{\text{Mean peak area of Set 2}}{\text{Mean peak area of Set 1}} - 1 \right) \times 100$$

##### 28.5.4.3. Acceptance Criteria

28.5.4.3.1. The average suppression/enhancement of the analyte's target ion (or ion transition)  $\leq \pm 25\%$  or the %CV of the suppression/enhancement  $\leq 20\%$ .

28.5.4.3.2. If the average suppression/enhancement exceeds  $\pm 25\%$ , the influence on these parameters will be assessed by at least tripling the number of different sources of blank matrices as applicable.

#### 28.5.5. Processed sample stability (if applicable)

##### 28.5.5.1. Procedure



- 28.5.5.1.1. If processed samples cannot be analyzed within a reasonable amount of time due to atypical event (e.g., instrument failure or power loss), the stability of the analyte in the processed samples over that time period should be evaluated.
  - 28.5.5.1.1.1. Determine the point in the procedure at which a break may occur and how long the break may be.
  - 28.5.5.1.1.2. Prepare blank matrix samples fortified at the cutoff calibrator and positive control concentrations in a large enough volume to complete the studies.
  - 28.5.5.1.1.3. A single source of blank matrix may be used to evaluate processed sample stability.
  - 28.5.5.1.1.4. Extract multiple aliquots from each concentration set using the method under validation.
  - 28.5.5.1.1.5. Combine, mix, and divide the processed samples for a given concentration pool into different autosampler vials for instrument analysis.
  - 28.5.5.1.1.6. Analyze the first vials of each concentration in triplicate to establish the time zero responses.
  - 28.5.5.1.1.7. Analyze the remaining vials in triplicate at different time intervals that represent the typical time range expected for processed samples to wait before being injected into the instrument.
- 28.5.5.2. Data analysis
  - 28.5.5.2.1. Monitor the responses of the processed samples stored at different time intervals and compare them to the time zero responses.
- 28.5.5.3. Acceptance criteria
  - 28.5.5.3.1. The average signal (peak area or ratios of peak area of analyte to internal standard) must be  $\pm 20\%$  of the time zero average signal.

## **28.6. Documentation requirements**

- 28.6.1. The method validation records shall include a summary of the validation studies and their results. The summary shall include the following:
  - 28.6.1.1. Scope
  - 28.6.1.2. Validation plan
  - 28.6.1.3. Description of all the parameters evaluated, if any of the parameters were not evaluated, then the reason shall be stated or justified
  - 28.6.1.4. Sample preparation steps to include concentrations and matrices
  - 28.6.1.5. Raw data or reference to where the raw data are stored
  - 28.6.1.6. Results and calculations
  - 28.6.1.7. Conclusions
  - 28.6.1.8. References
  - 28.6.1.9. Documentation of management review and approval
  - 28.6.1.10. Individuals involved in the method validation





28.6.1.11. Specific instrumentation

28.6.1.12. Dates

28.6.2. Method validation documentation shall also include a copy of the newly developed analytical method or a reference to its location. Further, validation documentation should be permanently retained according to the HFSC Record Retention Policy.

## **28.7. References**

28.7.1. ASB Standard 036, First Edition, 2019. Standard Practices for Method Validation in Forensic Toxicology.



## 29. Retrograde Extrapolation and the Widmark Equation

### 29.1. Purpose

29.1.1. This procedure outlines the process of performing retrograde extrapolations and calculations using the Widmark equation.

### 29.2. Scope

29.2.1. 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.

### 29.3. Equations and Calculations

#### 29.3.1. Retrograde Extrapolation

$$BAC_E = [(BAC_K - UM) + (t \times e_{low})] \text{ to } [(BAC_K + UM) + (t \times e_{high})]$$

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 (0.010 g/100 mL/hour); and  $e_{high}$  = elimination rate range high limit (0.025 g/100 mL/hour).

#### 29.3.2. The Widmark Equation

$$a = BAC \times \rho \times V_{d,low} \text{ to } BAC \times \rho \times V_{d,high}$$

OR

$$BAC = a/(\rho \times V_{d,high}) \text{ to } a/(\rho \times V_{d,low})$$

Where:  $a$  = estimated amount of alcohol in body (g);  $BAC$  = blood ethanol concentration, either known, estimated, or hypothetical (g/100 mL);  $\rho$  = weight (g)  $V_{d,low}$  = volume of distribution low limit (0.45 L/kg = 0.0045 mL/g); and  $V_{d,high}$  = volume of distribution high limit (0.81 L/kg = 0.0081 mL/g).

#### 29.3.3. Number of standard drinks (N)

$$N = a/14$$

OR

$$N = (BAC \times \rho \times V_d)/14$$

#### 29.3.4. 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)

#### 29.3.5. Facts

##### 29.3.5.1. 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

##### 29.3.5.2. Facts needed in order to use the Widmark equation:

- BAC or Number of drink(s)
- Weight (lb)

29.3.5.3. Additional facts such as time of the last meal, height, age, sex, 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; number of drink(s) is not used in retrograde extrapolation.

#### 29.3.6. Assumptions and Limitations

29.3.6.1. Retrograde extrapolation may only be calculated if the individual is solely undergoing elimination of ethanol at the time of the stop (i.e., in the post-absorptive phase).

29.3.6.1.1. 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.

29.3.6.2. A retrograde extrapolation will not be conducted if the facts in 29.3.5.1 are not given by the requestor unless it is a hypothetical scenario with disclosed assumptions.

29.3.6.3. A retrograde extrapolation will not be conducted if the  $BAC_k$  is less than 0.02 g/100 mL.

29.3.6.4. It is assumed the individual falls within the elimination rates 0.010 and 0.025 g/100 mL/h. The range includes the majority of the population regardless of age, sex, and drinking experience. Extreme cases, such as alcoholics during detoxification and ultra-rapid metabolizers or some cases of extreme dietary or liver conditions, may have an elimination rate greater than 0.025 g/100 mL/h or less than 0.01 g/100 mL/h, respectively (29.4.2 and 29.4.3).

29.3.6.5. The Widmark equation will not be used, and the number of standard drinks cannot be calculated, if the facts in 29.3.5.2 are not given by the requestor.



- 29.3.6.6. It is assumed the individual falls within the  $V_d$  values 0.45 and 0.81 L/kg. This range includes the majority of the population, both male and female, with different body mass index (BMI) values (29.4.7).
- 29.3.6.7. First pass metabolism will not be considered.
- 29.3.6.8. Gastroesophageal Reflux Disease (GERD) will not be considered, but an individual with GERD may absorb alcohol for more than 2 hours (29.4.6).
- 29.3.6.9. 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 29.4.6 and 29.4.7.
- 29.3.6.10. Type of food: carbohydrates (fructose) and amino acids (glycine) may slow the rate of ethanol absorption.
- 29.3.6.11. Type of drink: drinks with high ethanol concentrations (liquor) have absorption compared to wine or beer.
- 29.3.6.12. Trauma, shock, and massive blood loss decrease the ethanol absorption rate.
- 29.3.6.13. Gastric surgery increases the ethanol absorption rate.
- 29.3.6.14. Drugs affecting gastric emptying may change the ethanol absorption rate.
- 29.3.7. Performing calculations
- 29.3.7.1. A requestor may submit facts for a specific case using LAB-088 (Retrograde Extrapolation and Widmark Calculation Request), or an equivalent method. The analyst will use LAB-087 (Retrograde Extrapolation and Widmark Calculation Worksheet), or an equivalent method, to conduct the calculations. LAB-088 or other retrograde extrapolation or the Widmark calculation-related documents provided by the requestor will become part of the case record.
- 29.3.7.1.1. Performing alcohol calculations is a forensic service request and a request for calculation should be submitted ahead of trial and technically reviewed. Calculations will not be performed for the first time during live testimony.
- 29.3.7.2. When calculating retrograde extrapolation estimations, the lower and upper range values will be rounded to two decimals.
- 29.3.7.3. When calculating the estimated amount of alcohol in the body (in number of drinks) using the Widmark equation, the lower and upper range values will be rounded to one decimal.
- 29.3.7.4. When calculating the estimated BAC using the Widmark equation, the lower and upper range values will be rounded to two decimals.
- 29.3.7.5. When calculating the estimated number of standard drinks using the Widmark equation, the result will be in a whole number range, encompassing the calculated numbers (e.g., if  $N = 1.6$  and  $2.5$ , the range will be reported as 1-3).



29.3.7.6. An elimination rate calculated from two or more test results shall not be used in place of the elimination rate range of 0.010 - 0.025 g/100 mL/h.

#### 29.4. References

- 29.4.1. Jones, AW. Body Mass Index and Blood-Alcohol Calculations. *Journal of Analytical Toxicology*. 2007;31:177-178.
- 29.4.2. 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.
- 29.4.3. 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.
- 29.4.4. 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.
- 29.4.5. 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.
- 29.4.6. Booker, JL and Renfro, K. The Effects of Gastroesophageal Reflux Disease on Forensic Breath Alcohol Testing. *Journal of Forensic Sciences*. 2015;60:1516-1522.
- 29.4.7. Maskell, P and Cooper, G. The contribution of body mass and volume of distribution to the estimated uncertainty associated with the Widmark Equation. *Journal of Forensic Sciences*. 2020;65:1676-1684.
- 29.4.8. Caplan, YH and Goldberger, BA., eds., *Garriott's Medicolegal Aspects of Alcohol*, 6th ed. Tucson, AZ: Lawyers & Judges Publishing Company, Inc., 2015.
- 29.4.9. OSAC 2020-S-0003 Guidelines for Performing Alcohol Calculations in Forensic Toxicology



## 30. Estimation of Uncertainty of Measurement (UM)

### 30.1. Purpose

30.1.1. This procedure describes an estimation of UM, a statistical calculation of known variables that contribute to the inherent variance of the overall result at a desired confidence level.

### 30.2. Scope

30.2.1. 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.

30.2.2. The UM is not calculated for qualitative methods whose results are reported positive or negative rather than numerical values.

### 30.3. Calculating and Reporting of UM

30.3.1. For the Volatiles analysis, the confidence level used is 99.73%,  $k = 3$ .

30.3.2. For the GC/LC-MS analysis, the confidence level used is 95.45%,  $k = 2$ .

30.3.3. The expanded uncertainty is rounded to two significant digits (e.g., 9.4% or 24%), which will be then used to calculate the UM value associated with a quantitative test result.

30.3.3.1. The reported expanded uncertainty values are on the UM packets and/or LAB-079 for drug methods and the UM packet for the alcohol method.

30.3.4. UM is reported in the same units and decimal places as the test results.

30.3.5. Report UM will be limited to at most two significant digits, unless there is a documented rationale for reporting additional significant digits.

30.3.6. Reported UM is calculated using the following formula:

$$\text{Reported UM} = \pm (\text{Reported concentration} \times \text{UM}\%)$$

30.3.6.1. For the Volatiles analysis, reported UM is rounded to three decimal places for blood alcohol samples except for diluted samples (e.g., liquids), which will follow the decimal place of the reported test result after incorporation of the dilution factor.

30.3.6.2. For the GC/LC-MS analysis, reported UM is rounded to one decimal place if the test result is  $< 10$  and to the whole number if the test result is  $\geq 10$ .

30.3.7. The uncertainty of measurement is evaluated every two years at the minimum.

30.3.8. The Uncertainty of Measurement Spreadsheets are available in the laboratory in a retrievable format.

### 30.4. Uncertainty Components

30.4.1.1. Measurement Reproducibility – accounts for the control with the largest Percent Relative Standard Deviation or pooled %RSD based on historical control data.



$$\%RSD_{\text{pooled}} = \sqrt{\frac{(n^1 - 1)\%RSD_1^2 + (n^2 - 1)\%RSD_2^2 + \dots + (n^k - 1)\%RSD_k^2}{n^1 + n^2 + \dots + n^k - k}}$$

k is the number of pooled control lots and n is the number of measurements obtained from a specific lot.

- 30.4.1.2. CRM Uncertainty – accounts for the CRM used as a calibrator with the largest reported uncertainty.
- 30.4.1.3. 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.
- 30.4.1.4. Duplicates (Volatiles) – accounts for the maximum allowed difference between analytically-obtained values of duplicate case samples.
- 30.4.1.5. 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.
- 30.4.1.6. 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.

### 30.5. Re-calculation

- 30.5.1. UM will be reviewed and if needed, recalculated every two years at the minimum.
- 30.5.2. Approval dates of re-calculated UM values by the Toxicology section and Quality division may precede their effective dates as the section coordinates associated documents and/or practice. UM effective dates will be noted in the file name of UM packets.

### 30.6. References

- 30.6.1. National Institute of Standards and Technology. SOP No. 29. Standard Operating Procedure for the Assignment of Uncertainty. 2014.
- 30.6.2. ASCLD/LAB Guidance on the Estimation of Measurement Uncertainty - Annex A. Details on the NIST 8 Step Process. 2011.



## **31. Opinions and Testimony**

### **31.1. Purpose**

31.1.1. This procedure describes the acceptable extent and contents of expert opinions and testimony provided by a forensic analyst/toxicologist. The requirements listed in this procedure follow and were adapted from the ANSI/ASB Best Practice Recommendation 037, First Edition 2019: Guidelines for Opinions and Testimony in Forensic Toxicology.

### **31.2. Scope**

31.2.1. It is intended for written and oral expert toxicological opinions regarding the interpretation of analytical toxicology findings.

### **31.3. Written and Oral Opinions**

31.3.1. Written expert toxicological opinions regarding the interpretation of analytical toxicology findings should not be part of the basic analytical toxicology report. A separate expert report or other communication format (e.g., email) should be used to convey such opinions.

31.3.2. Written expert toxicological opinions should include a comment that states that the opinions may be subject to change based upon new information that becomes available (e.g., case history, additional analytical testing, new research findings and publications, etc.).

31.3.3. An expert toxicological opinion, whether written or oral, should:

31.3.3.1. be expressed in a clear, coherent manner;

31.3.3.2. be based on established scientific principles and foundations;

31.3.3.3. be based on the totality of information available, including case history, observations, circumstances, and other relevant information, and not based solely on analytical results;

31.3.3.4. include information on case specific documents and records reviewed;

31.3.3.5. have references that support the opinion;

31.3.3.5.1. References should be provided either in the expert report or made available upon request.

31.3.3.6. clearly state any assumptions made; and

31.3.3.7. clearly state any known limitations of the opinion.

### **31.4. Appropriate Opinions and Testimony by a Toxicologist**

31.4.1. Through testimony and offering an expert toxicological opinion, it is generally appropriate for a toxicologist to:

31.4.1.1. discuss a laboratory report and any analytical work that supports that report. Applicable limitations should also be addressed.

31.4.1.2. qualify a reported concentration in the context of a given case as subtherapeutic, therapeutic, toxic or lethal when that statement can be backed by appropriate references, databases and/or other relevant information.





- 31.4.1.3. address the pharmacokinetics/toxicokinetics, as well as the pharmacodynamics/toxicodynamics of drugs or other chemicals.
- 31.4.1.4. discuss the toxicological impact of the presence, absence and/or stability of drugs or other chemicals.
- 31.4.1.5. address impairment for the average individual to the extent that effects are consistent with documented pharmacodynamic and toxicodynamic properties of the substance and within the context of a given case.
- 31.4.1.6. perform or discuss toxicological calculations that are generally accepted in the field and can be supported by research and references, provided appropriate limitations are cited. For example, ethanol back extrapolation calculations may be performed.

### **31.5. Inappropriate Opinions and Testimony by a Toxicologist**

- 31.5.1. The following are considered to generally be inappropriate opinions and/or testimony for a toxicologist to offer, as they currently lack consensus within the scientific community or are generally beyond the scope of the toxicologist's expertise.
  - 31.5.1.1. A toxicologist should not opine as to the absolute cause of death of an individual. This does not preclude a toxicologist from addressing the toxicological impact of any substances found in the toxicological analysis of specimens from the case.
  - 31.5.1.2. A toxicologist should not address behavioral intent based solely upon a drug concentration.
  - 31.5.1.3. A toxicologist should not opine as to a specific individual's degree of impairment based solely on a quantitative result.
  - 31.5.1.4. A toxicologist should not imply impairment of an individual based on analytical findings from urine, hair or other matrices unless supported by the literature.
  - 31.5.1.5. A toxicologist should not opine as to the absolute cause of an accident.
  - 31.5.1.6. A toxicologist should not perform extrapolation calculations for drugs other than ethanol.
  - 31.5.1.7. A toxicologist should not calculate the dose of a drug based on a postmortem drug concentration in blood.
  - 31.5.1.8. A toxicologist should not calculate the dose of a drug (with the exception of ethanol) through body burden calculations.
  - 31.5.1.9. A toxicologist should not opine as to the effects of a drug or combination of drugs on a specific individual without context of a given case. This does not preclude a toxicologist from addressing general effects of drugs at varying concentrations (Section 30.4).
  - 31.5.1.10. A toxicologist should not use words such as "scientific certainty" or "reasonable degree of scientific certainty", unless required by jurisdictional regulations.

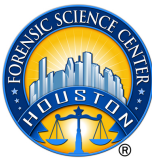
### **31.6. Documented Review**

- 31.6.1. **Prior to testifying to findings of another scientist (i.e., the testifying scientist did not serve as the certifying scientist, case technical reviewer, or case administrative reviewer), the testifying scientist will complete a documented review (e.g., an entry in LIMS) of relevant case and examination records.**



### **31.7. References**

- 31.7.1. ANSI/ASB Best Practice Recommendation 037, First Edition, 2019. Guidelines for Opinions and Testimony in Forensic Toxicology.



## 32. Appendix 1. Abbreviations

=	equal
+/- or ±	plus or minus
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
%	percent
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
AMDIS	automated mass spectral deconvolution and identification system
AMP	amphetamines confirmation or immunoassay
AMU	atomic mass units
AN	acid-neutral
AR	administrative review
Avg. Abs	average absorbance
BAC	blood alcohol concentration
BAN	basic, acidic, and neutral drug screen
BARB	barbiturates
BAR	barbiturates immunoassay
BCR	blood collection report located on inner plastic box
BE or BZE	benzoylecgonine
BH	biohazard
BNZ	benzodiazepines/zolpidem confirmation or benzodiazepines immunoassay
BQC	whole blood ethanol control
BSD	GCMS drug screen and qualitative confirmation
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BZ or BENZO	benzodiazepines
BUP	buprenorphine or buprenorphine immunoassay
c	concentration
cal	calibrator
CAR or CARISO	carisoprodol or carisoprodol immunoassay
CE	cocaethylene
C/M	carisoprodol/meprobamate
CI	confidence interval



CNS	central nervous system
COA	certificate of analysis
COC	cocaine, cocaine confirmation, cocaine and metabolites immunoassay, <b>chain of custody, or certificate of calibration</b>
COQC	carryover quality control
CRM	certified reference material
CV	coefficient of variation
DCM	dichloromethane or methylene chloride
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
FEN	fentanyl immunoassay
FENT	fentanyl
FID	flame ionization detector
FN	false negative
FP	false positive
g	grams
GC	gas chromatograph/chromatography
H <sub>2</sub> O	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
h/hr(s)	hour(s)
HS	headspace
HSA	hexane saturated with acetonitrile
IB	inner box



IPA	Isopropanol
I.S., IS, ISTD	internal standard
ISV	insufficient sample volume
kg	kilogram
L	liter
lb	pound
LC	liquid chromatography
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
MET	methamphetamine immunoassay
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/spectrometry
MS/MS	tandem mass spectrometer/spectrometry
MTDN	methadone
MU, UM	measurement uncertainty or uncertainty of measurement
N/A, NA, or na	not applicable
NAM	not acceptable match
NBZ	novel benzodiazepines confirmation
ND	not detected
Neg	negative
NF	not found
ng	nanogram
NPD	nitrogen phosphorus detector
NQC	negative quality control
OB	outer box
OFC or Ofc	officer



OPI	opioids confirmation or immunoassay
OXY	oxycodone or oxycodone immunoassay
oz	ounce
PBS	phosphate buffer saline
PCP	phencyclidine, PCP confirmation, or PCP immunoassay
PFFA (PFAA)	pentafluoropropionic anhydride (pentafluoropropionic acid anhydride)
PFTBA	perfluorotributylamine
Pkg	package
Pos	positive
PQC	positive quality control
PWP	package with parent
QC	quality control
QNS	quantity not sufficient
QQQ	triple quadruple mass spectrometer/spectrometry; interchangeable with MS/MS
QS	quantum satis (bring to volume)
RCF	relative centrifugal force
RE	relative error
RL	reporting limit
Rpt	reporting
RRT	relative retention time
RT	retention time
RSD	relative standard deviation
SCR	Single case report
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
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
TR	technical review
ULOQ	upper limit of quantification
UM	uncertainty of measurement
UNK or unk	unknown



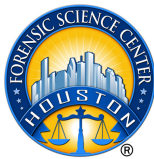
$V_d$	volume of distribution
w/	with
w/out	without
ZOL or ZOLP	zolpidem



### 33. 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.