Department of Forensic Science

TOXICOLOGY PROCEDURES MANUAL

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1 INTRODUCTION

1.1 Introduction

The Section analyzes blood and other biological samples for the presence of alcohol, drugs and poisons. Types of cases analyzed include DUI/DUID (Driving under the Influence/ Driving under the Influence of Drugs), drug-facilitated crimes, death investigations, non-implied consent cases (possession, manslaughter and search warrants), and alcoholic beverage control (ABC) investigations.

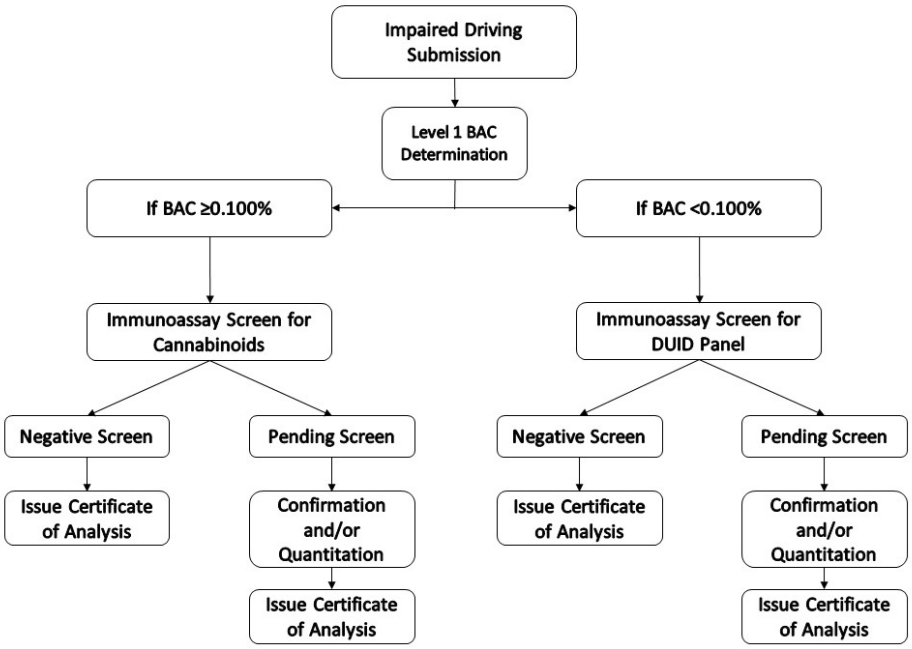
1.2 Toxicology Analytical Schemes

1.2.1 The following are general analytical schemes to be used for toxicology cases. Medical Examiner (OCME) and Toxicology – Other (TO) case testing is decided based on information on the Request for Laboratory Examination Form and case history. The cases are too diverse to have a specific analytical scheme; however, all cases follow a general analytical protocol. The impaired driving protocol is designed to identify alcohol and drugs that can impair driving. Once potentially impairing levels of alcohol or drugs have been identified, the testing may be stopped and a Certificate of Analysis is generated. Exceptions may be necessary due to customer requests for additional testing.

1.2.2 Testing Workflows

Medical Examiner / Toxicology-Other Cases: (e.g., non-DUI/DUID cases, maiming, manslaughter, sexual assault, poisoning, impaired driving cases where samples are collected via search warrant not pursuant to §§18.2-268.2 - 18.2-268.6) Testing may be assigned based on case information, submitter request, or other agreements with customers. Assigned testing may include, but is not limited to, alcohol/volatiles, immunoassay or other drug screening, carbon monoxide analysis, or other confirmatory tests listed in this manual.

1.2.3 Impaired Driving (Implied and Non-Implied (Search Warrant) Consent) Cases: Cases where blood is collected via implied consent or search warrant pursuant to §§18.2-268.2 – 18.2-268.6.



Additional testing may be conducted at the discretion of a Toxicologist, Supervisor, or Group Supervisor.

2 TOXICOLOGY QUALITY GUIDELINES

2.1 Summary

The following toxicology quality guidelines apply for the analysis and subsequent accepting and reporting of toxicology results, unless otherwise specified in a specific method SOP.

2.1.1 Minor deviations and exceptions shall be authorized by the section supervisor or group supervisor and documented in the case file. If the section supervisor or group supervisor is not available, the Toxicology Program Manager may approve deviations.

2.1.2 Major deviations and exceptions shall be authorized by the Toxicology Program Manager and documented in the case file with an MFR.

2.1.3 When such deviations and exceptions affect the original contract, they shall be communicated to the customer and this communication shall be documented in the case file.

2.1.4 For examination documentation that is scientifically analyzed and/or reviewed electronically, the individual preparing the document or the issuing examiner shall utilize the Comment tool in Adobe Acrobat to make corrections, add text, interlineations, or other notations. For all contemporaneous changes, the individual shall conclude these changes with an Adobe Dynamic Stamp (or equivalent electronic marking that includes the identity of the individual and a timestamp to include the date). For any non-contemporaneous changes, the individual shall use another Adobe Dynamic Stamp for the new changes.

2.2 Guidelines for Confirming Positive Results

2.2.1 As a general matter of forensic principle, the detection of drugs and other toxins should be confirmed (whenever possible) by a second technique based on a different chemical principle.

2.2.2 If a second technique is not available, the identification must be confirmed on a different aliquot of the same specimen or in a second specimen.

2.2.3 Whenever possible, the confirmatory (second) test should be more specific and sensitive than the first test for the target analyte. Mass spectrometry is recommended as the confirmatory technique. Exceptions include analytes which are not readily analyzed by mass spectrometry such as carbon monoxide, volatile hydrocarbons (alcohols), and heavy metals.

2.2.4 The following are acceptable confirmatory practices in order of preference. At least one condition shall be satisfied in order to identify and report a drug.

2.2.4.1 Identification of the substance/substance class and specific identification of the substance in a different aliquot by a different chemical principle (e.g., base screen or immunoassay followed by LCMSMS quantitation).

2.2.4.2 Identification of the substance in more than one aliquot by different chemical principles (e.g., base screen identification of antidepressant by GC-MS followed by quantitation by GC-NPD or selective immunoassay for acetaminophen or salicylate followed by quantitation and confirmation by LCMSMS).

2.2.4.3 Identification of the substance in different biological samples by one or more chemical principles (e.g., positive immunoassay for opiates in blood, confirmation of morphine in vitreous humor).

2.2.4.4 Identification of the substance in one biological sample using two separate aliquots and one chemical principle (e.g., ethanol analysis by headspace GC-FID).

2.2.4.5 Identification of an incidental substance by mass spectrometry in one aliquot and case history verifies the identification (e.g., lidocaine confirmed in base screen by mass spectrometry in postmortem case involving medical resuscitation).

2.3 Guidelines for Batch Analysis

2.3.1 In order to maximize efficiency within the toxicology laboratory, it is common to group specimens into batches. The following are general guidelines for batch analysis, unless otherwise specified in a method SOP.

2.3.2 The transfer, handling and aliquoting of specimens is documented with the analyst's signature, date, and time on the batch summary worksheet. The signature and date may be indicated by a secure digital signature. Each batch worksheet also contains the unique FS lab number where the original raw data (including chromatograms of calibrators/controls, calibration curves, instrument sequence lists, etc.) shall be stored. The analysis start and end dates are defined on the Toxicology Batch Worksheet (or equivalent document). The start of the analysis date is the analyst custody date on the form. The analysis completion date is the date of data review noted on the Toxicology Batch Worksheet.

2.3.3 Each batch analysis must contain a sufficient number of controls to monitor the performance of the assay; however, the total number of controls will depend on the size of the batch and the nature of the test.

2.3.3.1 For qualitative confirmations, the batch should contain the appropriate batch summary worksheet and a minimum of one negative control and one positive control/reference standard.

2.3.3.2 For quantitative analysis, each batch of specimens should include 10% controls, including at a minimum, one positive and one negative control.

2.3.3.3 A solvent blank (double blank), a matrix blank (blank blood or matrix with no standards or internal standards), or negative control (blank blood or matrix with internal standard) should be run after the highest spiked standard for methods that utilize a high threshold/calibrator for reporting to monitor for method carryover. This does not apply to blood alcohol analyses, ELISA, acid/base/neutral screens, or other qualitative screens (dependent on control concentration).

2.3.4 For batch analyses that utilize instrument autosamplers:

2.3.4.1 Case samples shall be bracketed by an acceptable calibrator or positive control. Refer to ¶ 7.7.5 for alcohol/volatile analysis control bracketing.

2.3.4.2 The identity of each vial in the autosampler must be verified by an independent analyst with the autosampler sequence list and/or worksheet. This vial verification is documented with initials and date on the sequence table. The initials and date may be indicated by an electronic signature (or equivalent electronic marking that includes the identity of the individual and a timestamp to include the date).

2.3.5 If a batch analysis fails to meet the QA/QC acceptance criteria, at minimum, a copy of the batch worksheet containing documentation of specimen aliquots and the reason for the batch failure shall be included in each case file. These batches are exempt from being reviewed by an independent analyst.

2.3.6 For quality assurance purposes, each batch analysis must be reviewed by an independent analyst and this review is documented with a signature and date on the batch worksheet. The signature and date may be indicated by a secure digital signature. The batch analysis review process includes a review of the following items:

2.3.6.1 Batch summary worksheet with chain of custody and raw data file location.

2.3.6.2 Documentation of vial verification

2.3.6.3 Autosampler sequence list

2.3.6.4 Calibrator chromatograms

2.3.6.5 Control chromatograms

2.3.6.6 Calibration tables and curves (for quantitative assays)

2.3.6.7 QA/QC summary sheets and UoM worksheets if applicable. These sheets must be reviewed for content, accuracy and data transfers. This review is documented with signature and date on the QA/QC summary sheet or batch sheet (for MassHunter, or equivalent, reports). The signature and date may be indicated by a secure digital signature.

2.3.6.8 Instrumental parameters, except Immunoassay and UV Spectrophotometry

2.3.6.9 All case sample chromatograms.

2.3.7 Once the batch summary review process is complete, a photocopy or an electronic copy of the batch worksheet, QA/QC summary sheet for positive results (if applicable), and UoM worksheets (if applicable) are placed in each case file or the appropriate Laboratory Information Management System (LIMS) object repository with the case sample chromatograms.

2.4 Quality Assurance and Quality Control

2.4.1 Definitions (Note: Some variations may exist in specific methods.)

2.4.1.1 Linear range: For most chromatographic assays, the Limit of Quantitation (LOQ) and Upper Limit of Quantitation (ULOQ) are administratively defined in terms of the concentration of the lowest and highest calibrator used in the calibration response curve.

2.4.1.2 LOQ: The LOQ is the lowest calibrator concentration included in the calibration response curve that satisfies RT, ion ratio, and chromatography reporting criteria and must back calculate within ±30% of target concentration. If the lowest calibrator does not meet these criteria, then the new LOQ is the next lowest calibrator that satisfies these criteria or the assay is repeated.

2.4.1.3 ULOQ: The ULOQ is the highest calibrator concentration included in the calibration response curve that satisfies RT, ion ratio, and chromatography reporting criteria and whose back-calculated concentration falls within ±20% of target concentration.

2.4.1.4 Negative Control: The negative control shall not contain the analyte of interest, satisfy RT, ion ratio, and chromatography reporting criteria at a response that is greater than 10% of the LOQ absolute response in a quantitative assay or threshold control in a qualitative assay.

2.4.1.5 Positive Control: The positive control contains the analyte of interest, satisfies RT, ion ratio, and chromatography reporting criteria and has an analyte concentration within the designated acceptable concentration range.

2.4.1.6 Threshold Control: Administratively determined cutoff concentration that is above or equal to the method's limit of detection and typically less than the limit of quantitation. It is used to determine reporting protocols between negative results and positive results. Exceptions for administratively determined threshold controls may be noted within individual methods or be approved by the Toxicology Program Manager based upon validation data.

Note: For methods without validated limits of detection, the thresholds are established from historical practices.

2.4.2 Qualitative Assays

Analyze a minimum of one negative and at least one positive control/reference standard along with unknowns in each chromatographic and rapid presumptive screening test.

2.4.3 Quantitative Assays

2.4.3.1 Calibrators: Solutions that are prepared from a certified reference material that are used to calibrate an assay. Note: Calibrator, internal standard, and control solutions should be prepared in the most appropriate solvent (e.g., purchased CRM is in acetonitrile, calibrator should be prepared in acetonitrile) for the stability of the analytes. The volume of calibrator standard and working solutions may be modified from the preparation delineated in the Procedures Manual if the glassware utilized is NIST traceable and the final concentration is the same.

2.4.3.2 Prepare a minimum of three different calibrators in each quantitative assay or as specified in the method SOP. The concentration of the calibrators should be such that they bracket the anticipated concentration of the unknown specimen(s).

2.4.3.3 Prepare a response curve of area (height) of analyte to area (height) of internal standard ratio versus calibrator concentration. Calculate the analyte concentration by interpolation of the linear plot. It is acknowledged that some assays are inherently non-linear (e.g., LCMSMS) and the use of quadratic models may be necessary and appropriate, and should be verified using low, medium and high controls. The response curve and determined unknown specimen concentration(s) are generated by the instrument software.

2.4.3.4 Calculate the coefficient of determination (r^2) for the curve. For most applications, an r^2 of greater than 0.990 is acceptable (unless otherwise indicated in a specific method SOP).

2.4.3.5 Evaluate the curve by back-calculating calibrator concentrations against the curve. Values of ± 30% from the target calibrator concentration are acceptable for the lowest calibrator. All other calibrators must fall within ±20% of target calibrator concentration unless otherwise indicated in a specific method SOP.

2.4.3.6 Calibration curve data point inclusion guidelines:

- At least 3 calibrators must be included in the calibration curve.
- All positive controls must be greater than the LOQ and less than the ULOQ.
- No more than 3 calibrators between the LOQ and ULOQ may be removed from the calibration curve. Note: The reason for removing the calibrator shall be clearly indicated through notations from the examiner/analyst or by the computer software on the data.

2.4.3.7 If the lowest calibrators are removed from the curve, this change in LOQ may require a repeat analysis of case specimens below the new LOQ. Alternatively, the change in LOQ should be communicated to the customer and documented with an MFR or the change can be communicated on the CoA by reporting “none detected at (new LOQ)” or “Present, less than (new LOQ).”

2.4.3.8 If more than 3 calibrators are removed from the curve, this exception and supporting justification shall be authorized by the supervisor or group supervisor and documented in the case file with an MFR.

2.4.4 Internal standards: Internal standards are required for chromatographic assays.

2.4.4.1 It is preferable to use an internal standard with similar extraction, derivatization, and chromatographic properties to the analyte(s) of interest.

2.4.4.2 The use of stable isotope internal standards for selected ion monitoring GC-MS and LCMSMS is encouraged but not required since well-chosen non-deuterated internal standards may give similar performance.

2.4.4.3 The volume of internal standard solution prepared may be modified from the volume indicated in the methods herein, however the concentration of the internal standard must remain the same as directed.

2.4.4.4 The internal standard recovery as measured by peak area/height or ion abundance should be monitored for calibrators, controls and case specimens. Internal standard response should be between approximately 50-200% of the IS response of the calibrators for quantitative assays (100% is approximately equal to the average calibrator IS response). This may also be denoted in other software as -50% to +100% of the IS response of the calibrators for quantitative assays (0% is approximately equal to the average calibrator IS response).

2.4.4.5 To maintain method reliability, the internal standard (IS) recovery of the samples should meet the above requirements (2.4.4.4). During data review, the examiner and reviewer should be mindful of these requirements and remediate accordingly.

Remediation actions for low or high IS recovery include, but are not limited to the following:

Assess the performance of the assay and other IS within the sample. For IS recovery above the acceptable range and the IS is an isotopically-labeled, matched standard – no further action is necessary.

Reinjection or reextract the specimen.

Document a reinjection on the chromatogram with “reinjection”, “R”, or another marking to indicate the reinjected data. Upon reinjection, if the internal standard recovery still falls outside the acceptable range:

The drug may be reported as “present” if it has been confirmed. The reason for reporting “present” shall be documented in the case file and, if appropriate, communicated to the customer.

The case specimen may be reported as “unsuitable for” the particular “analysis.” This exception shall be authorized by the section supervisor or group supervisor, documented in the case file, and communicated to the customer.

For methods utilizing the MassHunter software, note the S/N calculated by the instrument software. If the S/N of the quantitation transition/ion is greater than 10:1, the data may be accepted. This acceptance will be recorded on the data. If the S/N is less than 10:1, continue with remediation actions.

2.4.5 Controls: Controls in quantitative assays may be purchased or prepared in-house. In-house prepared controls should be prepared from a different manufacturer or different lot of standard material than used in calibrators. If this is not possible, controls should be prepared from a different weighing or dilution from the calibrators, preferably by another analyst. Results from quantitative quality control materials are recorded in control charts to readily detect trends such as deterioration of reagents, calibrators and controls.

2.4.5.1 Analyze a negative control and at least one positive control with each quantitative procedure.

2.4.5.2 In-house controls (prepared in either methanol or acetonitrile) fortified in matrix:

- Positive control range is ± 20% from the target concentration.
- All staff are responsible for entering new values into the statewide control charts, if applicable.
- The Research Section Supervisor or designee should review the statewide control charts each month to ensure consistency between laboratories and communicate results with the Toxicology Program Manager, section supervisors, and group supervisors as necessary.

2.4.5.3 Statewide methanolic controls fortified in matrix:

- Positive control range is ± 20% from the target concentration.
- The control is verified with six replicates against calibrators prepared from another source before being placed in service.
- If acceptable, the preparer or Chemistry Research Section Supervisor or designee will put the lot into service. The Chemistry Research Section Supervisor, the Toxicology Program Manager, or designee will create the control chart for the new lot. The Chemistry Research Section Supervisor, Toxicology Program Manager or designee will notify toxicology personnel that the new control is in service.
- All staff are responsible for entering new values into the statewide control charts.
- The Research Section Supervisor or designee should review the statewide control charts each month to ensure consistency between laboratories and communicate results with the Toxicology Program Manager, section supervisors, and group supervisors as necessary.

2.4.5.4 External purchased controls (i.e., UTAK or equivalent):

- Positive control range is ± 20% from the mean concentration established by statewide analysis. Instrumental data analysis methods that are used for setting the external or statewide control value should be updated when a new lot of control is put into use.
- Three replicates from each laboratory shall be used to set the DFS mean, unless otherwise specified. For controls used in the "Ethanol Content of Alcoholic Beverages by Headspace GC" analysis (¶ 23), the DFS mean is established from three replicates from four batches for a total of twelve values.
- The Research Section Supervisor or designee will put the lot into service and create the control chart on the shared drive.
- All staff are responsible for entering new values into the statewide control charts.
- The Research Section Supervisor or designee should review the statewide control charts each month to ensure consistency between laboratories and communicate results with the Toxicology Program Manager, section supervisors, and group supervisors as necessary.

2.4.5.5 The target analyte concentrations of positive controls must be between the lowest and highest calibrator. For assays using one positive control, it is preferred that the control concentration is approximately midrange of the curve. If multiple positive controls are used, control

concentrations preferably should monitor the overall performance of the assay (low, medium and high controls).

2.4.5.6 Each quantitative batch analysis should contain at least 10% controls. When multiple positive controls are run within a batch, 2 out of 3 positive controls (or 67%) must fall within the acceptance criteria. If controls do not meet acceptance criteria, all positive case samples should be repeated. However, qualitative results may be reported when reanalysis is not possible or practical.

2.4.5.7 Qualitative analyses using multiple control levels should average the retention time and ion/transition ratios for comparison and identification, if possible.

2.4.6 Pipette Utilization

Toxicology personnel shall not pipette less than 10 µL for calibrators.

2.4.7 The Department's laboratory facilities provide sufficient environmental conditions to conduct all tests listed in this Procedures Manual with no further consideration required.

2.5 Chromatographic and Mass Spectral Quality Control

2.5.1 Chromatographic quality control.

Some toxicology casework may contain multiple drugs or co-eluting decomposition products that may prohibit adherence to some of the following chromatography guidelines. Exceptions shall be documented in case notes or on chromatograms.

2.5.1.1 Retention Time: Retention times listed in specific methods are provided for elution order reference and may shift due to instrument differences (e.g., maintenance, tubing length, GC column, etc.). Retention times for both analyte and internal standard must be within ± 2% of the retention time obtained from the average of the calibrators (or however the instrument software calculates the reference retention time). Larger deviations (±3%) are acceptable for assays based on LCMSMS. Individual methods may have more strict RT or RRT criteria listed in the procedures. If an analyte RT is outside of ±2-3%, RRT may be used for confirmation when compared to a calibrator or control (within ±2 (GC,GCMS) or ±3% (LCMSMS) RRT).

2.5.1.2 Peak Resolution: To the greatest extent possible, chromatographic peaks used for quantitative analyses should be resolved from interfering peaks such that the signal height of the valley between adjacent peaks is no greater than approximately 10% of the peak height of interest.

2.5.1.3 Peak Width: Measured at the base of the peak, chromatographic peaks of interest should be at least approximately 10% of SIM window width to permit diagnostic review.

2.5.1.4 Peak Symmetry: Peak shape should be reasonably symmetrical and return to at least 10% of peak height.

2.5.1.5 Manual Integration: If manual integration is utilized, the original integration and the manually integrated chromatograms shall be retained and included with all batch/sample documentation. If there is a batch wide issue with the integration it may not be practical to retain this information for all samples. A deviation from this requirement may be documented by an MFR from the section/group supervisor and must include an example of the original and manual integrations.

2.5.2 Mass spectral quality control.

2.5.2.1 Full scan mass spectral identification: No rigid mass spectral probability based match criteria are defined to identify a drug. Flexibility is given to the experienced interpreter because rigid criteria can lead to misidentification as well as under-identification. The experienced interpreter will base identification on a number of factors, such as retention time, unique ions, ion abundance, S/N and literature references as well as probability based matching scores.

- Whenever possible, the identification of a drug should be based on a spectral library match or comparison to an actual standard reference material. This standard reference material is required to verify the mass spectrum and retention time.

- For qualitative identification, the analyte should be compared to the threshold control, a positive control, or calibrator containing standard reference material (for mass spectrum and retention time verification) and a negative control. The case sample analyte peak should be well resolved, the retention time should match the standard and the mass spectrum should contain all of the major and diagnostic ions unique to the analyte. Missing ions or the presence of additional ions in the unknown sample is indicative of a weak signal, background noise or co-eluting substances.

- Selected ion monitoring (SIM) identification: When SIM is used for identification of an analyte, whether as part of a quantitative procedure or not, retention time match is required and the use of at least two analyte qualifying ions and one internal standard qualifying ion is preferred.

2.5.2.2 Multiple reaction monitoring (MRM) identification: In tandem mass spectrometry methods, when MRM mode is used for identification of an analyte, as part of a quantitative procedure or not, retention time match and the use of two analyte transitions and one internal standard transition is required.

2.5.2.3 For SIM and MRM analyses, it is recognized that precursor and product (or fragment) ion masses may differ slightly (i.e., ±0.2 amu) from the validated mass due to differences in optimization between LCMSMS and GCMS instruments. The acceptance criteria for ion and transition ratios are ± 20% or 2SD relative to the average ratio from all calibrators used in the calibration response curve. However, it is recognized that some ion ratios are concentration dependent and that comparison to a calibrator of similar concentration may be necessary rather than the average ratio for the curve. This exception shall be documented in case notes or batch summary raw data file.

2.5.2.4 Ion and transition ratios may also have a greater variance based on the relative intensity of the product ion/transition to the base peak or target ion/transition. Acceptable tolerances for those targets are listed below (ANSI/ASB Standard 098, 1st ed.) and may be applied for samples that do not meet the ±20% criteria. The relative intensity column is the average of the ion ratios for calibrators. Application of tolerances other than ±20% may also be noted in specific methods.

Relative Intensity (% of base peak or target ion)	Tolerances for Electron Impact Ionization (relative)	Tolerances for all Other Ionization Techniques (relative)
Greater than 50%	± 20%	± 20%
20 to 50%		± 25%
10 to 20%		± 30%
Less than 10%	± 50%	± 50%

2.5.3 Due to slight variability in instrument performance, GCMS and LCMSMS parameters such as the fragmentor voltage, collision energy, cell accelerator voltage, capillary voltage, EMV, time segments, column temperature, and dwell times may be optimized for improved sensitivity and resolution. Any

modifications to these parameters requires a verification (see ¶ 2.8) approved by the Toxicology Program Manager. Other method parameters such as columns, mobile phases, gradients, injection volumes, and ion masses may be modified only after consultation with the Toxicology Program Manager and Chemistry Research Section Supervisor.

2.5.4 Reinjection criteria and documentation: Occasionally, there may be situations in which calibrators, controls and case samples need to be reinjected on the chromatographic instrument. If reinjection is not possible (e.g., stability or volume limitations), the analysis should be repeated. Common reasons for reinjection include the following:

2.5.4.1 Poor analyte or internal standard recovery (2.4.4.5). The vial is reinjected to determine whether poor recovery is due to the injection or extraction.

2.5.4.2 Carryover. Carryover may occur due to the use of automated chromatographic injection systems or due to the extreme range of drug concentrations detected in toxicological specimens. Regardless of cause, extreme caution is warranted when carryover is detected. Carryover is assessed using the criteria for a negative control. Appropriate actions may include the steps listed below.

If a sample is run directly after a sample which has an analyte concentration greater than the ULOQ, follow the below directions:

- If a solvent or matrix blank was run after the sample with the analyte concentration greater than the ULOQ and no carryover is detected, no further action is necessary.
- If the following sample satisfies the criteria for a negative control for the analyte of concern, no further action is necessary.
- If the following sample has a signal that indicates potential carryover of the analyte of concern, inject the following to assess carryover: negative control, sample(s) with potential carryover, and a positive control (may also include the sample with the concentration >ULOQ and a matrix/solvent blank). If the reinjection is added to the end of a currently running sequence/worklist, the negative control is optional.
- Instances of confirmed carryover requires supervisor notification, guidance and review of the analytical results. The supervisor shall document their review of the carryover and the appropriate response on an MFR to be included with batch summary raw data file and affected individual case files.

2.5.4.3 Documentation of reinjection.

- Document on the original chromatogram that the original injection was unacceptable using language such as “Not used due to (reason for reinjection).”
- Document on the reinjected chromatogram, “reinjection” or other suitable notation/abbreviation.

2.6 Criteria for Reporting Toxicology Case Results

2.6.1 Drug reporting guidelines: Report drug concentrations in mg/L on the certificate unless otherwise stated in a specific method SOP.

2.6.1.1 Results shall be reported to two (2) significant figures with the exception of ethanol, acetone, isopropanol, and methanol which are reported to three (3) decimal places. Results shall be rounded using conventional rounding rules (see Quality Manual ¶ 5.4.6.3).

The expanded Measurement Uncertainty shall be rounded using conventional rounding rules and reported (see ¶ 5.4). The reported measurement uncertainty shall be rounded to the same level of significance (i.e., decimal places) as the reported concentration.

2.6.1.2 For samples analyzed more than once, replicates must agree within ± 20% of the mean except for alcohols (see Alcohols by Headspace Gas Chromatography). Report the average of the replicates.

2.6.1.3 For an assay, if there are more than two replicate values, an exception to exclude one replicate from the mean ± 20% range may be made if that value causes the mean and ± 20% range to be unacceptable for all replicates. Document the exception in the case file.

2.6.1.4 If multiple dilutions are analyzed, report the least dilute sample that falls within the quantitation range of the assay. Maximum allowed dilutions are listed in the chapters. If the maximum allowed dilution is exceeded, positive results may be reported qualitatively (refer to 3.2.2 for screening dilution requirements).

2.6.1.5 If biological fluids or tissues are diluted prior to the analysis, all digits will be carried through the calculation and the final result will be rounded following conventional rounding rules.

2.6.1.6 Urine results are typically reported as "present" only since the concentration provides little interpretive value.

2.6.1.7 Enantiomers of targets may not be distinguishable, or reported on the COA, unless specifically noted in the procedure.

2.6.1.8 Acquire data with at least one additional significant figure than what is reported. For drugs with low concentrations (THC and fentanyl) it may be advantageous to collect data in µg/L or ng/mL and then convert to mg/L for reporting.

2.6.1.9 A drug may be reported as "Present" for a number of reasons including specific reporting guidelines in a method SOP, quantitative procedures were not performed or available or a quantitative procedure was performed but acceptance criteria were not satisfied and reanalysis is not possible or practical.

- To report a drug as "Present," it must be confirmed (see ¶ 2.2).
- Confirmed drugs not specifically requested for testing by the customer or listed in the case history may be reported "Present" without notification of the customer.
- Confirmed drugs requested by the customer or listed in the case history require prior notification to the customer to be reported "Present" and shall be appropriately documented in the case file.
- "Present" may be used to report the presence of drugs in different tissues for consistency and support of analytical findings (e.g., report a case with postmortem methadone findings of blood 1.3 mg/L, liver 2 mg/kg and urine "present").

2.6.1.10 Report a confirmed drug as "Present, less than (LOQ concentration)" if the concentration is less than the LOQ and greater than or equal to the threshold control of the batch. If a dilution factor was used in the analysis, multiply the dilution factor by the LOQ to determine the reported LOQ. Exceptions may be approved by the supervisor, group supervisor, or toxicologist on a case-by-case basis.

2.6.1.11 Report a confirmed drug as "Present, greater than (ULOQ concentration)" if the concentration is greater than the ULOQ of the assay and reanalysis is not possible or practical. If a dilution

factor was used in the analysis, multiply the dilution factor by the ULOQ to determine the reported ULOQ.

2.6.1.12 Report as "None detected" or "None Detected at (LOQ or threshold control concentration)" if a quantitative analysis was attempted that did not satisfy the criteria for confirmation or was below the LOQ or threshold control. If a dilution factor was used in the analysis, multiply the dilution factor by the LOQ to determine the reported LOQ.

2.6.1.13 Report as "Unsuitable for analysis": The quality of the sample is such that it cannot be sampled or an analysis or multiple analyses were attempted and the results are unacceptable. This may be reported as "Unsuitable for analysis" and shall include the reason it is unsuitable. Supervisor or group supervisor approval is required. If the supervisor or group supervisor are unavailable, the Toxicology Program Manager may approve.

2.6.1.14 Report as "Unsuitable for (method/drug/class) analysis". An analysis was attempted however the quality of the results did not meet QA/QC criteria, chromatography was unacceptable, or other quality issues were present. This may be reported as "Unsuitable for (method/drug/class) analysis" and shall include the reason it is unsuitable and the inclusion of the method reporting wording. The "(method/drug/class)" should be filled in with the target(s) or method of concern. This requires the approval of the supervisor or group supervisor. If the supervisor or group supervisor are unavailable, the Toxicology Program Manager may approve.

2.6.1.15 Report as "Inconclusive for the confirmation of [insert here] due to [insert reason here]". An analysis was attempted however the quality of the results prevents the examiner from reaching a conclusion as to the confirmation of an analyte, drug class, or method. The reason for using this statement may include a failure to meet QA/QC criteria, co-eluting compounds, poor chromatography, etc. This statement requires the method line to be included below the statement.

2.6.1.16 If an observation, data, or calculation is rejected, the reason, the identity of the individual taking the action and the date shall be recorded.

2.7 Report Wording

2.7.1 Only positive, confirmed results will be reported with the exceptions noted above for reporting "Present, Less than_," "Present Greater than_," and "None Detected at_,". Additional reporting language may be used to report uncommon scenarios (e.g., "none confirmed", "Quantity insufficient for further analysis").

The following language shall be added after the results for each item when there is no other confirmatory testing completed or the screen results are negative for all other analytes. Choose one of the following statements:

2.7.1.1 "No drugs and/or drug classes were detected"

2.7.1.2 "No other drugs and/or drug classes were confirmed." – This should be used in the scenario where there may be indications of drug presence however it was not confirmed.

2.7.1.3 "No other drugs and/or drug classes were detected." – This should be used in the scenario where there are positive, confirmed results and all other screening shows negative results. This may be used for other instances (e.g., cannabinoids inconclusive).

2.7.1.4 "No drugs and/or drug classes were confirmed." – This should be used in the scenario where screening is completed in one item and confirmed in another item. This may also be used for other instances (e.g., no standard available, non-relevant compounds, no validated method available, inconclusive results).

2.7.2 The examiner shall report the method used to evaluate the specimen(s) along with the associated analyte(s).

2.7.2.1 For the Acid/Base/Neutral Drug Screen and Quantitation by GC and GCMS method, the analytes shall be specified for base screen ("alkaline-extractable drugs.") or acid/neutral screens ("acid/neutral drugs."). This shall be followed by the statement: "Supporting documentation is maintained in the case file and current methods can be found in the Toxicology Procedures Manual."

2.7.2.2 For the Volatile Screen and Confirmation by Headspace GC and GCMS, the analytes shall be listed as "volatile organic substances.". This shall be followed by the statement: "Supporting documentation is maintained in the case file and current methods can be found in the Toxicology Procedures Manual."

2.7.3 If any of the methods in the case were performed at a laboratory other than the originating laboratory, refer to the Quality Manual for reporting requirements.

2.7.4 The examiner shall include the date range of testing to encompass the start date (date of toxicology accessioning on the summary worksheet) and the end date (date of examination) in the following format:

"Date(s) of Testing: [insert start date] – [insert end date]"

2.7.5 On DUI and DUID case types, if two vials of blood are received and only one vial of blood analyzed, the examiner shall add the following:

"A second vial of the whole blood sample was received and was not opened or analyzed."

2.7.6 The examiner shall add the following language to the end of the report:

"Supporting examination documentation is maintained in the case file. The above-listed methods are those approved for use at the time of analysis. Current methods can be found in the Toxicology Procedures Manual which can be found at www.dfs.virginia.gov/documentation-publications/manuals/. Measurement uncertainty is reported at a 95.45% level of confidence for all toxicological analyses except ethanol which is reported at a 99.73% level of confidence."

For reports that include deviations to the methodology and procedures in this manual, use the following:

"Supporting examination documentation, including a method deviation, is maintained in the case file. The above-listed methods are those approved for use at the time of analysis. Current methods can be found in the Toxicology Procedures Manual which can be found at www.dfs.virginia.gov/documentation-publications/manuals/. Measurement uncertainty is reported at a 95.45% level of confidence for all toxicological analyses except ethanol which is reported at a 99.73% level of confidence."

For ethanol content of alcoholic beverages cases, use the following:

"Supporting examination documentation is maintained in the case file. The above-listed methods are those approved for use at the time of analysis. Current methods can be found in the Toxicology Procedures Manual which can be found at www.dfs.virginia.gov/documentation-publications/manuals/. Measurement uncertainty is reported at a 95.45% level of confidence."

Note: If no uncertainty values are reported, the last sentence of the above paragraphs may be removed. The methodology-related statements may be removed in instances where no method is listed in the Results and Interpretations section.

2.7.7 For specimens where GHB was requested but testing was not performed, the following language may be used as a guide to report that GHB was not pursued:

"Item(s) [XX] was/were not analyzed for the GHB panel due to the delay in collection of the sample(s) from the reported time of the alleged incident. Due to this delay, the results of the analysis may not be of toxicological significance."

"No timeline was provided between the alleged incident and the sample collection. Without this information, the results of the GHB panel analysis may not be of toxicological significance."

2.8 Guidelines for Method Validation and Verification

Where possible, all validations shall be conducted in accordance with the ANSI/ASB 036 Standard Practices for Method Validation in Forensic Toxicology with the following guidelines. Experiments should be conducted on all available models of instrumentation intended for validation. When developing new methods for quantitation and identification, isotope or adduct ions should be avoided as qualifier ions.

2.8.1 Qualitative Method Validation

2.8.1.1 When a compound is to be added to an existing method for qualitative identification purposes or a new qualitative method is to be validated, a written validation plan shall be proposed to the Section Supervisor, Toxicology Program Manager, and Research Section Supervisor through the Qualtrax EAGeR workflow. After review, the validation plan will be submitted for approval by the TRT (or designees). This plan should address the following criteria (Note: Criteria may be methodology-dependent, exceptions may be approved by the Toxicology Program Manager):

List the analyte of interest, methodology, internal standards, and any expected concentrations of analytes or internal standards.

Sensitivity (Estimated Limit of Detection): The estimated limit of detection shall be defined as an administratively-defined decision point (threshold concentration). The administratively-defined decision point shall be established using two concentrations. The concentrations to be evaluated are the threshold concentration and 50% below the threshold concentration within the method. The decision point shall be evaluated by fortifying, at minimum, three different blank matrix sources, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver). The three different matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met.

Predetermined acceptance criteria:
Retention Time: ±3% (for LCMSMS) or ±2% (for GC or GCMS)
Qualifier Ion/Transition Ratio (if applicable): ±20%
Signal-to-Noise: ≥3.3

Interferences:

Endogenous Compounds: Where possible, a minimum of ten negative matrix sources, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) shall be analyzed without the addition of internal standard.

Internal Standard: To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) sample, per matrix type shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard (concentration delivered within method) without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) sample, per matrix type shall be evaluated.

Commonly Encountered Analytes: Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current methods).

In addition to commonly encountered analytes, each compound and internal standard will be evaluated individually.

Carryover: To evaluate for carryover, blank matrix samples immediately following progressively higher concentrations of fortified matrix within an injection sequence shall be analyzed. The concentrations selected should be based upon knowledge of typical concentrations or expected concentrations. At minimum, two concentrations shall be evaluated for carryover using triplicate analysis with a minimum of three sources per matrix type.

Stability: The stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. At minimum, a single blank matrix source, per matrix type, shall be extracted at two concentrations (high and low) and analyzed at minimum every twenty-four hours for a seven-day period with triplicate injections at each time point. The instrumental response for analyte and internal standard shall be evaluated.

Ionization Suppression/Enhancement (where applicable): Ionization suppression and enhancement shall be addressed with neat standards and post-extraction fortified samples. Two different sets of samples shall be prepared and their peak areas compared between sets. Neat standards, at low and high concentration, shall be prepared in neat extraction solvent and injected a minimum of six times each. A minimum of ten duplicates of post-extraction fortified samples (matrix that is extracted and then fortified), per matrix type, will be prepared to compare to the neat standards. If the ionization suppression or enhancement exceeds ±25% or the coefficient of variation exceeds ±20%, an evaluation of the effect on limit of detection shall be evaluated. The influence on the parameters shall be assessed by at least tripling the number of different sources of blank matrices used in the evaluation.

Note: The low and high concentrations should be based upon knowledge of typical concentrations, expected concentrations, or instrumental limits.

2.8.1.2 Upon completion of the validation, a validation summary shall be generated that summarizes the validation data. The validation summary shall be proposed to the Toxicology Program Manager and Research Section Supervisor for review. The validation summary will also be reviewed by the TRT (or designees).

2.8.1.3 A binder or electronic record of the finalized validation shall be created to contain the method development documents, approved validation plan, approved validation summary, draft SOP (if applicable), batch worksheets, and any other documents associated with the validation. The binder or electronic record shall then be sent to the Toxicology Program Manager.

2.8.1.4 If the validation is the addition of a compound or compounds to existing methodology, an MFR containing extraction and instrumental parameters shall be generated requesting the approval to use the method prior to the method/compound(s) being added to the Toxicology

Procedures Manual. Upon approval of the MFR, the method is then able to be used in any Toxicology section following a Qualitative Method Verification.

2.8.2 Qualitative Method Verification

When a laboratory, other than the laboratory where the validation took place, wants to utilize this method it will need to be verified as fit for use. This verification will consist of a batch analysis of the analyte (w/ IS), a blank, threshold control, and a double blank (no analyte or IS). If the procedure requires that matrix matched controls shall be prepared (e.g., GHB, GBL, 1,4-butanediol Quantitation and Confirmation by LCMSMS), verifications shall be performed for each matrix (e.g., blood and urine) prior to reporting casework in that matrix. Meeting batch acceptance criteria will demonstrate that the method is fit for use in the laboratory. The verification shall include a review of the software methods. Upon successful completion of the verification, the section supervisor (or designee) will send an MFR indicating that it is fit for use and requesting approval to use this method to the Toxicology Program Manager. This verification (data and MFR) will be retained within the laboratory completing the verification (either in hardcopy or electronic format). Note: for efficiency, the above-listed standards for verification can be combined into a casework batch analysis which shall undergo all review processes. If verification is combined with casework, no casework utilizing the new methodology may be released until the method is approved as fit for use by the Toxicology Program Manager.

2.8.3 Quantitative Method Validation

2.8.3.1 When a compound or compounds are to be added to an existing method for quantitative purposes or a new quantitative method is to be validated, a written validation plan shall be proposed to the Section Supervisor, Toxicology Program Manager, and Research Section Supervisor through the Qualtrax EAGeR workflow. After review, the validation plan will be submitted for approval by the TRT (or designees). This plan should address the following areas and appropriate acceptance criteria, if applicable (Note: Criteria may be methodology-dependent, exceptions may be approved by the Toxicology Program Manager):

List the analyte(s) of interest, methodology, internal standards, and any expected concentrations of analytes or internal standards.

Bias and Precision: Bias and precision shall be measured using fortified matrix samples. To evaluate, a minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The low concentration shall be three times the lowest end of the working range of the method and the high concentration shall be within approximately 80% of the highest end of the working range. The medium concentration shall be between the low and the high concentrations. Bias, within-run precision and intermediate precision shall be calculated from the five batch analyses.

Sensitivity:

Estimated Limit of Detection: The estimated limit of detection shall be defined as an administratively-defined decision point (threshold concentration). The administratively-defined decision point shall be established using two concentrations. The concentrations to be evaluated are 50% of the lowest calibrator concentration (threshold concentration) and 50% below the threshold concentration within the method. The decision point shall be evaluated by fortifying, at minimum, three different blank matrix sources, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver). The three different matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met.

Predetermined acceptance criteria:
Retention Time: ±3% (for LCMSMS) or ±2% (for GC or GCMS)
Qualifier Ion/Transition Ratio (if applicable): ±20%
Signal-to-Noise (if applicable): ≥3.3

Lower Limit of Quantitation:

The lower limit of quantitation shall be established by evaluating the lowest non-zero calibrator for the method. For each matrix type, a minimum of three different matrix sources shall be fortified at the lowest calibrator concentration and analyzed over a minimum of three analyses. A minimum of nine replicates per matrix sources (27 replicates per matrix type) will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met.

Predetermined acceptance criteria:
Retention Time: ±3% (for LCMSMS) or ±2% (for GC or GCMS)
Qualifier Ion/Transition Ratio (if applicable): ±20%
Signal-to-Noise (if applicable): ≥3.3
Back Calculated Concentration: ±20%

Calibration Model: The calibration model shall be established by determining the working range of analyte concentration over which the method shall be used. A minimum of six different non-zero concentrations shall be used to evaluate the calibration model. A minimum of five replicate determinations from five different batches will be utilized. The model will be established by residual analysis and statistical comparisons (ANOVA) between the model fits.

Note: All calibration curves generated during the validation shall be utilized to determine the best fit calibration model. No calibration data may be excluded without consultation with the Research Section Supervisor and Toxicology Program Manager.

Ionization Suppression/Enhancement: Ionization suppression and enhancement shall be addressed with neat standards and post-extraction fortified samples. Two different sets of samples shall be prepared and their peak areas compared between sets. Neat standards, at low and high concentrations, shall be prepared in neat extraction solvent and injected a minimum of six times each. Duplicate analyses, a minimum of ten matrix sources, of post-extraction fortified samples (matrix that is extracted and then fortified), per matrix type, will be prepared to compare to the neat standards. If the ionization suppression or enhancement exceeds ±25% or the coefficient of variation exceeds ±20%, an evaluation of the effect on limit of detection shall be performed. The influence on the parameters shall be assessed by at least tripling the number of different sources of blank matrices used in the evaluation.

Note: The low and high concentrations should be the low and high concentrations used for bias and precision evaluations.

Carryover: To evaluate for carryover, blank matrix samples immediately following progressively higher concentrations of fortified matrix within an injection sequence shall be analyzed. The concentrations selected should be the highest calibrator concentration and a concentration greater than the highest calibrator concentration. At minimum, two concentrations shall be evaluated for carryover using triplicate analysis with a minimum of three sources per matrix type.

Interferences:
Endogenous Compounds: Where possible, a minimum of ten negative matrix sources, per matrix type (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) shall be analyzed without the addition of internal standard.

Internal Standard: To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) sample, per matrix type shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard (concentration delivered within method) without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, postmortem blood, antemortem blood, urine, and liver) sample, per matrix type shall be evaluated.

Commonly Encountered Analytes: Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current methods).

In addition to commonly encountered analytes, each compound and internal standard will be evaluated individually through the extraction process. Each analyte and internal standard will be spiked into separate extraction tubes/vials and processed through the entire procedure. Analysis on the instrument will be used to determine whether the analyte or internal standard contribute to other target signals. Any contribution will be evaluated for the significance of interference.

Dilution Integrity: The dilution integrity shall be assessed for scenarios including concentrations above the upper limit of quantitation with sufficient sample volume (large volume) and when limited sample volume is available (small volume), where applicable. Common dilution ratios shall be evaluated for bias and precision per matrix type utilizing the experiments delineated in bias and precision.

Stability: The stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. At minimum, a single blank matrix source, per matrix type, shall be extracted at two concentrations (high and low) and analyzed at minimum every twenty-four hours for a seven-day period with triplicate injections at each time point. The instrumental response for analyte and internal standard shall be evaluated.

2.8.3.2 Upon completion of the validation plan, a validation summary shall be generated that summarizes the data. The validation summary shall be proposed to the Toxicology Program Manager and Research Section Supervisor for review. The validation summary will also be reviewed by the TRT (or designees).

2.8.3.3 A binder or electronic record of the finalized validation shall be created to contain the method development documents, approved validation plan, approved validation summary, draft SOP (if applicable), batch worksheets, and any other documents associated with the validation. The binder or electronic record will then be sent to the Toxicology Program Manager.

2.8.4 Quantitative Method Verification

When a laboratory, other than the laboratory where the validation took place, wants to utilize a validated method it will need to be verified as fit for use. The verification, at a minimum, should include the calibration curve, a threshold control (if applicable), a blank, a double blank, and controls. If the procedure requires that matrix matched calibrators and controls shall be prepared (e.g., GHB, GBL, 1,4-butanediol Quantitation and Confirmation by LCMSMS), verifications shall be performed for each matrix (e.g., blood and urine) prior to reporting casework in that matrix. Meeting batch acceptance criteria will demonstrate that the method is fit for use in the laboratory. The verification shall include a review of the software methods. Upon successful completion of the verification, the section supervisor (or designee) will send an MFR indicating that it is fit for use and requesting approval to use this method to the Toxicology Program Manager. This verification (data and MFR) will be retained within the laboratory completing the verification (either in hardcopy or electronic format). Note: For efficiency, the above-listed standards for verification can be combined into a casework batch analysis which shall

undergo all review processes. If verification is combined with casework, no casework utilizing the new methodology may be released until the method is approved as fit for use by the Toxicology Program Manager.

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3 SAMPLING PROCEDURE

3.1 Sampling evidence is critical in toxicological analysis. One must be sure that what is sampled is truly representative of the total sample submitted. The analyst must take into consideration the homogeneity (or lack thereof) among submitted biological specimens. In order to perform a toxicological analysis, a representative sample shall be removed from the biological specimen.

3.2 Biological fluids should be placed on a rocker or inverted several times to ensure sample homogeneity prior to removal of an aliquot for analysis.

3.2.1 Biological fluids may be diluted prior to analysis for a number of reasons (e.g., small sample volume or analyte concentration is greater than ULOQ). Refer to the Quality Control section of specific methods for instructions and limitations.

3.2.2 For screening purposes, no less than one half of the SOP required specimen volume should be sampled.

3.2.3 For quantitations and confirmations that require dilution due to the analyte concentration exceeding the ULOQ, refer to the Quality Control section of specific methods for instructions and limitations. For methods without explicit instructions, a minimum of 100 µL of fluid should be diluted as necessary in an appropriate blank matrix such as blank blood or water (for vitreous, urine, tissue homogenates).

3.2.4 Biological fluids may be clotty. These samples may be homogenized prior to sampling.

3.3 Tissues (liver, brain, spleen) are generally considered to be homogeneous throughout the tissue: therefore, a portion of the tissue may be sampled and analyzed.

3.3.1 Weigh approximately 1 gram tissue. Add appropriate volume of water to obtain final dilution (e.g., add 4 mL water for a final dilution of 1:4 or 1/5). Homogenize sample in a homogenizer or blender.

3.3.2 Analyze 1-2 ml of tissue homogenate (or as described in specific method SOP).

3.3.3 Multiply the analyte concentration by dilution factor and report tissue concentration in mg/Kg.

3.3.4 Tissue fluid is generally considered to be homogenous and may also be analyzed. If tissue fluid is analyzed instead of actual tissue, this should be documented in the case notes and reported as such on the Certificate of Analysis.

3.4 Gastric contents may contain pills, pill fragments and/or partially digested food material and therefore are NOT homogeneous samples.

3.4.1 Prior to the analysis of gastric contents, weigh the total gastric contents. Record total weight.

3.4.2 Homogenize the gastric contents prior to sampling to ensure homogeneity.

3.4.3 Weigh or pipette (1-2 gm or mL) of the gastric homogenate and dilute with water to achieve the desired dilution factor.

3.4.4 Analyze the diluted gastric homogenate and multiply the analyte concentration by the dilution factor.

3.4.5 Multiply the analyte concentration by the total gastric contents weight and report gastric contents results as total mg analyte per total (gm) gastric contents submitted.

4 EVIDENCE HANDLING AND STORAGE

4.1 Evidence Submission

The proper selection, collection, submission and storage of biological specimens for toxicological analysis are important if the analytical results are to be accurate and their subsequent interpretations scientifically sound.

4.1.1 A minimum of 10 mL of blood, serum or plasma should be submitted for non-implied consent cases requiring comprehensive toxicological analyses. If less than 10 mL sample is submitted, the analyses shall be prioritized in order to maximize the value of the toxicological analyses. If less than 10 mL of sample is submitted, DFS may not be able to complete all of the requested examinations.

4.1.2 Blood samples should be collected in gray top tubes (or equivalent) containing potassium oxalate and sodium fluoride to preserve the samples. DFS provides gray top tubes (DUI Kits) and clear blood vials (to the OCME for specimen collection) containing these preservatives. If specimens are submitted in tube/vial types other than those routinely provided by DFS, it will be noted/documented in the case file.

4.1.3 It is recognized that hospital or clinical specimens collected pursuant to medical treatment may be collected in blood vials with or without preservatives. Such exceptions shall be noted in the case file with an appropriate description of the evidence (e.g., purple top, red top, green top, yellow top SST, serum, plasma etc).

4.1.3.1 If the hospital or clinical specimens differ in the collection times and the differences could affect the interpretation of the results, then the times should be recorded in the case file and reported on the Certificate of Analysis.

4.1.3.2 In general, the specimens with the earliest collection times should be analyzed whenever possible.

4.1.4 Postmortem samples should be labeled with type (e.g., blood, bile, urine, liver) and location of blood sample collection (e.g., iliac, heart, subclavian). Failure to label postmortem evidence appropriately limits the value and interpretation of toxicological results.

4.1.5 GHB panel testing will not be pursued if more than 15 hours (or 8 hours) had passed between the alleged incident (ingestion) and the collection of urine (or blood) specimen(s). This provision addresses the pharmacokinetic properties of GHB/GHB analogues and the toxicological significance of analytical results that may contribute to the investigation of a drug facilitated crime. Exceptions may be made by a toxicologist, group supervisor, or section supervisor on a case-by-case basis.

4.2 Evidence Receipt

At the time of receipt, the specimen label information should be inspected and compared to RFLE (if available) to verify that the information matches. Any discrepancies should be documented on a toxicology worksheet.

4.2.1 A DUI or toxicology worksheet is prepared for each case file.

4.2.2 The DUI or toxicology worksheets should document unique specimen identifiers and the evidence should be labeled accordingly.

4.2.2.1 For postmortem cases, a toxicology worksheet is prepared which documents the numbers and types of specimens along with their unique identifiers (e.g., two vials of iliac blood, uniquely identified as TX1A and TX1B). The specimens are labeled with the unique DFS laboratory number, unique item/subitem designation and initials of the analyst labeling the evidence (in accordance with QM).

4.2.3 The DUI and toxicology worksheets are used to document additional information such as deficiencies in external packaging, evidence seals, and unusual types or conditions of specimens.

4.3 Storage of Toxicology Biological Evidence

4.3.1 Specimens received by evidence receiving and the toxicology laboratory should, as appropriate, be refrigerated (2-8°C) as soon as possible to preserve their condition. Specimens may also be stored frozen (<-10ºC).

4.3.2 Whenever evidence is not actively being analyzed, it should be stored in secured evidence refrigerators. Access to the refrigerators should be limited to toxicology personnel.

4.3.2.1 Evidence in the process of examination generally will not remain in short term storage for longer than 180 days.

4.3.3 Evidence custody for the receipt into the section and placement into administrative storage shall be documented in accordance with the Quality Manual. Evidence custody for accessioning of items/subitems shall be documented on toxicology worksheets with FS number, item/sub-item designation, date, and analyst's signature. Time in/out of administrative custody will be recorded when sampling evidence.

4.3.3.1 If evidence is transferred between analysts while it is not in the locked toxicology administrative storage, the transfer will be documented on the Toxicology Item Chain of Custody Form. The analysts will indicate the affected items and will document the transfer of custody with their signatures, dates, and times.

4.3.4 Upon completion of a case, OCME and non-implied consent cases are resealed and retained in access-controlled evidence refrigerators until they are returned to the submitting agency. Completed DUI cases are stored at least 120 days to ensure compliance with the Code of Virginia §18.2-268.7 (see DUI evidence handling section).

4.3.5 If three vials of identical postmortem blood specimens are received, deidentified aliquots of postmortem samples, from one tube, may be reserved for quality assurance purposes. The aliquotting of these samples shall be recorded on a Batch Worksheet.

4.3.5.1 Tissue homogenates prepared by DFS may be deidentified and reserved for quality assurance purposes.

4.3.6 DUI and Toxicology-Other samples that use vacuum blood collection tubes are submitted with the original rubber stopper. The rubber stoppers may be removed during the course of sampling, discarded, and replaced with disposable caps/tops.

4.4 DUI Evidence Handling

4.4.1 The following guidelines apply to the receiving, processing, storage and destruction of driving under the influence (DUI) samples pursuant to the Code of Virginia §18.2-266, 268.6 and 268.7 or obtained via search warrant pursuant to the Code of Virginia §18.2-268.5-18.2-268.7.

4.4.2 Evidence Receipt

4.4.2.1 Evidence is typically submitted by mail through a carrier service (United States Postal Service, UPS or Federal Express) and received by DFS Evidence Receiving staff. The unopened container is then transferred to the Toxicology Section. Evidence may also be received directly (hand to hand) by Evidence Receiving staff from submitting officers. All evidence is placed directly into toxicology administrative storage.

4.4.2.2 DUI evidence can be accessioned using one of two methods which shall be documented on the DUI/DUID Summary Worksheet:

Method 1: One DUI kit is accessioned individually and processed to completion as described in Section 4.4.2.3 to Section 4.4.2.7.

Method 2: Several DUI kits are accessioned simultaneously as described in Section 4.4.2.3 to Section 4.4.2.7.

4.4.2.3 The blood vials are removed from the mailing container and the mailing container is discarded. The blood vials are inspected for any discrepancies (e.g., evidence not sealed, vials not provided by DFS or Certificates of Blood Withdrawal (CBWs) not attached to vials). Any discrepancy is noted in the case file.

4.4.2.4 The receiving employee initials both blood vials and affixes the LIMS-generated barcodes with the FS lab number to each vial. If an RFLE is received, then a LIMS-generated barcode with the FS number is affixed to the RFLE and the receiving employee signs and dates the RFLE.

4.4.2.5 The receiving employee shall create a DUI/DUID summary worksheet for each case. The DUI/DUID worksheet includes FS lab number, suspect name, description of number of blood vials and their corresponding vial numbers and the signature of the employee that opened the DUI kit.

4.4.2.6 One of the blood vials is selected for analysis by DFS (typically the blood vial with the greatest quantity of blood). The receiving employee removes the CBW from this vial and labels the CBW with the FS number, their initials and the date. The removed CBWs are stored until the Certificates of Analysis are generated. All blood vials are stored in access-controlled refrigerators.

4.4.2.6.1 If accessioning Method 2 is utilized, an independent person will verify the following:

- The FS Lab # on each blood vial is consistent with the FS Lab # written on the DUI/DUID Summary Worksheet, the RFLE, if available, and the information in FA.
- The name on the Certificate of Blood Withdrawal is consistent with the name written on the DUI/DUID Summary Worksheet and RFLE.
- The vial number on each blood vial is consistent with the vial numbers written on the DUI/DUID Summary Worksheet.

The verification shall be documented on the DUI/DUID Summary Worksheet with initials and date.

4.4.2.6.2 If additional blood is required for analysis, the second tube may be utilized. If opened, the second CBW will be removed from the vial and labeled with the FS number, initials and the date. The removed CBWs will be stored together.

4.4.2.7 Upon completion of analysis, all blood vials will be sealed and retained in long term storage for a minimum of 120 days after the completion/release of the certificate of analysis (see ¶ 4.3.4). The samples will not be destroyed and will be returned to the submitting agency if the Department receives a written request from the Commonwealth during the examination and subsequent 90 day period.

4.4.3 DUI Evidence Processing and Storage

4.4.3.1 The analyst who analyzes the blood for ethanol content is the analyst that initially breaks the blood vial seal.

4.4.3.2 Once testing is completed, a Certificate of Analysis is generated and the stored CBW is affixed to the Certificate of Analysis. The blood vial is then resealed and partnered with the second vial of blood in the refrigerator upon completion/release of the Certificate of Analysis in LIMS. The sealed vial(s) should be stored for a minimum of 120 days following the completion/release of the certificate of analysis.

4.4.4 Motions and Court Orders to Transmit Blood Samples to Independent Laboratories

4.4.4.1 All motions and court orders to transmit blood samples are given to a designated DFS employee.

4.4.4.2 Each motion and court order is labeled with the date of receipt (or electronic record) and shall be sent to the Administration/Legal section. Once the motion/order is received into Toxicology from Administration/Legal, add the FS Lab number (if needed).

4.4.4.3 Receipted motions and orders are documented in the DUI excel spreadsheet for electronic tracking.

4.4.4.4 Upon receipt of motion or order, the corresponding blood vials are identified for eventual court ordered release.

4.4.4.5 Motions are stored in the motion file until the corresponding court order is received.

4.4.4.6 Once a court order (signed by a judge) is received, the order is sent to the appropriate DFS personnel who will generate a packet containing a certified mail mailing envelope, partially completed transfer of custody form and a copy of the court order.

4.4.4.7 When the packet is received, a designated toxicology employee or examiner will fill out remaining sections of the transfer of custody form. The sealed blood vials are placed into a DUI shipping container and subsequently placed into the mailing envelope with the copy of the court order and mailed via a carrier service that utilizes a package tracking system which includes documentation confirming delivery. Documentation confirming delivery is attached to the corresponding transfer of custody forms and stored within each case file.

4.4.4.8 When blood samples are mailed, document “transfer out” in LIMS. In comments, document that pursuant to a court order, blood samples were sent to independent laboratory (specify lab).

4.4.5 Requests from Commonwealth’s Attorneys to Return Blood Samples.

4.4.5.1 All written requests to return blood samples are given to a designated DFS employee.

4.4.5.2 Each request is labeled with the date of receipt, name or initials of employee receiving the request, and corresponding FS lab number (if available). A copy of the request, and any subsequent withdrawals of that request, if received will be printed and placed in the case file.

4.4.5.3 Received requests are documented in the DUI excel spreadsheet for electronic tracking.

4.4.5.4 Upon receipt of a request, the corresponding blood vials are identified for eventual return. After the 90 day post-examination period has ended and no request for independent testing has been received, the request is sent to the appropriate DFS personnel who will generate a packet containing a partially completed transfer of custody form and a copy of the request and provide it to toxicology staff.

4.4.5.5 When the packet is received, a designated toxicology employee or examiner will fill out remaining sections of the transfer of custody form. The evidence will be prepared for return to the agency via the initial submission process (e.g., hand delivery, mail carrier) unless another method of return has been requested.

4.4.5.5.1 For evidence that is being returned by mail, the sealed blood vials are placed into a DUI shipping container (or other means of containment such as a heat seal bag) and subsequently placed into a mailing envelope with the copy of the request and mailed via a carrier service that utilizes a package tracking system which includes documentation confirming delivery. Documentation confirming delivery is attached to the corresponding transfer of custody forms and stored within each case file. When blood samples are mailed, document "transfer out" in LIMS. In comments, document that pursuant to a request, the blood samples were returned to the submitting agency.

4.4.5.5.2 For evidence that is being returned by hand delivery, the sealed blood vials are placed into a DUI shipping package (or other means of containment such as a heat seal bag) and transferred to the Evidence Receiving section. If the evidence was originally submitted without a Request for Laboratory Examination (RFLE), a RFLE will be completed by the toxicology employee or examiner. The evidence is then returned to the submitting agency.

4.4.5.5.3 Note: The "DUI shipping container (or other means of containment such as a heat seal bag)" does not need to have a container created for it in LIMS. Items not in a container may be returned to Evidence Receiving or via mail service.

4.4.5.6 In accordance with VA Code 18.2-268.7 B, if requests for both the return of the samples to agencies and to transmit the samples to an independent laboratory are received by toxicology, the request to transmit the samples to an independent laboratory will take priority.

4.4.6 Destroying DUI Evidence

4.4.6.1 If, after 90 days following the release of the CoA, no motions or court orders have been received on a particular case, the blood samples shall be destroyed after 120 days to ensure compliance with the Code of Virginia §18.2-268.7.

4.4.6.2 A Tox DUID Destruction Report (called a destruction list or other named report of samples that can be destroyed) will be generated.

4.4.6.3 Before the destruction of samples, a second person shall verify that samples with pending holds/blood motions/agency return requests or which have extended destruction dates (e.g., supplemental testing performed) are excluded from the destruction list. The verifier's initials and date on the destruction list will denote this verification.

4.4.6.4 The physical evidence will be collected (or barcode scanned into a LIMS batch) and then verified by a second person against the destruction list. The verifier's initials and date on the destruction list will denote this verification. Alternatively, the verifier may enter their password in LIMS as a "witness"; password entry in this context will denote that the verification was performed.

4.4.6.5 After the destruction list and physical evidence have been verified, the physical evidence will be destroyed by removing any remaining Certificates of Blood Withdrawal to deidentify the samples. The blood vials will then be placed in the biohazard trash.

4.4.6.6 A deidentified aliquot may be reserved for quality assurance purposes.

4.4.6.7 Document destruction of blood vials in LIMS and retain the destruction lists according to the Department's record retention policy.

5 ESTIMATION OF THE MEASUREMENT UNCERTAINTY

5.1 Scope

An estimation of the measurement uncertainty shall be determined for all analytical procedures in which a quantitative measurement is reported on the Certificate of Analysis.

5.2 Documentation

Calculations related to the reported estimation of measurement uncertainty shall be maintained in the individual case file in which the measurements are made. Uncertainty budgets are maintained electronically by the Program Manager. Some analytes may require the use of "220-F135 Toxicology Day of UoM Worksheet(s)" or "220-F161 Toxicology ABC Day of UoM Worksheet" which shall be maintained in the affected individual case file(s).

5.3 Measurement Uncertainty

5.3.1 Uncertainty Budget

5.3.1.1 Estimations of the measurement uncertainty shall be conducted and documented using an uncertainty budget.

5.3.1.2 The uncertainty budget for a given procedure shall include both random (Type A) uncertainties and systematic (Type B) uncertainties.

5.3.1.3 Since the measurement uncertainty is only an estimate, generally uncertainties shall not exceed two significant figures.

5.3.1.4 Calculations used to estimate the uncertainty shall be rounded using conventional rounding rules (see Quality Manual).

5.3.1.5 In order to combine the uncertainty, the uncertainty values should be measured in the same units. Typically, it is beneficial to express all uncertainty values in % to eliminate the necessity to convert measurements to the same units.

5.3.2 Type A Standard Uncertainty

5.3.2.1 Random (Type A) uncertainty results from measurement values being scattered in a random fashion due to laws of chance and thus has a normal or Gaussian shaped distribution.

5.3.2.2 Random (Type A) uncertainty is best determined by historical data of repeated measurements.

5.3.2.3 Control charts are used to establish the standard deviation and relative standard deviation for common quantitative procedures.

5.3.2.3.1 Examples of control data include the following:

- Lipomed BAC controls
- UTAK controls
- Statewide controls
- In-house prepared controls

5.3.2.3.2 A control chart is a graphical representation of statistically analyzed data and is designed to monitor an analytical process to verify that it is operating within an expected or desired range.

5.3.2.3.3 Once the control chart is established, all future analyses shall be plotted on the appropriate control chart and monitored for trends.

5.3.2.3.4 The estimation of measurement uncertainty shall only utilize control chart data from the previous two years, if available.

5.3.2.3.5 The relative standard deviation used for uncertainty calculations shall be calculated annually using the previous two years of control data, if available.

5.3.2.4 For newly implemented methods that lack two years of control data, the Type A data can be obtained from the validation, verification, and from on-going control data until the two year interval is met.

5.3.2.5 For quantitative analyses which may not be able to establish an annual uncertainty of measurement value, six control replicates shall be analyzed to determine the relative standard deviation of the mean. Control acceptance criteria can be found in ¶ 2.

5.3.2.5.1 All six control measurements shall be recorded in the "220-F135 Toxicology Day of UoM Worksheet" or "220-F161 Toxicology ABC Day of UoM Worksheet".

5.3.2.5.1.1 These replicates are considered to be normally distributed with a divisor of 1.

5.3.2.5.2 The control accuracy of ± 20% will also be utilized in calculating the measurement uncertainty.

5.3.2.5.2.1 Control accuracy is considered to be a rectangular distribution and therefore is divided by the √3.

5.3.2.5.3 The root sum squared method shall be used to calculate the combined uncertainty.

5.3.2.5.4 The expanded uncertainty shall be calculated using the student's t-table for n = 6 (2.65) at 95.45% level of confidence.

5.3.2.6 When multiple measurements are performed on case specimens (e.g., BAC analysis performed in duplicate) the measurements shall be averaged.

5.3.2.6.1 When multiple measurements are performed, as specifically required by the Procedures Manual, uncertainty is calculated using the standard deviation of the mean (σ_{mean}) which is calculated by dividing the historical standard deviation (σ) by the square root of the number of measurements (n).

5.3.2.6.2 In all other instances where multiple measurements are performed a conservative approach shall be taken by using a divisor of 1.

5.3.3 Type B Standard Uncertainty

5.3.3.1 Systematic uncertainty results from the inherent biases in measuring systems and quantitative analytical methods. These uncertainties may be reduced by optimizing the method or measuring system, but can never be completely eliminated.

5.3.3.2 Examples of systematic uncertainties are:

5.3.3.2.1 Preparation of calibrator, internal standard or control solution using 10 mL volumetric flask.

5.3.3.2.2 Using an analytical pipette to prepare calibrator or controls.

5.3.3.2.3 Using an analytical pipette to sample 2 mL of specimen.

5.3.3.2.4 Using a Hamilton diluter to prepare calibrators, controls and specimens for BAC analysis by headspace GC.

5.3.3.2.5 Using a repeater pipette to dispense internal standard into all calibrators, controls and case specimens.

5.3.3.2.6 Uncertainty associated with Certificates of Analysis on analytical standards (e.g., Cerilliant Certificate of Analysis on Ethanol-50 standard indicating ethanol concentration of 50 ± 1.55 mg/dL at 95.45% level of confidence). Note: A 95.45% level of confidence is equivalent to 2σ and should be divided by 2 for use in uncertainty calculations (which calculates uncertainty based on 1σ).

5.3.3.3 The scope of the control data used in the estimation of Type A uncertainty (e.g., control data gathered by all four DFS toxicology laboratories) already encompasses some associated Type B uncertainties including instruments, maintenance, and analysts.

5.3.3.4 The use of an internal standard for quantitative analysis minimizes other sources of uncertainty including instrumental factors such as the injector, GC column and detector. These miscellaneous uncertainty factors have no significant impact on the overall estimation of uncertainty and are therefore not included in the calculation.

5.3.3.5 Systematic (Type B) uncertainties resulting from measurement bias typically have an equal chance of falling within a range and therefore follow a rectangular or random distribution.

5.3.3.5.1 With rectangular distribution, the range (± a) of the outer limits is used to estimate the standard deviation (σ) using the equation $\sigma = a/\sqrt{3}$.

5.3.3.5.2 For example, a 10 mL volumetric flask has a tolerance of ± 0.02 mL. The calculated uncertainty associated with this measurement is $0.02/\sqrt{3}$ or 0.01.

5.3.4 Combination of uncertainties

Uncertainties are combined using the Root Sum Squares technique

$$U_{combined} = \sqrt{(U_1^2 + U_2^2 + U_3^2 + U_4^2 ....)}$$

5.3.5 Determination of confidence

5.3.5.1 In order to determine the expanded uncertainty, the combined uncertainty is multiplied by the coverage factor (k) using the equation $U_{expanded} = U_{combined} \times k$.

5.3.5.2 The coverage factor shall be determined from the Student's t-table for coverage factors of 95.45% and 99.73% using the degrees of freedom (n-1) for each analytes' Type A control data.

5.4 Reporting the Estimated Measurement Uncertainty

5.4.1 The "Toxicology Uncertainty of Measurement Reporting" (or similarly named) worksheet shall be used to generate the UOM/MU reporting values.

5.4.2 The values generated at the annual evaluation will be updated into the reporting worksheet.

5.4.3 All cases examined after the publication of the reporting worksheet will use the updated values.

5.4.4 Since the measurement uncertainty is only an estimate, it shall be rounded and limited to two significant figures (except for ethanol, acetone, isopropanol, and methanol which are rounded to three (3) decimal places). The reported measurement uncertainty shall be rounded to the same level of significance (i.e., decimal places) as the reported concentration. Measurement uncertainty is reported at a 95.45% level of confidence for all toxicological analyses except ethanol or blood alcohol which is reported at a 99.73% level of confidence.

5.4.4.1 Example 1: Ethanol 0.050 ± 0.003% by weight by volume

5.4.4.2 Example 2: Blood Alcohol Content 0.080 ± 0.004% by weight by volume

5.4.4.3 Example 3: Fentanyl 0.0020 ± 0.0002 mg/L

5.5 Measurement Traceability

Measurement Traceability is an essential element of the Department's measurement assurance program and is required for all measurements where measurement uncertainty is reported. Measurement traceability for common toxicological analyses is typically accomplished in the following manner.

5.5.1 Measurement Traceability (Drug Compounds):

5.5.1.1 Calibrators: Traceability is established by the procurement of calibrators from an ISO/IEC 17025 and ISO 17034:2016 accredited reference material provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of Analysis for each reference material are kept in the laboratory or electronically.

5.5.1.2 NIST Traceable Volumetric Flasks: Traceability is maintained for the calibrators by preparing the calibrator stock solutions using NIST traceable volumetric flasks. The certificate of traceability for each piece of glassware is kept in the laboratory or electronically.

5.5.1.3 Calibration of Pipettes: Traceability is maintained for the preparation of calibrator stock solutions by utilizing mechanical pipettes externally calibrated annually by an approved ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of calibration for each pipette are kept in the laboratory or electronically.

5.5.1.4 Balance Traceability:

5.5.1.4.1 Balance Calibration: The traceability is established by the annual, external calibration of the balances by an ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of calibration for each balance are kept in the laboratory or electronically.

5.5.1.4.2 Steel Weight Calibration: Additional traceability is established by the use of steel calibration weights to check the balance calibration monthly. The weights are calibrated every three years by an external, ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of calibration for each weight and weight set are kept in the laboratory or electronically.

5.5.2 Measurement Traceability (Blood Alcohols):

5.5.2.1 Alcohol Calibrators: Traceability is established by the procurement of calibrators from an ISO/IEC 17025 and ISO 17034:2016 accredited reference material provider. The certificate

and scope of the provider is kept with the Quality Assurance Section. Certificates of Analysis for each reference material are kept in the laboratory or electronically.

5.5.2.2 Alcohol Controls: Additional traceability may be established by the procurement of controls from an ISO/IEC 17025 and ISO 17034:2016 accredited reference material provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of Analysis for each reference material are kept in the laboratory or electronically.

5.5.2.3 Calibration of Hamilton Microlab Diluter: Traceability is maintained by utilization of Hamilton Microlab Diluters that are externally calibrated annually by an approved ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificate of calibration for each diluter are kept in the laboratory or electronically.

5.5.3 Measurement Traceability (Alcoholic Beverages):

5.5.3.1 Calibrators: Traceability is established by the procurement of a calibrator solution that is a NIST Standard Reference Material. Certificates of Analysis for the reference material is kept in the laboratory or electronically. The certificate and scope of the provider is kept with the Quality Assurance Section.

5.5.3.2 NIST Traceable Volumetric Flasks: Traceability is maintained for the calibrators by preparing the calibrator solutions using NIST traceable volumetric flasks. The certificate of traceability for each piece of glassware is kept in the laboratory or electronically.

5.5.3.3 Calibration of Pipettes: Traceability is maintained for the preparation of calibrator solutions by utilizing mechanical pipettes externally calibrated annually by an approved ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of calibration for each pipette are kept in the laboratory or electronically.

5.5.3.4 Alcohol Controls: Additional traceability is established by the procurement of controls from NIST or an ISO/IEC 17025 and ISO 17034:2016 accredited reference material provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of Analysis for each reference material are kept in the laboratory or electronically.

5.5.3.5 Calibration of Hamilton Microlab Diluter: Traceability is maintained by utilization of a Hamilton Microlab Diluter that is externally calibrated annually by an approved ISO/IEC 17025 accredited calibration provider. The certificate and scope of the provider is kept with the Quality Assurance Section. Certificates of calibration are maintained in the laboratory or electronically.

5.6 References

5.6.1 ASCLD/LAB Policy on Measurement Uncertainty. (AL-PD-3060 Ver. 1.1)

5.6.2 ASCLD/LAB Guidance on the Estimation of Measurement Uncertainty – ANNEX D, Toxicology Testing Discipline. (AL-PD-3065 Ver. 1.0)

6 QUALITY ASSURANCE

6.1 Introduction

6.1.1 The purpose of this section is to provide a uniform Quality Assurance Program for the Section. In combination with the toxicology quality guidelines, it is designed to ensure that the parameters of the testing process are routinely monitored in a manner that maintains the success and reliability of the analytical results.

6.1.2 Since most forensic toxicology specimens are limited in quantity or volume, it is highly desirable to minimize the need for repeat analysis due to the failure of equipment, materials or reagents. The focus of the quality assurance program is to prevent problems before they occur rather than address the failures after they happen.

6.1.3 It is expected that the analyst will report any unacceptable or anomalous behavior of any analytical system immediately to their supervisor. It is further expected that appropriate actions will follow as soon as possible and be properly documented.

6.2 Reagents

6.2.1 Chemicals used in qualitative and quantitative analyses should be of at least ACS reagent grade or better.

6.2.2 Solvents shall be high quality, low residue solvents (e.g., HPLC grade, Omnisolv, Optima etc).

6.2.3 Water used in reagent preparation should be either deionized or reverse osmosis (abbreviated throughout this manual as dH_2O).

6.2.4 Upon receipt of all reagents, chemicals and supplies that could potentially affect test results, the packing slip will be checked for agreement with items received and this review is documented with the signature of the receiver on the packing slip.

6.2.4.1 The signature of the Supervisor / Program Manager / Director on the purchase request signifies that the supply ordered meets the quality specifications listed above and in the toxicology quality guidelines.

6.2.4.2 A copy of the signed purchase request and a signed copy of the packing slip shall be maintained by the Section Supervisor, or designee, for at least six years.

6.2.5 The following information shall be recorded for all purchased reagents and reference materials, either on the bottle or in a log (hardcopy or electronic) with a reference to the bottle:

6.2.5.1 Date of receipt

6.2.5.2 Date opened

6.2.5.3 Date of verification (if appropriate)

6.2.5.4 The initials of the person opening the bottle

6.2.5.5 The initials of the person performing the verification, if different

6.2.5.6 The expiration date, if necessary

6.2.6 Reagents, chemicals and supplies shall be handled, transported, stored and used in a manner that maintains their quality at an acceptable level. In general, the manufacturer's recommendations for storage conditions as specified on the product label should be followed.

6.2.7 All laboratory prepared reagents, solutions and standards shall be prepared using good laboratory practices.

6.2.8 All prepared reagents shall be documented on the *Reagent Preparation Log*, the *Toxicology Reagent Preparation Log*, or in an electronic log system (e.g., Resource Manager). Hardcopy forms shall be maintained in a reagent preparation logbook (Note: exception for freshly prepared reagents). Reagents that are prepared fresh/consumed on the date of production may be documented on the Toxicology Batch Lot Summary sheet and do not need to be documented on the Reagent Preparation Log or electronically. A different final volume of reagent not listed in a particular reagent preparation instruction may be made as long as the preparation and the final volume prepared are documented on the reagent log.

6.2.8.1 A reagent preparation form is created with the individual reagent, preparation instructions and chemical names. Upon completion, the accuracy of the individual form is reviewed by a supervisor or their designee to ensure that the preparation instructions and QC are in compliance with the most current version of the toxicology procedures manual. This review process is documented with initials and date.

6.2.8.2 All reagents must be quality control tested for reliable performance. The QC check is typically performed within a batch of samples as evidenced by the acceptable performance of the calibrators and controls with the particular reagent. As such, the documentation of the QC check is typically the unique FS lab file number that contains the batch analysis raw data.

6.2.8.3 All laboratory prepared reagents/solutions will be clearly labeled to include at a minimum reagent identity, preparer's initials and date of preparation or lot number.

6.2.9 In general, all solutions and reagents (unless otherwise indicated in a specific SOP) may be stored at room temperature for up to 2 years after preparation date or when the solution/reagents fails the quality control check (whichever comes first).

6.2.10 All chemicals and commercial reagents should be replaced when their stated expiration date or shelf life has expired and/or when they fail the quality check.

6.3 Preparation of Blank Blood

6.3.1 Obtain human packed blood cells from a blood bank.

6.3.2 Empty packed blood cells into a large glass jar/container with a lid.

6.3.3 Add approximately 100 mL dH_2O and approximately 100 mL 1% sodium fluoride (10 gm sodium fluoride dissolved in 1 L dH_2O) to achieve similar consistency/fluidity as whole blood.

6.3.4 Label the prepared blank blood with a unique lot number, preparation date and the initials of the preparer.

6.3.5 Analyze each lot of blank blood using an Immunoassay control panel, base screen, and other appropriate assays not covered by immunoassay. The approving analyst shall clearly note on the data and the bottle any analyte for which the lot is positive.

6.3.6 The lot of blank blood shall not be used for assays that tested positive for the analyte of interest. If additional analyses are to be performed, the blank blood is only acceptable as a negative control if the analyte of interest is shown to be absent during analysis.

6.3.7 Records from blank blood preparation and screening shall be maintained in the section for a minimum of ten years after they are removed from service.

6.3.8 Store at 2-8°C for up to two years.

6.4 Reference Materials

6.4.1 Reference materials shall be at least of United States Pharmacopeia – National Formulary (USP-NF) quality and are used to prepare calibrators or controls.

6.4.1.1 Whenever possible, certified reference materials should be acquired from vendors accredited to ISO/IEC 17025 and ISO 17034:2016 and include the supplier's Certificate of Analysis to document traceability, purity, accuracy, precision and homogeneity. If the above requirement cannot be met, it is acceptable to establish traceability through ISO 17034:2016.

6.4.1.1.1 For instances where traceability cannot be established using certified reference materials (carboxyhemoglobin analysis) other approved vendors may be used to purchase reference materials.

6.4.1.2 Patented reference materials may be obtained directly from the pharmaceutical manufacturer.

6.4.1.3 Reference materials used in casework are considered critical supplies and shall be purchased from manufacturers/vendors/suppliers approved by the Program Manager.

6.4.1.4 The following manufacturers/vendors/suppliers are pre-approved for the purchase of new reference materials:

6.4.1.4.1 Calibrator solutions shall be prepared using certified reference materials from ISO/IEC 17025 and ISO 17034:2016 approved vendors (refer to ¶ 5.5.1.1):

- Cerilliant (may also be listed on COAs as Supelco or Sigma-Aldrich)
- RTC
- Cayman Chemical
- LGC
- Lipomed
- Restek
- SPEX Certiprep

6.4.1.4.2 Non-calibrator solutions may be made from the above mentioned list (¶ 6.4.1.4.1) and from the following approved vendors:

- UTAK Laboratories, Inc
- Immunalysis
- RNA Medical
- IL CO controls
- Fisher
- SIGMA-ALDRICH (non-CRM materials)
- USP (United States Pharmacopeia)
- Oakwood Chemical
- Santa Cruz Biotechnologies
- MP Biomedicals

6.4.1.5 Reference materials not verified by the vendor must be verified and documented prior to use or concurrently with casework. Whenever possible, verification should include full spectrum GC-MS analysis with comparison to library spectra and the absence of additional/interfering chromatographic peaks or the use of other analytical techniques as necessary (LCMSMS or UV-VIS spectrophotometry) to generate suitable definitive instrumental data.

6.4.1.5.1 Verification data should be labeled with analyst's initials and date.

6.4.1.5.2 Store the verification data in the drug standard logbook containing corresponding Certificates of Analysis from drug manufacturers. Drug manufacturer Certificates of Analysis can be stored electronically in appropriately labeled folders.

6.4.1.5.3 Due to the nature of the work within the Section, verification may be performed within a batch of samples when the standard is used as a calibrator or control. As such, the documentation of verification may be stored with the unique FS lab file number that contains the batch analysis raw data with the FS number noted within the drug standard records.

6.4.1.6 Reference materials shall be stored in a manner that maintains their quality. In general, powders are stored at room temperature, aqueous solutions are stored at 2-8°C and solvent-based standards are stored at -10 to -20°C, unless otherwise indicated by the supplier or in a specific method SOP. Allow all reference materials and reagents to come to room temperature prior to starting procedures.

6.4.1.7 After opening the CRM manufacturer's ampoule, transfer contents to an airtight container for storage in the appropriate conditions.

6.4.1.8 DFS assigned expiration dates for multi-component reference material solutions shall be set for two years from date of preparation or for the earliest expiration of a reference material component, whichever is shorter. Solutions may be retested to extend the DFS expiration date for at most one additional year. Exceptions to assigned expirations and/or extensions may be approved by the Program Manager or noted within specific procedures. DFS assigned expiration dates for blood alcohol CRMs are delineated in the Alcohols by Headspace Gas Chromatography method. DFS assigned expiration dates for alcoholic beverage CRMs are delineated in the Ethanol Content of Alcoholic Beverages by Headspace GC method.

6.4.2 Records (may be recorded in hardcopy or in an electronic format)

6.4.2.1 Drug reference materials and quantities will be tracked pursuant to all legal requirements. Tracking of other reference materials may be conducted in the laboratory for supply chain purposes.

6.4.2.2 The *Drug Stock Standard Preparation Log* will be used to track the preparation of stock standards. If the stock standard is used as a calibrator, or used to create a calibrator, the serial number of the calibrated glassware used shall be documented on the preparation log.

6.4.2.3 The *Multi-component Standard, Calibrator and Control Preparation Log* will be used to track the preparation and verification of multi-component standard, calibrator and control solutions. If the solution is used as a calibrator, or used to create a calibrator, the serial number of the calibrated glassware used shall be documented on the preparation log. The preparation log may be stored electronically.

6.5 Reference Collections

6.5.1 Reference collections of data or materials used for identification, comparison or interpretation shall be fully documented, uniquely identified and properly controlled.

6.5.2 Purchased data libraries (reference collections) are fully documented and uniquely identified. No changes may be made to purchased reference collections. Examples of such libraries include GC-MS NIST, GC-MS Pfleger/Maurer/Weber, and Wiley.

6.5.3 Data libraries obtained from reputable forensic sources are fully documented and uniquely identified. No changes may be made to these reference collections. The addition or removal of forensic libraries must be approved by the Program Manager. Current forensic libraries approved for use:

- GC-MS AAFS
- GC-MS NIST
- GC-MS Wiley
- GC-MS ENFSI
- GC-MS TIAFT
- GC-MS SWGDRUG
- GC-MS Cayman Chemical
- GC-MS Pfleger/Maurer/Weber
- GCMS NPS Discovery Library

6.5.4 For in-house libraries, each entry is automatically identified by a unique tracking number generated by the instrument software. These libraries should be generated or modified by an instrument operator or by a designee of the Section Supervisor.

6.5.4.1 At a minimum the following information should be included with each new entry into in-house data libraries:

- Compound name
- Drug standard identifier (lot number)
- Date
- Initials of person entering data

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6.6 Balances

6.6.1 All analytical balances will be checked monthly for accuracy using Class 1 weights or better. Record the weights in the balance logbook with the date and analyst's initials. The logbook for balances may be hardcopy or in an electronic format (e.g., Resource Manager).

6.6.2 Weights used to check balance accuracy shall be re-certified every three years by an ISO/IEC 17025 accredited vendor whose scope of accreditation covers the certification performed.

6.6.3 The below listed balances are intended as examples of a balance class of type with appropriate check weights. If the individual balance does not fit into these categories, use three weights within its range or as approved by the Program Manager.

Balance Type	Balance Examples	Check Weights
Analytical (dual range)	Mettler XS 105	0.01000 (± 0.00005) g 20.00000 (± 0.00020) g 50.0000 (± 0.0005) g 100.0000 (± 0.0005) g
Analytical	Mettler AE 160 Mettler AG204 Ohaus E-10640	50.0 (± 0.2) mg 100.0 (± 0.2) mg 1000.0 (± 0.2) mg
Top Loading (± 0.01) gram	Mettler PE 2000 Mettler PB303 Mettler PE1600 Sartorius 2100	5.00 (± 0.02) g 10.00 (± 0.03) g 100.00 (± 0.03) g
Top Loading (± 0.001) gram	Mettler PC303 Ohaus Explorer	0.500 (± 0.002) g 1.000 (± 0.002) g 100.000 (± 0.005) g

6.6.4 Accuracy must be established for monthly balance checks or after a balance has been put into service after purchase, maintenance or repair. The following are guidelines for performing balance checks.

6.6.4.1 The check weights listed in the table in 6.6.3 are weighed and recorded on the balance logsheet.

6.6.4.2 The accuracy of each weight should meet the criteria in 6.6.3

6.6.4.3 If the accuracy of a weight is outside the acceptable range, ensure that the balance is level and clean prior to rechecking.

6.6.4.4 Perform balance performance check again. If the balance accuracy fails again, use the internal calibration function to recalibrate balance. Repeat balance check. Record all pre and post calibration measurements on logsheet.

6.6.4.5 If, after the above mentioned actions, the result of the balance check is still outside of the acceptable range, re-calibrate the balance for a second time. Repeat balance check. If it fails again, the balance shall be taken out of service (and labeled as such) until maintenance and/or calibration are performed by a qualified and approved vendor.

6.6.4.5.1 Record the service call on the analytical balance QC sheet kept with each analytical balance.

6.6.4.5.2 If the balance is taken out of service for repair/maintenance, perform a balance check prior to putting the balance back into service.

6.6.5 All balances are cleaned, serviced and calibrated annually by an outside vendor that is accredited to ISO/IEC 17025 and whose scope of accreditation covers the calibration performed. Record the service call on the analytical balance QC sheet kept with each analytical balance. Each balance shall be labeled with the date of calibration and when the next calibration is due.

6.6.6 Minimum balance loads:

6.6.6.1 5-Place balance = 0.00450 gram

6.6.6.2 4-Place balance = 0.0300 gram

6.6.6.3 3-Place Balance = 0.150 gram

6.6.6.4 2-Place balance = 0.90 gram

6.6.6.5 High Capacity (g) Balance = 27.0 grams

6.7 pH Meters

6.7.1 Calibrate the pH meter prior to each use. Refer to the individual pH meter's instrument manual for these procedures.

6.7.1.1 The reference buffers chosen should bracket the expected pH value range of the solution, if possible.

6.7.1.2 The pH values must be within ± 0.1 units of the pH value stated on each individual reference buffer's labeling.

6.7.1.3 If the calibration values are within the accepted limits, the pH meter is ready to use for reagents.

6.7.1.4 If the calibration values are not within the accepted limits, rerun and/or troubleshoot as necessary.

6.7.2 Rinse the electrode with dH_2O, as appropriate.

6.7.3 Reference buffers shall be replaced when they expire.

6.7.3.1 Keep the buffer bottle tightly sealed and free of contamination.

6.7.3.2 Do not reuse an aliquot of buffer or return it to the original bottle.

6.7.4 Refer to the pH meter instrument manuals for recommendations on proper use and storage, good laboratory practices, correct applications, problem samples and trouble shooting.

6.7.5 For non-critical pH measurements, an approximate pH may be determined using pH paper.

6.8 Pipettes

6.8.1 Fixed volume, variable volume, multichannel repeater pipettes and Hamilton diluters shall have their calibration evaluated and certified annually by an approved ISO/IEC 17025 accredited vendor whose scope of accreditation covers the calibration performed.

6.8.2 Maintain repair documentation and calibration certificates generated by the vendor in the pipette logbook. The calibration certificates or labeling of successful calibration from the vendor on the pipettes demonstrate that the pipette has passed QC prior to being placed into or back into service. The logbook for pipettes may be hardcopy or in an electronic format (e.g., Resource Manager).

6.8.3 As needed, clean the inside and outside of pipettes with isopropanol and check the seal.

6.8.4 If a pipette appears to be out of calibration between normally scheduled performance/calibration checks, the pipette will be sent to an authorized vendor for repair.

6.8.4.1 The maintenance/repair shall be documented in the pipette logbook along with the calibration certificate generated by the vendor.

6.8.5 All pipettes shall be uniquely identified and appropriately labeled with the date of calibration and when the next calibration is due.

6.9 Refrigerators/Freezers

All refrigerators and freezers that are used to store biological evidence or critical reagents should be monitored triweekly to ensure the appropriate storage temperature. This includes refrigerators and freezers within the section and those used by evidence receiving for the temporary storage of toxicology evidence.

6.9.1 Post a temperature log on each refrigerator or freezer. The log may be maintained in hardcopy or in an electronic format (e.g., Resource Manager).

6.9.2 For refrigerators/freezers without a digital external temperature monitor, place a thermometer in each refrigerator or freezer. All thermometers should be NIST traceable or verified annually against a NIST traceable thermometer (see thermometer section).

6.9.3 Read and record the thermometer temperature of refrigerators and freezers triweekly (Monday, Wednesday and Friday). If the temperature checks cannot be performed on Monday, Wednesday or Friday due to a holiday or other closure, they should be made on the first regularly scheduled workday following the holiday or other closure.

6.9.4 Record temperatures on the temperature log along with the initials of the staff member performing the temperature check.

6.9.4.1 For refrigerators, the temperature should fall between 2-8°C.

6.9.4.2 For freezers, the temperature should fall below -10°C.

6.9.5 If the temperature should fall just outside the acceptable range, the thermostat should be adjusted accordingly to bring the temperature back into the acceptable range. Document this adjustment on the temperature log and continue to monitor the temperature of the unit daily for the following week to ensure the thermostat adjustment was effective.

6.9.6 For extreme temperature changes (e.g., freezer above 0°C, refrigerator below 0° C or greater than room temperature), all biological evidence and critical reagents should be removed immediately from the affected unit and placed in alternative refrigerators and/or freezers. The refrigerator or freezer should be placed out of service, labeled as such until it can be repaired and the repair should be documented on the temperature log.

6.9.6.1 The quality of critical reagents exposed to extreme temperatures may be compromised and the affected reagents should undergo a performance check or verification prior to their use on casework.

6.9.7 Maintain temperature logs for all refrigerators and freezers for at least six years.

6.10 Heat Blocks

6.10.1 Heat blocks are generally used for the evaporation or derivatization of samples.

6.10.2 With each use, the temperature of the heat block should be checked with a thermometer to ensure the temperature falls within the recommended approximate temperature range.

6.10.2.1 The thermometer should be NIST traceable or verified annually against a NIST traceable thermometer.

6.10.2.2 Adjust the thermostat as necessary to achieve the desired temperature.

6.10.2.3 The temperature should fall within 2°C of the recommended temperature range.

6.10.2.4 If the correct temperature cannot be achieved, remove the heat block from service and label it as such until it can be repaired. Document the repair in the heat block instrument logbook. The log may be maintained in hardcopy or in an electronic format (e.g., Resource Manager).

6.10.3 Documentation of the performance of the heat block is evidenced by the acceptable performance of the calibrators and controls with each batch analysis.

6.11 Evaporators

6.11.1 The temperature of the evaporator should be checked using a thermometer to ensure the temperature falls within the recommended approximate temperature range.

6.11.1.1 The thermometer should be NIST traceable or verified annually against a NIST traceable thermometer.

6.11.1.2 Adjust the thermostat as necessary to achieve the desired temperature.

6.11.1.3 The temperature should fall within 2°C of the recommended temperature range.

6.11.1.4 If the correct temperature cannot be achieved, remove the evaporator from service and label it as such until it can be repaired. Document the repair in the evaporator instrument logbook. The log may be maintained in hardcopy or in an electronic format (e.g., Resource Manager).

6.11.2 Documentation of the performance of the evaporator is evidenced by the acceptable performance of the calibrators and controls with each batch analysis.

6.11.3 Check the nitrogen gas supply and replace as needed.

6.11.4 Add water and anti-algae drops as needed (to turbovap).

6.12 Centrifuges

6.12.1 All recommended centrifuge speeds are approximate in order to achieve the appropriate separation of layers. Therefore, no intermediate tachometer checks to verify the speed of the rotor are required.

6.12.2 Clean centrifuge as needed.

6.12.3 If a centrifuge is taken off line for repair/maintenance, it should be labeled as out of service until it is repaired. Document repair/maintenance in the centrifuge maintenance log. The log may be maintained in a hardcopy or in an electronic format (e.g., Resource Manager).

6.13 Thermometers

6.13.1 All thermometers used to check temperatures on refrigerators, freezers, heat blocks and evaporators should be certified by NIST or NIST traceable; or they may be checked for accuracy against a NIST or NIST traceable thermometer annually.

6.13.2 NIST or NIST traceable thermometers are good for 2 years from their original date of calibration. Purchase a new NIST or NIST traceable thermometer when the unit needs to be recalibrated. Maintain documentation of the calibration of the NIST traceable thermometer.

6.13.3 The following are guidelines to verify thermometer accuracy against a NIST or NIST traceable thermometer.

6.13.3.1 Tag each thermometer with an identifying number.

6.13.3.2 Thermometers should be checked for accuracy at temperatures similar to those of their intended use.

6.13.3.3 The easiest way to verify the accuracy of a thermometer is to simply compare its reading to that of a NIST or NIST traceable thermometer under the same conditions (e.g., place a NIST or NIST traceable thermometer in refrigerator with the thermometer requiring verification). Record the temperatures of the thermometer and the NIST or NIST traceable thermometer.

6.13.3.4 To verify the accuracy of digital thermometers on refrigerators and freezers, simply place a NIST or NIST traceable thermometer in the refrigerator or freezer. Record the temperatures of the thermometer and the digital readout.

6.13.3.5 To verify the accuracy of multiple thermometers at one time use one of the following procedures:

6.13.3.5.1 For thermometers used in the range of 2-8°C (refrigerator), prepare an ice/water slurry in a beaker. Place a NIST traceable thermometer and all other thermometers in the beaker. Record the temperature of each thermometer.

6.13.3.5.2 For thermometers used below 0°C, prepare an ice/water slurry in a beaker and add salt to lower the temperature below 0°C. Place a NIST traceable thermometer and all other thermometers in the beaker. Record the temperature of each thermometer.

6.13.3.5.3 For thermometers used in the high temperature range (heat blocks and evaporators), partially fill the wells of a heat block with sand or fill a test tube with glycerol. Turn on the heat block to be in range of 50-80°C. Place a NIST traceable thermometer and all other thermometers in the heat block or glycerol. Record the temperature of each thermometer.

6.13.4 All thermometers should be within ± 2°C of a NIST or NIST traceable thermometer within the range of intended use. Any thermometers that vary more than 2°C from a NIST or NIST traceable thermometer will not be used.

6.13.5 Record the date/initials of the annual thermometer accuracy check on the refrigerator log and on the unique identifying tag on each thermometer. The log may be maintained in hardcopy or in an electronic format (e.g., Resource Manager).

6.14 Incubators (Ovens)

Incubators (ovens) are only used to dry glassware therefore it is not necessary to verify the temperature of the oven.

6.15 Fume Hoods and Biological Safety Cabinets

Refer to the Department Safety Manual.

6.16 UV-VIS Spectrophotometer

6.16.1 Day-of-Use

6.16.1.1 Perform the wavelength analysis. This is not a function on the Agilent Cary 60, therefore this is not required.

6.16.1.2 As necessary, clean sample compartment.

6.16.2 Biannually (every two years per the manufacturer's recommendation and the service contract)

6.16.2.1 Schedule an on-site preventive maintenance service call as per the service agreement.

6.16.2.2 Maintain preventive maintenance documentation in the instrument log. The log may be maintained in hardcopy or electronic format (e.g., Resource Manager).

6.17 Gas Chromatographs

6.17.1 Most toxicology procedures are performed in batch and therefore most maintenance procedures are performed prior to running a batch of samples. Record all maintenance in the Instrument Log (hardcopy or electronic (e.g., Resource Manager)) with the date and initials.

6.17.2 Day-of-Use

6.17.2.1 For MSD, perform Autotune. A copy (electronic or hardcopy) of the Autotune shall be maintained for at least six years. Autotune results shall meet the following criteria:

- The mass assignments shown in the upper profile part of the display should be within ± 0.2 amu of 69, 219 and 502.
- Inspect the mass peaks in the upper profile part of the display for good peak shape (no peak splitting and resolution between mass 502 and 503).
- The peak widths (PW) of the three peaks should be 0.6 ± 0.1 amu.
- The isotope ratio figures (indicating the relative abundances of the naturally occurring isotopes) should be within 25% of the theoretical values of 1.08 for m/z 69, 4.32 for m/z 219 and 10.09 for m/z 502.
- Air and water leaks (masses 28 and 18) should be minimal.

6.17.2.2 Run a Performance Check (e.g., a specific instrument check mix or a batch calibrator). Maintain a hardcopy or digital version in the laboratory.

6.17.3 As Needed

6.17.3.1 The following should be replaced as needed:

- Septa/Merlin Microseal
- Liner
- Gold seal
- Gap column
- Syringe
- Columns
- Gas filters

6.17.3.2 Check and replace gas cylinders.

6.17.3.3 Clip front portion of column and reinstall.

6.17.3.4 Silanize injection port liner (excluding instruments with NPD). An example of instrument parameter which may be used:

- Injector: Split Mode
- Gas Saver: Off
- Split Ratio: 200:1
- Injection port: 200ºC
- Oven: 200ºC
- Silanizing reagent: Silyl-8
- Injection volume: 2 µL
- Condition column after injection

6.17.3.5 Condition column

6.17.3.5.1 Short method example: Hold oven temperature 10 degrees above Final Temperature for 15 minutes

6.17.3.5.2 Long method example: Hold oven temperature 300 degrees for 600 minutes (overnight)

6.17.3.6 Detector maintenance

6.17.3.6.1 Clean MSD source

6.17.3.6.2 Replace NPD bead

6.17.3.6.3 Replace NPD ceramics

6.17.3.6.4 Clean FID

6.17.3.6.5 Replace or clean jet

6.17.4 Every six months

6.17.4.1 Computer maintenance

6.17.4.1.1 Archive methods, macros and data files onto long term storage media.

6.17.4.1.2 Once archived, data files and sequence files more than one month old may be deleted from the hard drive.

6.17.4.1.3 Perform appropriate disk clean-up and defragment the hard drive.

6.17.5 Annually

6.17.5.1 Schedule an on-site preventive maintenance service call as per the service agreement. Preventive maintenance shall be performed on instruments not covered under service contract in accordance with the manufacturer's recommendations.

6.17.6 After the instrument has been shut down or significant maintenance has been performed, verify that the instrument is fit for use by running a check mix solution, positive control or calibrator to ensure appropriate sensitivity, chromatography and separation of the components of the mixture.

6.17.6.1 Retain instrument verification documentation in the instrument logbook.

6.17.7 Hydrogen generator

6.17.7.1 Add dH_20 weekly or as needed.

6.17.7.2 Change moisture filter as needed.

6.17.7.3 Change deionizer bags at least annually.

6.17.8 Zero air generator

6.17.8.1 Replace filter cartridges annually.

6.17.8.2 Replace catalyst module, as needed.

6.18 Tandem Mass Spectrometer (QQQ)

6.18.1 Most toxicology procedures are performed in batch and therefore most maintenance procedures are performed prior to running a batch of samples. Record all maintenance in the Instrument Log (hardcopy or electronic (e.g., Resource Manager)) with the date and initials.

6.18.2 Day-of-Use (prior to each batch analysis)

6.18.2.1 For QQQ, perform Checktune. A file containing the Checktune parameters will be maintained in an electronic format for a minimum of six years. The Checktune report shall be assessed for the following:

6.18.2.1.1 The mass-to-charge (m/z) assignments and the full-width-at-half-maximum (FWHM) should be within the stated tolerance of the Checktune parameters.

6.18.2.1.1.1 Exceptions are made for the two highest m/z assignments.

6.18.2.1.1.2 If any of the other m/z assignments or FWHM are out of tolerance, the operator shall adjust the "Gain and Offset" in the manual tune mode. After completing this adjustment, the operator shall repeat the Checktune.

6.18.2.1.1.3 If the Checktune repeatedly fails, the operator shall run an Autotune.

6.18.2.1.2 The abundances should be compared to previous Checktunes and reviewed for any substantial deviations.

6.18.2.2 Clean the source, including the spray shield and spray chamber. The capillary cap can be cleaned, as needed.

6.18.2.3 Check solvent levels and replace as needed.

6.18.2.4 Run a Performance Check (e.g., a specific instrument check mix or a batch calibrator). Maintain a copy of the data in an electronic format for a minimum of six years.

6.18.3 As Needed

6.18.3.1 The following should be replaced as needed:

- PTFE filter
- Active inlet valve
- ALS needle and seat
- In-line filter
- Column

6.18.3.2 Replace and reseat pump seals

6.18.3.3 Backflush column

6.18.3.4 Water Purifier – perform maintenance as indicated by the manufacturer

6.18.3.5 Check and replace gas cylinders and gas filters

6.18.3.6 Check the demister on the rough pump and drain as needed.

6.18.3.7 Check the water drain reservoir from nitrogen generator and empty as needed.

6.18.4 Monthly

6.18.4.1 Perform an Autotune.

The operator shall ensure that m/z assignments, fragmentation patterns, and isotope separation are satisfactory. An electronic copy of the Autotune shall be maintained for a minimum of six years.

6.18.5 Every six months

6.18.5.1 Computer maintenance

6.18.5.1.1 Archive methods, tune files, and data files onto long term storage media.

6.18.5.1.2 Once archived, data files and sequence files more than one month old may be deleted from the hard drive.

6.18.6 Annually

Schedule an on-site preventive maintenance service call as per the service agreement.

6.18.7 After the instrument has been shut down or significant maintenance has been performed, verify that the instrument is fit for use by running a check mix solution, positive control or calibrator to ensure appropriate sensitivity, chromatography and separation of the components of the mixture.

6.18.7.1 Notate instrument verification in the instrument logbook and retain documentation in an electronic format.

6.18.8 Nitrogen generator

Change moisture filter as needed.

6.19 New Instrument Installation

6.19.1 Obtain documentation from the instrument service representative that demonstrates that the instrument performs to the manufacturer's specification.

6.19.2 Load methods, macros and libraries and test their functionality.

6.19.3 Perform self check or autotune (GC-MS, QQQ), as needed.

6.19.4 After methods have been loaded or created, run check solutions, positive controls or calibrators to demonstrate the instrument is fit for use (e.g., appropriate sensitivity, specificity, accuracy, precision, chromatography or identification of the components of the mixture).

6.19.5 Archive methods and data analysis macros to suitable long-term storage media.

6.19.6 Retain instrument verification documentation in the instrument logbook (hardcopy or electronic (e.g., Resource Manager)).

6.19.7 A summary of the verification shall be sent to the Program Manager for approval prior to placing the new instrument into service.

6.19.8 If the instrument does not meet expectations or acceptance criteria, label it as "not in service" and notify the Program Manager as soon as possible.

6.20 Immunalysis ELISA System

6.20.1 TECAN System. Logbook may be kept in a hardcopy or electronic format (e.g., Resource Manager). (Note: annual PMs may be performed by the manufacturer while the equipment is under warranty)

6.20.1.1 Daily (with each use)

6.20.1.1.1 Clean the surfaces with isopropanol.

6.20.1.1.2 Clean the probe with isopropanol.

6.20.1.1.3 Check fluid levels and empty waste containers.

6.20.1.2 Weekly

6.20.1.2.1 Check tubing for leaks.

6.20.1.2.2 Clean liquid reservoirs with mild soap solution and rinse thoroughly.

6.20.1.3 Monthly

6.20.1.3.1 Perform acid/base wash of system.

6.20.1.3.1.1 Prepare 100 mL of a 1 N HCl solution and place the system intake tubing into the acid solution.

6.20.1.3.1.2 Flush the instrument with approximately 50 mL of acidic solution.

6.20.1.3.1.3 Let the solution stand in system for approximately 15-20 minutes.

6.20.1.3.1.4 Place the system intake tubing into a beaker containing dH_20 and flush the instrument with approximately 50 mL of dH_2O.

6.20.1.3.1.5 Prepare a 1 N NaOH solution and place the system intake tubing into the basic solution.

6.20.1.3.1.6 Flush the instrument with approximately 50 mL of basic solution.

6.20.1.3.1.7 Let the solution stand in the system for approximately 15-20 minutes.

6.20.1.3.1.8 Remove tubing from basic solution and place into beaker containing dH_20.

6.20.1.3.1.9 Flush the instrument with approximately 50 mL dH_20.

6.20.1.3.1.10 Replace the intake tubing back into the liquid reservoir and flush instrument 3 times with approximately 50 mL dH_2O.

6.20.1.4 Annually

6.20.1.4.1 Replace sampling probe, syringe cap, pump valves, probe tubing, reagent tubing and waste tubing as needed.

6.20.1.4.2 Perform performance check, only if the annual preventive maintenance check does not include a precision test of the dispensing system.

6.20.1.4.2.1 Place Fisher pH 7 buffer (yellow solution) in reagent, TMB or substrate trough.

6.20.1.4.2.2 Pipette 100 µL of the solution into all wells of a blank 96 well plate.

6.20.1.4.2.3 Read absorbance at 450 nm. Calculate CV for each absorbance reading.

6.20.1.4.2.4 Turn plate 180° and reread plate. Calculate CV for each absorbance reading.

6.20.1.4.2.5 The CV for each well across the plate should be less than 10%. If the CV exceeds 10%, contact Immunalysis for service.

6.20.2 Plate washer

6.20.2.1 Daily (with each use)

6.20.2.1.1 Clean surfaces with isopropanol.

6.20.2.1.2 Perform Rinse/Night with dH_2O daily.

6.20.2.2 Monthly

6.20.2.2.1 Disassemble manifold and sonicate in methanol. Reassemble. (as needed)

6.20.2.2.2 Clean dispensing needles with cleaning needles.

6.20.2.2.3 Check liquid filters in wash bottles for particles and rinse liquid filters with dH_2O.

6.20.2.2.4 As needed, acid/base wash the plate washer mirroring the procedure listed for the Tecan.

6.20.2.3 Annually

6.20.2.3.1 Disinfect instrument with 10% bleach solution.

6.20.2.3.2 Replace all aspirating and dispensing tubes, as needed.

6.20.2.3.3 Replace all liquid filters, as needed.

6.20.2.3.4 Replace manifold sealing, as needed.

6.20.2.3.5 Recalibrate the dispensing pump, as needed.

6.20.3 Plate reader

6.20.3.1 Daily (with each use)

Clean surfaces with isopropanol.

6.20.3.2 Every six months

Clean the filters.

6.20.3.3 Yearly – only required when an instrument is not covered under the manufacturer's warranty (typically three years after purchase). Perform or schedule the manufacturer's preventive maintenance.

6.21 Glassware

Volumetric glassware used to prepare calibrators shall be NIST traceable and visually inspected prior to each use. NIST traceable volumetric flasks shall be replaced every ten years.

6.22 Automated Liquid Handling System

6.22.1 Hamilton MicroLab STAR

Given the variability of system use, the weekly and bi-weekly maintenance can be performed prior to each use if the system is not utilized within the delineated time intervals (e.g., used once per month).

In addition to the weekly and bi-weekly maintenance, a performance check of the vacuum manifolds shall be performed if the system is used infrequently. If the system has not been used for more than two weeks or not all vacuum manifolds are used for more than two weeks, this performance task shall be performed. This check includes manually checking each manifold to ensure it can meet vacuum requirements prior to use. Vacuum manifold checks can be completed by plugging the holes in the tube adapter plate and placing that on the manifold. Then the vacuum can be turned on manually and the vacuum pressure reduction can be monitored to show that a vacuum seal is being made successfully.

6.22.1.1 Weekly

6.22.1.1.1 Power cycle the system. This allows the system to "re-home" (reset) the coordinates of the moving parts of the system. This may be done more often as necessary.

6.22.1.2 Bi-Weekly

6.22.1.2.1 Wipe down interior of instrument with cleaning wipes (e.g., bleach based wipes). This cleaning should focus on portions exposed to biological fluids (vacuum well chambers, waste chambers, etc.).

6.22.1.2.2 Flush vacuum reservoirs by pouring appropriate volumes of water and/or methanol through the vacuum manifold. Allow to drain.

6.22.1.2.3 Check the volume of waste and remove as necessary.

6.22.1.3 Semi-annually (approximately every six months)

6.22.1.3.1 Schedule preventive maintenance from the manufacturer per the service agreement

6.23 Equipment Records

6.23.1 Equipment records may be maintained electronically or as a hardcopy.

6.23.2 The identity of the equipment may be maintained on the maintenance log and as a label on the equipment.

6.23.3 The manufacturer name, type identification, and serial numbers may be maintained on a maintenance log, server file of equipment, or other log file.

6.23.4 Checks for compliance with laboratory specifications includes verification documentation for analytical instruments and calibration certificates for other equipment such as pipettes, diluters, and balances.

6.23.5 The location of equipment within the Toxicology section is assumed to be within the laboratory (unless a pipette has been sent for calibration). Toxicology equipment is not removed from the laboratory for outside testing.

6.23.6 The manufacturer's instructions are maintained within the laboratory if they are received as a hardcopy. Many manufacturers provide electronic instructions on their websites therefore some equipment may not have instructions within the laboratory.

6.23.7 Documentation of calibrations and/or adjustments may be maintained electronically on a server file if hardcopies are not provided by the calibration provider. All calibrated equipment shall also be labeled with the appropriate calibration information.

6.23.8 Maintenance plans are covered in preceding sections of the Toxicology Procedures Manual. Any maintenance that is performed is recorded in the instrument log including any preventive maintenance, damage, malfunctions, or repairs to the instruments.

7 ALCOHOLS BY HEADSPACE GAS CHROMATOGRAPHY

7.1 Summary

An aliquot of each biological specimen is diluted semi-automatically with an internal standard solution into a glass vial, sealed, and placed in a heated headspace autosampler. Positive cases are confirmed in a second run. The concentration of ethanol or other volatiles in a dilute aqueous biological sample is directly proportional to the concentration of these compounds in the gas phase (headspace). A portion of the resultant headspace vapor above the liquid is automatically injected into a dual column gas chromatograph (GC) equipped with dual flame ionization detectors (FID). Ethanol, methanol, acetone and isopropanol are identified by retention time and the concentrations of these volatile compounds are calculated automatically by the software based on linear regression of the calibration curve.

7.2 Specimen Requirements

Approximately 100 µL of blood or other fluids or 1-2 g tissue.

7.3 Reagents and Standards

- NIST traceable ethanol standards for use as calibrators (0.20% and 0.50% by weight by volume (w/v)), stored at 2-8°C.
- NIST traceable multicomponent alcohol mixes for use as calibrators containing 0.01%, 0.05% and 0.10% w/v acetone, methanol, ethanol and isopropanol, stored at 2-8°C.
- n-Propanol
- NIST traceable ethanol standards for use as controls (0.05%, 0.08%, 0.10%, 0.20%, and 0.30% w/v), stored at 2-8°C.
- NIST traceable multicomponent alcohol mixes for use as a control containing 0.05% w/v ethanol, methanol, isopropanol, and acetone

7.4 Calibrators, Controls and Internal Standards

7.4.1 Calibrators

7.4.1.1 Calibrators shall be purchased as NIST traceable standards.

7.4.1.2 Purchased calibrators

7.4.1.2.1 0.50% and 0.20% ethanol standards.

7.4.1.2.2 0.10%, 0.05% and 0.01% multicomponent alcohol mix standards.

7.4.2 Internal standard (IS) preparation: n-propanol can be a putrefactive product. If n-propanol contamination is suspected, other internal standards such as methyl ethyl ketone or t-butanol may be used. Document this exception in the case file with a MFR.

7.4.2.1 0.03% (v/v) n-propanol internal standard solution. Pipette 300 µL n-propanol into a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to one year.

7.4.3 Controls

7.4.3.1 Positive controls within the batch shall be purchased from a source different from the calibrators. Alternatively, if no second source is readily available, positive controls within the batch must be of a different lot than the calibrators used.

7.4.3.2 NIST traceable ethanol controls: 0.10%, 0.20%, 0.30%, 0.05% and 0.08% w/v. If the multicomponent control (0.05% w/v) contains ethanol, a separate ethanol control at 0.05% is not required.

7.4.3.3 NIST traceable multicomponent alcohol control containing 0.05% w/v acetone, methanol, ethanol and isopropanol.

7.4.3.4 Negative control: prepared from dH_2O or blood.

7.4.4 After opening the CRM manufacturer's ampoule, transfer all contents to an airtight container for storage in the appropriate conditions. Airtight containers may be stored and used for up to four weeks from opening.

7.5 Apparatus

7.5.1 Gas chromatograph with data system, dual columns, dual flame ionization detectors and a headspace autosampler

7.5.2 Columns. Restek Rtx-BAC 1 and Rtx-BAC 2 or Agilent DB-ALC1 and Agilent DB-ALC2 capillary columns or Agilent DB-BAC1 UI and DB-BAC2 UI capillary columns

7.5.3 Glass 20 mL (23 x 75 mm) headspace vials with Teflon or Butyl septa and aluminum seals

7.5.4 Hamilton Microlab Diluter

7.5.5 Vial seal crimper

7.5.6 Test tubes or sample cups

7.5.7 Headspace Sampler Operational Parameters. The following conditions are recommended starting parameters. Autosampler parameters may be adjusted to permit improved performance. Parameters for using nitrogen as the carrier gas are shown in parentheses.

- Sample Oven 70ºC
- Sample Valve 80ºC
- Transfer Line 90ºC
- Vial Pressurization Gas helium or nitrogen
- GC Cycle 4.5 min (5.5 min)
- Vial Equilibration 7.0 min
- Injection Duration 0.5 - 1.0 min
- Vial shaking Off
- Fill mode Default
- Loop Fill Pressure 15 psi
- Loop Fill Mode Custom
- Loop Ramp Rate 30 psi/min
- Loop Final Pressure 1.5 psi
- Loop Equilibration Time 0.05 min
- Extraction Mode Single
- Vent After Extraction ON
- Post Injection Purge 200 mL/min for 3 min

7.5.8 Gas Chromatograph Operational Parameters. The following conditions are recommended starting parameters. Instrument conditions may be adjusted to permit improved performance. Parameters for using nitrogen as the carrier gas are shown in parentheses.

- Inlet
  - Split
  - Split ratio 10:1 (7.5:1)
  - Split flow 70 mL/min (22.5 mL/min)

Total flow	80 mL/min (28.5 mL/min)
Pressure	24 psi (14 psi) constant pressure mode
Temperature	110˚C
Septum purge flow	3 mL/min

- Oven 40ºC Isothermal
- GC Column Flow 5-10 mL/min (3 mL/min)
- Detectors (FID) 250ºC

Hydrogen flow	30 mL/min
Air flow	400 mL/min
Make-up flow	25 mL/min or 45 mL/min
Make-up gas	helium or nitrogen

7.6 Procedure

At a minimum, calibrators, controls, and case specimens are analyzed singly. If a case specimen is positive, it will be realiquotted and confirmed on a second run. Confirmatory analyses run on an additional, different biological specimen or tissue may be run in duplicate on the same analytical run (see ¶ 2.2.4.3). Negative specimens may be reported with the quantitative results from the initial run.

7.6.1 Allow all case samples, calibrators and controls to come to room temperature before preparing the aliquots.

7.6.2 Mix biological fluids by placing samples on a rocker or inverting each sample several times. Pour approximately 0.2 mL of calibrator, control, blood or other biological fluid into a clean test tube or sample cup (this initial step enables visualization of any clots and prevents possible contamination of the original sample by the internal standard solution). For samples with small/limited volumes where pouring off may waste specimen, samples do not need to be poured off for sampling. For urine specimen cups or containers where pouring off is difficult, samples may be moved to another container with a transfer pipette.

7.6.3 Place the diluter delivery tip into the specimen, making sure its tip is below the surface of the sample. Activate the diluter. At this point, the diluter draws 50 µL of sample into its delivery tube.

7.6.4 Direct the delivery tip into the appropriately labeled headspace vial and activate the diluter. The diluter will dispense the specimen and 450 µL of IS solution into the vial.

7.6.5 Flush the diluter tube as necessary by activating the diluter one or more times or rinsing with dH_2O, depending on the viscosity or other nature of the specimen. Dispense washings into a waste beaker. Wipe withdraw tip with Kimwipe/tissue paper as needed between sampling and dispensing.

7.6.6 Stopper the headspace vial and place in the sample rack.

7.6.7 Repeat steps 7.6.1 – 7.6.6 for all calibrators, controls and specimens.

7.6.8 NOTE: If analyzing tissue, weigh approximately 0.5 g of tissue and transfer to a headspace vial. Record the weight of the tissue. Pipette 4.5 mL internal standard into the headspace vial. Stopper the headspace vial with the Teflon seal and place in the sample rack.

7.6.9 NOTE: If analyzing tissues as homogenates, weigh approximately 0.5 g of tissue on a balance. Record weight. Place tissue in homogenizer tube and add 4.5 mL of internal standard solution. Homogenize. Transfer 0.5 mL into a headspace vial and stopper with the Teflon or Butyl seal and place in the sample rack.

7.6.10 Seal all headspace vials by crimping the aluminum rings over the Teflon seals.

7.6.11 Load headspace vials in the headspace auto sampler.

7.6.12 Daily Calibration (Pre-run). The method is calibrated prior to each day's batch sample analysis. Analyze the calibrators, negative control, NIST traceable 0.08% w/v ethanol control and NIST traceable multicomponent alcohol control on the pre-run batch. Calibrate the method. If the calibrators and/or controls do not satisfy quality control criteria, rerun the affected calibrator or control. If, after data reprocessing, the calibrators and/or controls still do not satisfy quality control criteria, then appropriate measures must be taken to rectify the problem (instrument maintenance, open or prepare new calibrators or controls, etc). Document such actions and measures in the BAC instrument log.

7.6.13 A STAT case sample(s) may be analyzed within a pre-run sequence provided it is bracketed with acceptable calibrators and controls and that appropriate QA/QC measures are applied. Appropriate measures include a vial verification performed by an independent analyst; a documented review of all calibrators, controls and case samples by an independent analyst; and documentation of chain of custody and aliquots. Document this STAT exception, review and communication with the customer on an MFR in the case file.

7.6.14 Batch sample analysis. Headspace alcohol analysis is performed as a batch analysis. Analyze one control at the beginning of the batch and, at a minimum, after every 10 injections. With smaller batch sizes, controls may be run more often, for example after every 5 samples. The ethanol controls are: 0.00%, 0.05%, 0.10%, 0.20%, and 0.30% by weight by volume. All control levels shall be run within the batch.

7.6.15 Vial Verification. After the completion of the batch, the identity of each vial in the headspace sampler is verified with the sequence table and the Alcohol Batch Worksheet. Vial verification is performed by an analyst other than the operator and is documented by initials and date on the printed sequence table.

7.7 Calculation

7.7.1 Volatiles are identified based on relative retention times compared to calibrators for both columns. Identification is performed by instrument software. Retention times for both analyte and internal standard must be within ± 2% of the retention time obtained from the average of the calibrators.

7.7.2 Concentration is calculated automatically by the software based on linear regression of the 5 point calibration curve (3 points for methanol, acetone and isopropanol) based on peak area or peak height measurement. The data from the Rtx-BAC1 or Agilent DB-ALC1 or Agilent DB-BAC1 UI column is utilized for quantitative results.

7.7.3 Tissue concentration is calculated as follows:

$$\text{Chromatogram concentration} \times \frac{0.5\ \text{g}}{\text{weighed amount}} = \text{volatile tissue concentration \% (w/w)}$$

7.8 Quality Control

7.8.1 Daily Calibration (Pre-Run). Acceptable tolerance for ethanol calibrators is ± 6% of the target concentration or 0.0040% w/v, whichever is greater. Acceptable tolerance for methanol, acetone and isopropanol is ± 10% of the target concentration or 0.0050% w/v, whichever is greater. Samples may not be analyzed prior to an acceptable pre-run for a particular analyte.

7.8.2 Negative control. The negative control is injected immediately after the 0.50% calibrator within the pre-run and is used to check for carryover. An acceptable negative control may not contain ethanol and/or other target volatiles greater than 0.0020% w/v. If unacceptable, prepare a fresh negative control. Reinject the 0.50% calibrator followed by the new negative control. If ethanol and/or another volatile is still present, perform instrument maintenance to correct the problem and document actions in the BAC instrument log. A negative control is also run within each batch of samples. The negative control may be used for acceptable targets if one of the targets is considered unacceptable (e.g., acetone is >0.0020 %w/v in the negative control but ethanol, methanol, and isopropanol may be reported).

7.8.3 Positive controls. Acceptable tolerance for ethanol controls is ± 6% of the target concentration or 0.0040% w/v, whichever is greater. Acceptable tolerance for methanol, acetone and isopropanol controls is ± 10% of the target concentration or 0.0050% w/v, whichever is greater.

7.8.4 Case samples shall be bracketed by acceptable controls with positive ethanol and positive multi-component controls at the beginning and end of every batch. The Pre-run controls are not considered bracketing of the batch. If one control fails, repeat all positive case samples (for the failed target) not bracketed between acceptable controls. If more than one control of the same target (e.g., two ethanol controls) fails, all positive samples (for the failed target) in the batch must be repeated. Negative results may be reported. Document corrective actions and exceptions on the Alcohol QC Worksheet and the BAC control chart or BAC instrument log. In general, corrective actions for failed controls may include repeating the batch, recalibrating the instrument, opening new controls or making new calibrators.

7.8.5 At least two controls within the batch must be the multi-component alcohol control containing 0.05% w/v acetone, methanol and isopropanol in order to report any methanol, acetone or isopropanol positive cases. The two controls shall bracket the batch.

7.8.6 If the specified NIST traceable controls are temporarily not available (e.g., backorder), alternative concentrations may be substituted (e.g., substitute 0.08% w/v control for 0.10% w/v control) provided there is documentation, explanation and justification in all affected case files. If there is a systemic problem obtaining external controls, the Program Manager shall be notified such that an alternative supplier can be identified and appropriate changes made to this procedure.

7.8.7 Coefficient of determination (r^2). The r^2 value for the linear regression curve must be 0.995 or greater. The coefficient of determination is automatically printed on the calibration curves and tables.

7.8.8 Replicate tolerance. A minimum of two analyses are required to report a positive volatile. Positive single fluid/tissue cases must be analyzed on two separate batch runs. On cases that have multiple fluids/tissues, the second fluid/tissue may be analyzed on the same or separate batches (see ¶ 2.2.4.3). Determine the average and ± 5% range of replicates if the samples appear to be outside of this tolerance (this only needs to be shown when necessary to demonstrate a sample outside of the ± 5% range).

Replicates must be within the ± 5% range or within ± 0.0040% (w/v) of the mean, whichever is greater. Reanalyze the sample if it is outside of tolerance. Alternatively, the result may be reported qualitatively.

An exception to exclude one or more volatile replicates from the mean ± 5% or ± 0.0040% range may be made if the value(s) causes the mean and ± 5% or ± 0.0040% range to be unacceptable. There may also be a need to exclude a value where the replicates have two sets of data that are acceptable (e.g., replicates 1 & 3 are acceptable and replicates 2 & 3 are acceptable). Choose the set of replicates with the smallest difference between the values or, report the results as present with the permission of the customer. Document the exception in the case file.

7.8.9 New calibrator certification:NIST traceable (external) calibrators will be verified with an acceptable pre-run prior to the analysis of case samples.

7.9 Reporting

7.9.1 For biological fluids, report the volatile concentration of the average of the replicates from the Rtx®-BAC1 or DB-ALC1 or DB-BAC1 UI column data, rounded to three (3) decimal places in % by weight by volume.

7.9.2 For biological tissues, report the volatile concentration of the average of the replicates from the Rtx®-BAC1 or DB-ALC1 or DB-BAC1 UI column data, rounded to three (3) decimal places in % by weight by weight.

7.9.3 Concentrations of ethanol, methanol, isopropanol and acetone less than 0.010% w/v shall be reported as "None Detected."

7.9.4 Negative results may be reported from a single quantitative run.

7.9.5 The upper limit of quantitation (ULOQ) for ethanol is 0.50% w/v. Any ethanol results greater than 0.50% should be repeated using a 1:2 or 1:3 (or other as necessary) dilution with water. If the customer agrees with reporting a result of "present, greater than 0.50% w/v," samples do not need to be diluted and reanalyzed.

7.9.6 Methanol, isopropanol or acetone concentrations ≥ 0.10% may be reported as "present" (in contaminated or embalmed cases).

7.9.7 Analyze two different postmortem biological fluids in postmortem cases (e.g., blood and vitreous or blood and urine) with positive results, provided the samples have been submitted and there is sufficient sample for the analysis. Postmortem samples are utilized to assess postmortem ethanol formation. Two fluids are not necessary for antemortem samples. Tissues may be analyzed on a case by case basis. Document exceptions in case file, communication with the customer on an MFR or on the Certificate of Analysis (e.g., "quantity insufficient for analysis").

7.10 Note

This method may also be used on non-biological samples (e.g., breath alcohol simulator solutions, commercial products, and unknown liquids).

7.11 References

7.11.1 B.L. Levine, Principles of Forensic Toxicology (Third Edition), American Association for Clinical Chemistry, Inc., 2010, pp 175-190.

7.11.2 Agilent Technologies, "Analysis of blood alcohol concentration with an Agilent Intuvo 9000 GC System", Published 4/2/2020, Accessed 6/14/2022, https://www.agilent.com/cs/library/applications/application-blood-alcohol-intuvo-9000-gc-5991-8999en-agilent.pdf.

7.11.3 Agilent Technologies, "Determination of Blood Alcohol with Dual Column/Dual FID and the Agilent Intuvo 9000 GC." Published 4/9/2020, Accessed 6/14/2022, https://www.agilent.com/cs/library/applications/5991-7217EN.pdf.

7.11.4 Restek Corporation, "Rtx®-BAC1 and BAC2 Columns", #121-01[001], Revision Date: 5/01.

7.11.5 Historic References

7.11.5.1 L. C. Nickolls, "A Modified Cavett Method for the Determination of Alcohol in Body Fluids," Nov. 1960, Analyst, Vol. 85, pp 840-942.

7.11.5.2 K. M. Dubowski, "Manual for Analysis of Ethanol in Biological Liquids," Department of Transportation Report No. DOT TSC NHTSA-76-4, Jan 1977.

7.11.5.3 G. Machata, "Determination of Alcohol in Blood by Gas Chromatographic Head Space Analysis," Clin Chem. Newsletter, 4(1972), 29.

8 DRUG SCREENING BY ELISA

8.1 Summary

The Immunalysis Direct ELISA Kits are specific and sensitive in-vitro tests to detect the presence of drugs in forensic samples such as whole blood, serum, urine, vitreous and tissue homogenates. The Immunalysis Direct ELISA kits consist of microplates that are coated with a polyclonal antibody with high affinity for the target analytes. These antibodies display cross-reactivity with related drugs within a drug class. An aliquot of the diluted unknown specimen is incubated with a dilution of enzyme-labeled drug derivative in microplate wells coated with fixed amounts of oriented high affinity purified polyclonal antibody. A competitive binding for the antibody binding sites occurs between the enzyme-labeled drug and the drug in the forensic sample. The wells are washed thoroughly to remove any unbound sample or residual reagent and a chromogenic substrate is added. The color produced is stopped using a dilute acid stop solution and the wells are read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of the drug in the sample. The results obtained are presumptive, meaning that any positive result requires appropriate confirmation by a more specific analytical technique such as GC-MS.

8.2 Specimen Requirements

Approximately 50 µL of whole blood, biological fluid(s) or tissue dilutions/homogenates.

8.3 Reagents and Standards

- Immunalysis Direct ELISA Kits for Amphetamine, Acetaminophen, Barbiturates, Benzodiazepines Benzoylecgonine (Cocaine Metabolite), Buprenorphine, Carisoprodol, Fentanyl, Methadone, Methamphetamine, Opiates, Oxycodone/Oxymorphone, PCP, Salicylate, THC Carboxylic Acid, Zolpidem, Dextromethorphan, Diphenhydramine, Tramadol, and Tricyclic Antidepressants. Each kit contains:
    - 96 well microplates coated with polyclonal antibodies. The plates are sealed in a moisture and air barrier pouch with a desiccant. Plates and unused wells should be stored in this pouch according to the manufacturer's specifications and are stable until at least the expiration date.
    - Drug conjugate containing drug derivative labeled with horseradish peroxidase in a buffered protein solution with stabilizers containing azide free preservatives. The conjugate should be stored according to the manufacturer's specifications and is stable until at least the expiration date.
    - TMB chromogenic substrate containing 3, 3', 5, 5' tetramethylbenzidine and peroxide in buffer. The substrate is light sensitive and care should be taken to minimize its exposure to light. It should be stored according to the manufacturer's specifications and is stable until at least the expiration date.
    - Stop reagent, 1N hydrochloric acid. It should be stored according to the manufacturer's specifications and is stable until at least the expiration date.
    - Kit insert containing manufacturer-provided instructions and information.
    - Note: Immunalysis kits should be tracked by kit lot number. The conjugates and plates are lot number specific and must be matched with each other. TMB and stop solution are not lot number specific. Kits with identical lot numbers may be combined. Upon receipt in the laboratory, the kits should be opened and components labeled and separated for proper storage. The kit and component lot numbers should be recorded.
- Acetaminophen (APAP) powder
- Amphetamine, 1 mg/mL
- Butalbital, 1 mg/mL
- Buprenorphine, 100 µg/mL
- Clonazepam, 1 mg/mL
- Meprobamate, 1 mg/mL

- Benzoylecgonine, 1 mg/mL
- Fentanyl, 100 µg/mL
- Methadone, 1 mg/mL
- Methamphetamine, 1 mg/mL
- Morphine, 1 mg/mL
- Oxymorphone, 1 mg/mL
- Phencyclidine, 1 mg/mL
- Acetylsalicylic Acid (ASA) powder
- 9-Carboxy-11-nor-delta 9-THC (THC-COOH), 1 mg/mL
- Zolpidem, 1 mg/mL
- Dextromethorphan, 1 mg/mL
- Diphenhydramine, 1 mg/mL
- Tramadol, 1 mg/mL
- Nortriptyline, 1 mg/mL
- UTAK Drugs of Abuse Plus Level 1 Control – Negative Control
- UTAK Drugs of Abuse Plus Level 2 Control – Low Positive Control
- UTAK Drugs of Abuse Plus Level 3 Control – Positive Control
- UTAK Drugs of Abuse Plus Level 4 Control – High Positive Control

8.4 Solutions, Standards, Calibrators and Controls

8.4.1 External Calibrators and Controls

Whole blood calibrators/controls may be utilized from UTAK Laboratories which serve as the negative control (NC), low positive control (LPC), positive control (PC) and high positive control (HPC).

8.4.1.1 These are aliquotted, stored at -10 to -20°C and are stable until at least the expiration date. Once thawed, they must be used within 25 days.

8.4.1.2 Analyte concentrations for externally prepared controls:

Analyte	Level 1 – NC (mg/L)	Level 2 - LPC (mg/L)	Level 3 - PC (mg/L)	Level 4 - HPC (mg/L)
Acetaminophen	0	10	20	200
Amphetamine	0	0.025	0.05	0.5
Methamphetamine	0	0.025	0.05	0.5
Benzoylecgonine	0	0.025	0.05	0.5
Butalbital	0	0.5	1	10
Clonazepam	0	0.02	0.04	0.4
Fentanyl	0	0.001	0.002	0.02
Meprobamate	0	2	4	40
Methadone	0	0.025	0.05	0.5
Morphine	0	0.02	0.04	0.4
Oxymorphone	0	0.02	0.04	0.4
Phencyclidine	0	0.005	0.01	0.1
Salicylic Acid	0	25	50	500
THC-COOH	0	0.01	0.02	0.2
Zolpidem	0	0.025	0.05	0.5
Dextromethorphan	0	0.025	0.05	0.5
Diphenhydramine	0	0.05	0.1	1
Tramadol	0	0.125	0.25	2.5
Nortriptyline	0	0.025	0.05	0.5
Buprenorphine	0	0.001	0.002	0.020

8.4.2 Internal Calibrators and Controls

If external calibrators and controls are not available, they may be prepared in-house.

8.4.2.1 Blank blood negative control (**NC**). Blood bank blood previously determined not to contain drugs. Do not use blank blood containing azide as it may affect the ELISA assays.

8.4.2.2 Cutoff Reference Solutions:

8.4.2.2.1 Drugs of Abuse Cutoff Reference solution is prepared in one laboratory, assigned a lot number and distributed to all 4 DFS laboratories. A copy of the preparation records shall be distributed to each laboratory with the solution.

8.4.2.2.2 Add the following volumes to a 100 mL volumetric flask and qs to volume with methanol.

Drug	µL of 1 mg/mL standard	Final concentration (mg/L)
Amphetamine	50	0.5
Benzoylecgonine	50	0.5
Methadone	50	0.5
Methamphetamine	50	0.5
Zolpidem	50	0.5
Clonazepam	40	0.4
Morphine	40	0.4
Oxymorphone	40	0.4
THC-COOH	20	0.2
PCP	10	0.1
Butalbital	1000	10
Meprobamate	4000	40
Fentanyl	20 (100 µg/mL std)	0.02
Buprenorphine	20 (100 µg/mL std)	0.02
Dextromethorphan	50	0.5
Diphenhydramine	100	1.0
Tramadol	250	2.5
Nortriptyline	50	0.5

8.4.2.2.3 Acetaminophen/Salicylate Cutoff Reference Solution, 0.2 mg/mL and 0.5 mg/mL respectively: Weigh 20 mg acetaminophen and 50 mg acetylsalicylic acid. Transfer to a 100 mL volumetric flask and qs to volume with methanol.

8.4.2.2.3.1 This solution is prepared in one laboratory, assigned a lot number and distributed to all 4 DFS laboratories. A copy of the preparation records shall be distributed to each laboratory with the solution.

8.4.2.2.4 The cutoff reference solutions are stable in excess of 24 months when stored at -10 to -20°C.

8.4.2.3 Blood Positive Control (PC)

8.4.2.3.1 Prepare spiked blood PC by adding 100 µL of Drugs of Abuse Cutoff Reference Solution and 100 µL of Salicylate/Acetaminophen Cutoff Reference Solution (as needed) to appropriately labeled tube. Dry tube under nitrogen to evaporate methanol. Reconstitute in 1 mL blank blood to prepare the following drug cutoff

concentrations (mg/L). Vortex briefly. Once prepared, store at 2-8°C and use within 30 days.

Drug	Final Cutoff Concentration (mg/L)
Amphetamine	0.05
Benzoylecgonine	0.05
Methadone	0.05
Methamphetamine	0.05
Zolpidem	0.05
Clonazepam	0.04
Morphine	0.04
Oxymorphone	0.04
THC-COOH	0.02
PCP	0.01
Butalbital	1
Meprobamate	4
Fentanyl	0.002
Acetaminophen	20
Salicylate	50
Buprenorphine	0.002
Dextromethorphan	0.05
Diphenhydramine	0.10
Tramadol	0.25
Nortriptyline	0.05

8.4.2.4 Blood Low Positive Control (LPC)

8.4.2.4.1 Prepare spiked blood LPC by adding 50 µL of Drugs of Abuse Cutoff Reference Solution and 50 µL of Salicylate/Acetaminophen Cutoff Reference Solution (as needed) to appropriately labeled tube. Dry tube under nitrogen to evaporate methanol. Reconstitute in 1 mL blank blood. Vortex briefly. Once prepared, store at 2-8°C and use within 30 days. Final concentrations will be ½ the values listed in 8.4.2.3.1

8.4.2.5 Blood High Positive Control (HPC)

8.4.2.5.1 Prepare spiked blood HPC by adding 1 mL of Drugs of Abuse Cutoff Reference Solution and 1 mL of Salicylate/Acetaminophen Cutoff Reference Solution (as needed) to appropriately labeled tube. Dry tube under nitrogen to evaporate methanol. Reconstitute in 1 mL blank blood. Vortex briefly. Once prepared, store at 2-8°C and use within 30 days. Final concentrations will be ten times the values listed in 8.4.2.3.1

8.5 Apparatus

8.5.1 Test tubes

8.5.2 Screw cap test tubes, 16 x 100 mm disposable glass (for drug conjugates)

8.5.3 Vortex mixer

8.5.4 Micropipettes, 8 channel multichannel pipette, pipette tips

8.5.5 Timer

8.5.6 Automated liquid-handling robot (e.g., TECAN MiniPrep or TECAN EVO)

8.5.7 Micro-plate washer

8.5.8 Micro-plate Reader

8.5.9 Computer/printer with Magellan, TECAN and Access software

8.5.9.1 Drug Screening Panels (additional analyses may be added to any panel):

8.5.9.1.1 DUID Panel

Amphetamine
Barbiturates
Benzodiazepines
Buprenorphine
Carisoprodol
Cocaine metabolite
Fentanyl
Methadone
Methamphetamine/MDMA
Opiates
Oxycodone/Oxymorphone
PCP
THC-COOH
Zolpidem
Dextromethorphan
Diphenhydramine
Tramadol
Tricyclic Antidepressants

8.5.9.1.2 Abused Drug Panel

Cocaine metabolite
Opiates
Oxycodone/Oxymorphone
Methamphetamine/MDMA
PCP
Methadone
Fentanyl

8.5.9.1.3 Postmortem Tox panel

Barbiturates
Benzodiazepines
Buprenorphine
Carisoprodol
Cocaine metabolite
Fentanyl
Methadone
Methamphetamine/MDMA
Opiates
Oxycodone/Oxymorphone
PCP
Zolpidem

8.5.9.1.4 X (extra) panel

Amphetamine
Acetaminophen
Acetylsalicylic acid (salicylate)

8.5.9.1.5 Any analyte may be run individually by request or as needed

8.5.9.2 Assays, cutoffs and volumes of diluted samples for each assay

Assay	Cutoff (mg/L)	Sample Volume (µL)	Conjugate Volume (µL)
Acetaminophen	20	10	100
Amphetamine	0.05	10	100
Barbiturates	1	10	100
Benzodiazepines	0.04	40	100
Buprenorphine	0.002	100	100
Carisoprodol	4	10	100
Cocaine metabolite	0.05	20	100
Fentanyl	0.002	100	100
Methadone	0.05	20	100
Methamphetamine/MDMA	0.05	10	100
Opiates B	0.04	20	100
Oxycodone/Oxymorphone	0.04	10	100
PCP	0.01	20	100
Salicylate	50	10	100
THC-COOH	0.02	20	100
Zolpidem	0.05	20	100
Dextromethorphan	0.05	40	50
Diphenhydramine	0.10	40	50
Tramadol	0.25	75	50
Tricyclic Antidepressants	0.05	50	50

8.6 Procedure

8.6.1 Allow all biological samples and reagents come to room temperature before starting procedure.

8.6.2 Label glass disposable test tubes: NC, LPC, PC, HPC and case sample IDs. Prepare controls per ¶ 8.4.

8.6.3 Briefly mix each sample. Pour off approximately 100 µL sample into a clean test tube (this initial step enables visualization of any clots and prevents possible cross contamination of samples with micropipette or diluter). For samples with small/limited volumes where pouring off may waste specimen, samples do not need to be poured off for sampling. For urine specimen cups or containers where pouring off is difficult, samples may be moved to another container with a transfer pipette.

8.6.4 Dilute each sample 1/20 by mixing 50 µL of sample with 950 µL dH_2O in the appropriately labeled tubes, vortex briefly, and centrifuge if necessary. Depending on the quality or quantity of sample, other volumes may be used to achieve similar 1/20 dilutions.

8.6.5 The first four samples of each assay include the NC, LPC, PC and HPC which verify that all reagents and instruments are working properly prior to the analysis of case samples. A second HPC must also be run at the end of a batch to bracket case samples and ensure reagent and instrument reliability throughout the run. Important: no two samples or controls can have the same name. Two positive controls must have unique names, e.g., HPC-1 and HPC-2.

8.6.6 Place all diluted specimens in the appropriate location on TECAN microplate robot. Check samples for bubbles. If any bubbles are present, they must be minimized with a stick or pipette tip before the diluted samples are pipetted into the plates.

8.6.7 Create a sample dilution rack with unique ID name. Enter all sample ID's in the appropriate position with unique DFS forensic number and item number (as needed). Print sample rack list for vial verification. With every batch of samples run on TECAN robot, the identity of each diluted specimen tube is verified with the sample rack list and dilution rack location by an individual other than the operator. Vial verification is documented by initials on the sample rack list.

8.6.8 Fill 16 x 100 mm screw cap tubes with drug conjugates and place on TECAN robot. Conjugate and plate lot numbers must always be matched.

8.6.9 Add microplates containing strips with the corresponding assays to be run. Always fill the rows (with blank wells if necessary). When handling microplates, use caution not to touch the bottom of the microplate as this may interfere with the measurement of absorbance.

8.6.10 Start TECAN microplate robot to select method, sample dilution rack and destination plates (see TECAN operations manual for details).

8.6.11 Once TECAN robot has completed all sample and conjugate additions, remove microplates from the robot.

8.6.12 Incubate microplates for 1-3 hours at room temperature to allow competitive binding to occur.

8.6.13 Wash microplate wells 6 times with dH_2O using the microplate washer.

8.6.14 Invert plates and slap dry on absorbent paper to ensure all residual moisture is removed.

8.6.15 Using an 8 channel multichannel pipette, manually add 100 µL substrate to each well. Add substrate to plates in sequential order.

8.6.16 Incubate plates at room temperature in the dark until appropriate color development is achieved (usually 15-60 minutes).

8.6.17 In the same sequential order as above, add 100 µL stop solution to each well. This will change blue color to yellow.

8.6.18 Using a dual wavelength plate reader, read absorbance of each plate within 1 hour of yellow color development (See Operations Manual for details).

8.6.19 Print ELISA drug screening sample report for each case file.

8.7 Calculation

8.7.1 The ratio of the absorbances of the positive controls and samples (B) relative to the negative control (B_0) are multiplied by 100 to generate B/B_0 values.

8.7.1.1 If the sample B/B_0 is equal to or less than the B/B_0 of the PC, the sample is presumptive positive for that class of drugs and the result is listed as "pending" for confirmation.

8.7.1.2 If the sample B/B_0 falls between the LPC and PC, the sample could contain low concentrations of drugs. The result is listed as "review" such that the toxicologist, supervisor, or group supervisor will decide whether or not to pursue confirmation of the drug depending on case history.

8.7.1.3 If the sample B/B_0 is greater than the B/B_0 of the LPC, drugs were "none detected" in the sample and the result is listed as "NEG" or "ND."

8.8 Quality Control and Reporting

8.8.1 Quality Control Criteria

8.8.1.1 The NC must be negative relative to the PC (NC B/B_0 > LPC > PC). The absolute absorbance of the NC should be greater than 0.80 except for methadone and benzodiazepine. For methadone and benzodiazepine plates, the NC should be greater than 0.60.

8.8.1.2 The LPC must be negative relative to the PC (LPC B/B_0 < NC > PC).

8.8.1.3 Both HPC values must be positive relative to the PC (HPC B/B_0 < PC).

8.8.2 Corrective action for failed quality control

8.8.2.1 If NC is positive or less than 0.80 (or 0.60 for methadone and benzodiazepine plates), repeat all samples.

8.8.2.2 If the LPC is positive, repeat or send to confirmation all case samples with B/B_0 within 20% above the PC.

8.8.2.3 If either HPC is negative, repeat all case samples.

8.8.2.4 Exceptions to these guidelines must be authorized by the Program Manager and documented in the case file with an MFR.

8.8.3 All positive results are presumptive and must be confirmed by a more specific, selective and quantitative procedure.

8.8.4 Confirmation testing may be ordered even if the ELISA result is negative. For example, some benzodiazepines differ in their cross-reactivity with the benzodiazepine assay; if the benzodiazepine ELISA demonstrates an elevated response relative to the negative control, a benzodiazepine confirmation may be ordered to test for specific benzodiazepines. Any confirmation testing ordered from an elevated response will need two aliquots independent of the ELISA result.

8.8.5 Due to the specificity and cross reactivity of each assay (see Immunalysis Kit Inserts), the following nomenclature is used to report screened drugs and/or drug classes (in no specific order): cocaine/benzoylecgonine, opiates, oxycodone/oxymorphone, methamphetamine/methylenedioxymethamphetamine (MDMA), phencyclidine, barbiturates, benzodiazepines, carisoprodol/meprobamate, fentanyl, methadone, cannabinoids, zolpidem, diphenhydramine/cyclobenzaprine, dextromethorphan, tramadol, tricyclic antidepressants, buprenorphine/norbuprenorphine, amphetamine/methylenedioxyamphetamine (MDA).

8.8.5.1 The above-mentioned list is not inclusive of all possible screening assays or combinations of assays and can be modified to reflect the assays utilized.

8.8.5.2 Due to the cross reactivity of citalopram with the diphenhydramine assay, pending results from this assay can be used as confirmation.

8.9 Note

For each batch of samples run on the ELISA system, the worksheets and associated QC data are placed in one unique DFS case file that is referenced on each case sample report. The QC data pack must contain the specimen aliquot worksheet and associated technical review by an independent examiner, plate setup containing kit lot

numbers and the TECAN sample rack list including vial verification by an independent examiner and their corresponding initials.

8.10 References

8.10.1 Immunalysis ELISA Kit Inserts, Pomona CA.

8.10.2 TECAN Columbus Pro Washer Instruction Manual, 30008658 2004-12.

8.10.3 TECAN Hydroflex Washer Instruction Manual, 30086671, 2013-08.

8.10.4 TECAN Sunrise Absorbance Reader Instruction Manual, 30008746, 2005-02.

8.10.5 TECAN Miniprep Logic Manual, 160013, July 1999.

8.10.6 Freedom EVO75 Operating Manual, 30093968.00.

8.10.7 Evaluation of Immunalysis ELISA Assays for the Detection of Drugs of Abuse in Postmortem Bile and Urine. Patton, Isenschmid, Helpler and Schmidt. SOFT Annual Meeting, Portland, OR 2003.

8.10.8 Evaluation of Immunalysis ELISA Assays for the Detection of Drugs of Abuse in Postmortem Blood. Isenschmid, Patton, Helpler and Schmidt. TIAFT Annual Meeting, Melbourne, Australia 2003.

8.10.9 Validation of the Immunalysis Microplate ELISA for the Detection of Buprenorphine and its Metabolite Norbuprenorphine in Urine. Miller, Torrance and Oliver. JAT 30: 115-119, March 2006.

8.10.10 Validation of the Immunalysis Microplate ELISA for the Detection of Methamphetamine in Hair. Han, Miller, Lee, Park, Lim, Chung, Wylie and Oliver. JAT 30: 380-385, July 2006

8.10.11 Wagner, R. and McLean, L., in-house development and validation for dextromethorphan, diphenhydramine, tramadol, and tricyclic antidepressants.

9 ACID/NEUTRAL/BASE DRUG SCREEN AND QUANTITATION BY GC AND GC-MS

9.1 Summary

Acidic, neutral and basic drugs are extracted from biological fluids or tissues using solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by instrumental analysis with gas chromatography and/or gas chromatography-mass spectrometry (GC-MS). This procedure may employ several drug mixes, but at least one positive control and one negative control. The procedure may be used to screen for basic, acidic and neutral drugs. Once drugs have been confirmed, the procedure may be used to quantitate drugs provided at least 3 calibrators are used to generate a response curve.

9.2 Specimen Requirements

1-2 mL whole blood, urine, bile, gastric contents, other fluids or tissue homogenates.

9.3 Reagents and Standards

- Ammonium hydroxide
- Glacial Acetic Acid
- Potassium Hydroxide
- Potassium Phosphate
- Ethyl Acetate
- Methanol
- Dichloromethane
- Isopropyl alcohol
- Hexane
- Toluene
- Isoamyl alcohol
- Potassium or sodium phosphate buffer solution concentrate (1 M, pH 6.0, e.g., Fisher)
- Sodium phosphate, monobasic ($NaH_2PO_4 \bullet H_20$)
- Sodium phosphate, dibasic (Na_2HPO_4)
- Chloroform
- Hydrochloric Acid
- Sodium tetraborate decahydrate
- Sodium hydrogen carbonate
- Potassium carbonate
- Sulfuric acid

9.4 Solutions, Internal Standards, Calibrators and Controls

9.4.1 SPE Extraction

9.4.1.1 When using UCT CleanScreen® SPE Extraction columns, either sodium or potassium phosphate buffer may be used. However, the same buffer (sodium or potassium) must be used throughout the duration of the procedure.

9.4.1.1.1 0.1 M Potassium Phosphate Buffer, pH 6.0. Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M potassium hydroxide while stirring and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years. (Note: potential exothermic reaction, exercise caution when making this solution)

9.4.1.1.2 5 M Potassium Hydroxide: Add 28.05 g potassium hydroxide to ~80 mL dH_2O in a 100 mL volumetric flask, qs to volume with dH_2O. Solutions may also be

prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

OR

9.4.1.1.3 0.1 M Sodium Phosphate Buffer, pH 6.0. Weigh 1.70g Na_2HPO_4 and 12.14g $NaH_2PO_4 \cdot H_2O$ and transfer to a 1 L volumetric flask containing approximately 800 mL dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M sodium hydroxide and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years.

9.4.1.1.4 5 M Sodium Hydroxide: Add 20.0 g of sodium hydroxide to ~80 mL dH_2O in a 100 mL volumetric flask, qs to volume with dH_2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

9.4.1.2 1.0 M Acetic Acid. Add 100-200 mL dH_2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH_2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH_2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

9.4.1.3 Ethyl acetate/Hexane, 50:50 v/v. Mix 500 mL ethyl acetate with 500 mL hexane. Store at room temperature for up to two years.

9.4.1.4 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2). Mix 20 mL isopropanol with 2 mL ammonium hydroxide. Add 78 mL dichloromethane. Mix gently. PREPARE SOLUTION FRESH DAILY!

9.4.2 Solutions for liquid/liquid base extraction

9.4.2.1 Saturated borate buffer solution. Add sodium tetraborate decahydrate to dH_2O until no more dissolves after shaking vigorously. Store at room temperature for up to two years.

9.4.2.2 Toluene:Hexane:Isoamyl Alcohol (THIA) extraction solvent (78:20:2), v:v:v: Mix toluene (780 mL), hexane (200 mL), and isoamyl alcohol (20 mL). Store at room temperature for up to two years.

9.4.2.3 Sodium Hydrogen Carbonate/Potassium Carbonate (dry 3:2 w/w) Mix 300 g $NaHCO_3$ with 200 g K_2CO_3. Store at room temperature for up to two years.

9.4.2.4 0.5 N Sulfuric Acid: Add 13.8 mL concentrated sulfuric acid to a 1 L volumetric flask (partially filled with dH_2O) and qs to volume with dH_2O. Store at room temperature for up to two years.

9.4.3 Reagents for liquid/liquid acid/neutral extraction

9.4.3.1 1.0 M sodium phosphate buffer (pH 5.5). Weigh 13.8 g sodium phosphate monobasic, transfer to a 100 mL volumetric flask and qs to volume with dH_2O. Adjust pH to 5.5 with 5 M sodium hydroxide. Store at room temperature for up to two years.

9.4.3.2 0.1 N HCl. Pipette 8.3 mL concentrated hydrochloric acid into a 1 L volumetric flask (partially filled with dH_2O) and qs to volume with dH_2O. Store at room temperature for up to two years.

9.4.4 Internal Standard

Prepare internal standards from drug standards. The concentration of the internal standard should be approximately midrange of suspected analyte concentration. Suitable internal standards for basic drugs include Sertis, methapyrilene or mepivacaine. Suitable internal standards for acidic/neutral drugs include phensuximide, tolylbarbital, methaqualone, cyclopal or hexobarbital. Deuterated internal standards may also be used when performing analysis by GC-MS in SIM mode. The concentration of internal standard may vary depending on type of case analyzed (DUID vs. postmortem) and expected analyte concentrations.

9.4.5 Calibrators

9.4.5.1 Refer to the Toxicology Quality Guidelines for quality control criteria.

9.4.5.2 Due the wide variety of analytes and expected drug concentrations, it's not practical to have a single calibration curve for all analytes. However, the following are some suggested examples of how to prepare calibration curves to target low, intermediate and high analyte concentrations.

9.4.5.2.1 Dilute 1 mg/mL drug stock solutions to working stock solutions (typically 2, 10 or 100 µg/mL).

9.4.5.2.2 To prepare a low concentration calibration curve, pipette the following volumes of working stock solutions to appropriately labeled tubes. To eliminate a solvent effect, calibrators may be dried under nitrogen/air prior to the addition of blank blood. Add 2 mL blank blood to each tube.

0.05 mg/L calibrator	10 µL of 10 µg/mL working solution
0.10 mg/L calibrator	20 µL of 10 µg/mL working solution
0.20 mg/L calibrator	40 µL of 10 µg/mL working solution
0.50 mg/L calibrator	100 µL of 10 µg/mL working solution
1.0 mg/L calibrator	200 µL of 10 µg/mL working solution

9.4.5.2.3 To prepare a mid concentration calibration curve, pipette the following volumes of working stock solutions to appropriately labeled tubes. To eliminate a solvent effect, calibrators may be dried under nitrogen/air prior to the addition of blank blood. Add 2 mL blank blood to each tube.

0.10 mg/L calibrator	20 µL of 10 µg/mL working solution
0.20 mg/L calibrator	40 µL of 10 µg/mL working solution
0.50 mg/L calibrator	100 µL of 10 µg/mL working solution
1.0 mg/L calibrator	200 µL of 10 µg/mL working solution
2.0 mg/L calibrator	40 µL of 100 µg/mL working solution
4.0 mg/L calibrator	80 µL of 100 µg/mL working solution
6.0 mg/L calibrator	120 µL of 100 µg/mL working solution

9.4.5.2.4 To prepare a high concentration calibration curve (for acidic/neutral drugs), pipette the following volumes of working stock solutions to appropriately

labeled tubes. To eliminate a solvent effect, calibrators may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to each tube.

1.0 mg/L calibrator	10 µL of 100 µg/mL working solution
2.0 mg/L calibrator	20 µL of 100 µg/mL working solution
5.0 mg/L calibrator	50 µL of 100 µg/mL working solution
10 mg/L calibrator	100 µL of 100 µg/mL working solution
20 mg/L calibrator	200 µL of 100 µg/mL working solution
40 mg/L calibrator	400 µL of 100 µg/mL working solution
60 mg/L calibrator	600 µL of 100 µg/mL working solution

9.4.6 Controls

9.4.6.1 Controls may vary depending on type of case (DUID vs. postmortem). The positive control should contain frequently observed drugs at low concentrations to address sensitivity of the assay. In addition, the positive control should contain drugs of various chromatographic retention times (early and late eluting drugs) to ensure the chromatographic conditions are capable of detecting a number of drugs. The positive controls can be prepared in-house or purchased from an approved vendor (e.g., UTAK).

9.4.6.2 See Toxicology Quality Guidelines.

9.4.6.3 Negative Control. Blood bank blood previously determined not to contain reportable drugs (i.e., most bloods contain nicotine and caffeine but these drugs are not typically reported).

9.5 Apparatus

9.5.1 Agilent GC-FID, GC-MS and/or GC-NPD, manufacturer's software, compatible computer & printer

9.5.2 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

9.5.3 Test tubes, round bottom tubes, borosilicate glass

9.5.4 Test tubes, glass centrifuge, conical bottom

9.5.5 Test tubes, round bottom, screw cap tubes, borosilicate glass

9.5.6 Centrifuge capable of 2,000 – 3,000 rpm

9.5.7 Cleanscreen® Extraction Cartridges (ZSDAU020) from United Chemical Technologies (200 mg columns)

9.5.8 Solid phase extraction manifold

9.5.9 Vortex mixer

9.5.10 Evaporator/concentrator

9.5.11 GC autosampler vials and inserts

9.5.12 Test tube rotator

9.5.13 GC-NPD and GC-FID parameters. Instrument conditions may be changed to permit improved performance.

9.5.13.1 Oven program.

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1 minutes
- Ramp: 10°C/min
- Final Temp: 290°C

- Final Time: 10 minutes
- Run Time: 29 minutes

9.5.13.2 Inlet.

- Mode: Splitless
- Temperature: 270°C
- Constant pressure: 30 psi
- Purge flow: 60 mL/min
- Purge time: 0.75 min
- Total flow: 64.9 mL/min
- Injection volume: 2.0 µL

9.5.13.3 Detector.

- Temperature: 320°C
- Hydrogen flow: 3.0. mL/min
- Air flow: 60 mL/min
- Mode: Constant column + makeup flow
- Combined flow: 10.0 mL/min
- Injection volume: 2.0 µL
- Makeup flow: On

9.5.13.4 Column: HP-1MS or HP-5MS 30 m x 0.25 mm x 0.25 µm (or equivalent).

9.5.14 GC-MS parameters for screening. Instrument conditions may be changed to permit improved performance. Parameters for SIM quantitation will include necessary parameters below (adjusted for optimal performance) and will utilize the most appropriate ion fragments for the targets of interest.

9.5.14.1 Acquisition Mode: Scan (50 – 550 amu)

9.5.14.2 Column: HP-1MS or HP5MS 30 m x 0.25 mm x 0.25 µm (or equivalent)

9.5.14.3 Detector Temperature: 280°C

9.5.14.4 Basic drug screen.

9.5.14.4.1 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1 minutes

- Ramp: 10°C/min
- Final Temp: 290°C
- Final Time: 9 minutes
- Run Time: 28 minutes

9.5.14.4.2 Inlet

- Mode: Splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

9.5.14.5 Acidic/neutral drug screen.

9.5.14.5.1 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 120°C
- Initial time: 0 minutes
- Ramp 1: 10°C/min
- Final Temp 1: 260°C
- Final Time 1: 0 minutes
- Ramp 2: 30°C/min
- Final Temp 2: 300°C
- Final Time 2: 2.67 minutes
- Run Time: 18 minutes

9.5.14.5.2 Inlet

- Mode: Splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

9.6 Procedure

9.6.1 SPE Extraction Option.

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

9.6.1.2 Label clean screw cap tubes accordingly. Prepare calibrators and/or controls.

9.6.1.3 Pipette 2 mL of corresponding negative and positive control bloods and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes.

9.6.1.4 Pipette internal standard(s) into all tubes and vortex.

9.6.1.5 Add 4.0 mL deionized water to each tube. Mix, vortex briefly and let stand for 5 minutes.

9.6.1.6 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer supernatant to clean tubes and discard the tube with the remaining pellet.

9.6.1.7 Add 2.0 mL of pH 6 phosphate buffer, mix and vortex. As necessary adjust the pH to 5.5 to 6.5 with additional 0.1 M phosphate buffer. Note: larger volumes of water or phosphate buffer may be used to further dilute some specimens prior to SPE analysis

9.6.1.8 Solid phase extraction. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

9.6.1.8.1 Add 3 mL hexane to each column and aspirate.

9.6.1.8.2 Add 3 mL methanol to each column and aspirate.

9.6.1.8.3 Add 3 mL dH_2O and aspirate.

9.6.1.8.4 Add 1 mL of 0.1 M pH 6.0 phosphate buffer and aspirate.

9.6.1.8.5 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges under vacuum or positive pressure at approximately 1-2 mL/ minute flow.

9.6.1.8.6 Add 3 mL dH_2O and aspirate at ≤ 3inches of mercury or a low positive pressure (< ~10 psi).

9.6.1.8.7 Repeat the dH_2O wash.

9.6.1.8.8 Wash with 2.0 mL 1.0 M acetic acid and aspirate.

9.6.1.8.9 If only extracting basic drugs, add 3 mL methanol, aspirate under full vacuum/pressure for at least 2 minutes and skip to step 9.6.1.8.15.

9.6.1.8.10 If extracting acidic/neutral and basic drugs, dry columns under full vacuum/pressure for at least 2 minutes.

9.6.1.8.11 Add 2 mL hexane and aspirate.

9.6.1.8.12 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

9.6.1.8.13 Elute acid/neutral drugs by adding 3 mL of hexane/ethyl acetate (50:50 v/v) to each column. Collect eluate in conical test tubes by gentle column aspiration or gravity drain.

9.6.1.8.14 Remove acid/neutral conical test tubes. Add an additional 3 mL methanol to all SPE columns and aspirate to waste under full vacuum/pressure.

9.6.1.8.15 Add 2 mL hexane to each column. Dry columns at ≥ 10 inches of mercury or a high positive pressure (~50 psi) for five minutes.

9.6.1.8.16 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

9.6.1.8.17 Elute basic drugs by adding 3 mL of freshly prepared methylene chloride/isopropanol/ammonium hydroxide solution to each column. Collect eluate in conical test tubes by column aspiration or gravity drain. Elute at 1 - 2 mL/minute (no vacuum) and collect eluates.

9.6.1.9 Evaporate eluates to dryness at approximately 50°C under nitrogen.

9.6.1.10 Reconstitute the residue with 50-200 µL of toluene/ hexane/isoamyl alcohol (basic drugs) or ethyl acetate (basic or acidic/neutral drugs).

9.6.1.11 Vortex and transfer to autosampler vials.

9.6.1.12 Transfer autosampler vials to the GC and/or GC-MS. Drug retention time and GC-MS spectral match are used to identify drugs.

9.6.2 Basic LLE Extraction Option

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

9.6.2.2 Label clean screw cap tubes accordingly. Prepare calibrators and/or controls.

9.6.2.3 Pipette 2 mL of corresponding negative and positive control bloods and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes.

9.6.2.4 Pipette internal standard into all tubes and vortex.

9.6.2.5 Add 2 mL of saturated borate buffer to each tube.

9.6.2.6 Add 5 mL of toluene/hexane/isoamyl alcohol extraction solvent to each tube.

9.6.2.7 Rotate tubes for 20 minutes.

9.6.2.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.2.9 Transfer the top (organic) layer to appropriately labeled screw-cap test tubes. Discard lower (aqueous) layer.

9.6.2.10 Add 2 mL of 0.5N sulfuric acid to tubes. Cap and rotate 20 minutes. Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.2.11 Aspirate off top (organic) layer and discard.

9.6.2.12 Adjust aqueous layer to a basic pH by slowly adding solid 3:2 $NaHCO_3/K_2CO_3$ buffer until effervescence ceases. Then add approximately 0.3 g excess $NaHCO_3/K_2CO_3$ buffer to saturate the aqueous layer.

9.6.2.13 Add 200 µL of toluene/ hexane/isoamyl alcohol extraction solvent to each tube, cap tubes and vortex for 10-15 seconds. Centrifuge tubes at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.2.14 Transfer approximately 200 µL of top (organic) layer into GC autosampler vials.

9.6.2.15 Transfer autosampler vials to the GC and/or GC-MS. Drug retention time and GC-MS spectral match are used to identify drugs.

9.6.3 Acid/Neutral Extraction Option

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

9.6.3.2 Label clean screw cap tubes accordingly. Prepare calibrators and/or controls.

9.6.3.3 Pipette 1-2 mL of corresponding negative and positive control bloods and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes.

9.6.3.4 Pipette internal standard into all tubes and vortex.

9.6.3.5 Add 1 mL of pH 5.5 sodium phosphate buffer to each tube.

9.6.3.6 Add 5 mL of ethyl acetate to each tube.

9.6.3.7 Rotate tubes for 20 minutes.

9.6.3.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.3.9 Transfer the top (organic) layer to appropriately labeled screw-cap test tubes.

9.6.3.10 Evaporate to dryness at 50-60°C under nitrogen.

9.6.3.11 Reconstitute each sample with 0.5 mL hexane. Vortex briefly.

9.6.3.12 Add 2 mL 0.1 N HCl to each tube. Vortex for 30 seconds.

9.6.3.13 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.3.14 Aspirate and discard upper (organic) layer.

9.6.3.15 Add 5 mL chloroform to each tube. Vortex for 30 seconds.

9.6.3.16 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

9.6.3.17 Aspirate and discard upper layer.

9.6.3.18 Evaporate to dryness at 50-60°C under nitrogen.

9.6.3.19 Reconstitute samples with 50 µL ethyl acetate. (Note: Centrifugation may be necessary at this step)

9.6.3.20 Vortex briefly and transfer to autosampler vials.

9.6.3.21 Transfer autosampler vials to the GC and/or GC-MS. Drug retention time and GC-MS spectral match are used to identify drugs.

9.7 Calculation

9.7.1 GC-NPD and GC-FID Data.

9.7.1.1 Evaluate positive control to ensure efficiency of extraction and proper operation of the GC-NPD or GC-FID.

9.7.1.2 By comparing GC-NPD or GC-FID retention times to known retention times from retention time tables (i.e., caffeine, nicotine and cotinine) some cases may be determined to be “negative” for drugs. In cases with peaks indicating the presence of drugs other than caffeine, nicotine and cotinine, reinject the extracts with significant findings on the GC-MS for confirmation. Often, the GC-NPD results may be useful when attempting to confirm drugs by GC-MS.

9.7.1.3 Negative Control. The negative control is used as an interpretative aid in assessing internal standard recovery and identifying “junk” peaks that may be common in all samples.

9.7.1.4 For quantitative analysis, prepare a response curve of area (height) of analyte to area (height) of internal standard ratio versus calibrator concentration. Calculate the analyte concentration

by interpolation of the linear plot. It is acknowledged that some assays are inherently non-linear and the use of quadratic models may be necessary and appropriate, and should be verified using low, medium and high controls. The response curve and determined unknown specimen concentration(s) are generated by the instrument software.

9.7.1.5 See Toxicology Quality Guidelines.

9.7.2 GC-MS Data

9.7.2.1 Case samples. For screening of samples, take spectra of significant peaks on the TIC. Include spectra and spectral library matches for identified drugs or suspect compounds (excluding non-reported drugs such as caffeine, nicotine and cotinine). Do not include spectrums of "junk" peaks (e.g., fatty acids, phthalates, hydrocarbons, etc.). If needed, use extracted ion profiles to look for drugs indicated by history or GC-NPD results that are not significant peaks on the TIC. Label identified drugs on the TIC or NPD chromatogram.

9.7.2.1.1 For SIM quantitation criteria, refer to Toxicology Quality Guidelines.

9.7.2.2 Negative Control. The negative control is used as an interpretative aid in assessing internal standard recovery and identifying "junk" peaks that may be common in all samples.

9.7.3 See Toxicology Quality Guidelines.

9.8 Quality Control and Reporting

9.8.1 Negative control criteria for qualitative screening utilizing this method: The negative control shall not contain the analyte of interest, satisfy RT, and chromatography reporting criteria. If a blank matrix contains one or more analytes of interest, those analytes shall not be screened for or confirmed from that analysis.

9.8.2 Positive control: Refer to 9.4.6.1 for positive control(s) to be used.

See Toxicology Quality Guidelines.

9.9 References

9.9.1 T. Soriano, C. Jurado, M. Menendez and M. Repetto, "Improved Solid-Phase Extraction Method for Systematic Toxicological Analysis in Biological Fluids." J. Anal. Toxicol. 2001; March (25): 137-143.

9.9.2 W.H. Anderson and D.C. Fuller, "A Simplified Procedure for the Isolation, Characterization, and Identification of Weak Acid and Neutral Drugs from Whole Blood." J. Anal. Toxicol. 1987, Sep/Oct (11): 198-204.

9.9.3 Randall Edwards, in-house development.

10 CARBOXYHEMOGLOBIN SATURATION DETERMINATION BY SPECTROPHOTOMETRY WITH CONFIRMATION BY MICRODIFFUSION

10.1 Summary

Quantitation by UV-VIS Spectrophotometry

A dilute hemolysate of blood is treated with sodium dithionite to reduce oxyhemoglobin and/or methemoglobin; carboxyhemoglobin remains unaffected. The absorbance of this solution is scanned 650 nm to 500 nm and measured at 541 nm and 555 nm. The absorbance ratio of A_{541nm} / A_{555nm} is calculated and the percent carboxyhemoglobin is determined from the historical calibration curve.

Confirmation by Palladium Chloride Microdiffusion

Carbon monoxide is liberated from blood by strong acid in a microdiffusion cell and palladium chloride, in the center of the diffusion cell, is reduced to metallic palladium which has a silver appearance. The presence of CO in blood can thus be easily detected by the observation of the appearance of the silver film.

10.2 Specimen Requirements

Quantitation: Approximately 0.5 mL of whole blood

Confirmation: Approximately 2.0 mL of blood or mixed tissue containing sufficient quantity of hemoglobin

10.3 Reagents and Standards

Quantitation

- Ammonium hydroxide (NH_4OH), 0.4%. Pipette 15.9 mL of concentrated NH_4OH into a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.
- Sodium dithionite (sodium hydrosulfite) – lab-grade may be used in place of higher grades.

Confirmation

- Hydrochloric acid, concentrated
- Sulfuric acid, concentrated
- Palladium chloride
- Lead acetate
- Glacial acetic acid
- 0.1N Hydrochloric Acid: Cautiously add 8.3 mL of concentrated HCl to approximately 100 mL of dH_2O in a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.
- 10% (3.6 N) Sulfuric Acid: Cautiously add 10 mL of concentrated H_2SO_4 to approximately 70 mL of dH_2O in 100 mL volumetric flask. Cool and qs to volume with dH_2O. Store at room temperature for up to two years.
- 0.005 N Palladium Chloride Reagent: Weigh 0.22 g palladium chloride, transfer into a 250 mL volumetric flask and qs to volume with 0.1 N HCl and let stand overnight. Transfer to a 500 mL volumetric flask and qs to volume with 0.1 N HCl. Store at room temperature for up to two years.
- 10% Lead Acetate-Acetic Acid Solution: Add 10 mL of glacial acetic acid to a 100 mL volumetric flask and qs to volume with dH_2O. Saturate the solution with lead acetate by adding lead acetate until no more dissolves after mixing vigorously. Store at room temperature for up to two years.

10.4 Calibrators, Controls and Internal Standards

CO-Oximeter controls or similarly named controls from RNA Medical (Devens, MA) or Instrumentation Laboratory Company (Lexington, MA), stored at 2-8°C.

10.4.1 A certified reference material is not currently available to establish traceability for the calibrators and CO-oximeter controls.

10.5 Apparatus

Quantitation

10.5.1 1 cm UV-VIS cuvettes

10.5.2 Agilent Cary 60 and its associated software.

10.5.3 Method parameters for Agilent Cary 60.

- Wavelength range (nm): 650-500
- Measuring mode: Absorbance
- Scan speed: Any speed acceptable

Confirmation

10.5.4 Conway microdiffusion cells (2 well, with cover)

10.5.5 Sealant or dH_2O

10.6 Procedure

Quantitation

10.6.1 Allow all biological samples and reagents to come to room temperature before starting procedure unless otherwise noted by the manufacturer.

10.6.2 Prepare a baseline reference sample (blank) by adding sufficient volume of 0.4% NH_4OH solution and approximately 50 mg sodium dithionite to a 1 cm cuvette if using a single blank. Alternatively, two instrument blanks may be used in the run. One taken before the unreduced spectra that does not include the sodium dithionite above, then another blank taken with the sodium dithionite added to the 0.4% NH_4OH solution before collecting the reduced spectra.

10.6.3 Negative and positive blood control samples are prepared and analyzed as single, reduced samples, at a minimum. Case samples shall be prepared and analyzed to collect one unreduced spectrum and two reduced spectra, at a minimum.

10.6.4 To prepare unreduced controls and samples, add approximately one to three drops of case sample to a 1 cm cuvette containing sufficient volume of 0.4% NH_4OH solution (try to achieve a sufficient absorbance maximum between 0.80 and 1.2) and mix by inversion. Adjust concentrations of samples until absorbance criteria is met.

10.6.5 Scan the blank, controls, and samples to collect the unreduced spectra according to the “Analyze samples” section below.

After collecting the unreduced spectra, prepare the reduced samples. Add approximately ~50 mg sodium dithionite to above and mix by inversion. The amount of sodium dithionite may be measured using a scoop designed to deliver 50 mg of solid powders.

10.6.6 Rescan the cuvettes to collect the reduced spectra according to the “Analyze samples” section below.

10.6.7 Analyze samples

10.6.7.1 Prepare the instrument baseline correction (only needs to be performed once before sample analysis):

10.6.7.1.1 Enter method "setup" and go to "baseline" and select "baseline correction".

10.6.7.1.2 Ensure the sample compartment is empty (no cuvette) and click on "zero" to correct lamp intensity fluctuations.

10.6.7.1.3 Place the baseline reference sample into the sample compartment and click on "baseline" to correct the cuvette and solvent transmittance.

10.6.7.1.4 With the baseline reference sample in the sample compartment select "rapid result" and click on "blank". The instrument is now ready to analyze samples.

10.6.7.2 Scan the absorbance spectrum from 650 nm to 500 nm of each sample. Utilize the instrument software and reporting functions to determine the absorbances at 541 nm and 555 nm, print the absorbance spectrum, and carboxyhemoglobin saturation.

Confirmation (may not be performed if Quantitation results are < 10% saturation)

10.6.8 Prepare microdiffusion cell with sealant or dH_2O.

10.6.9 Add 2 mL of $PdCl_2$ reagent to center well of microdiffusion cell.

10.6.10 Add approximately 2 mL of blood to one side of the outer ring.

10.6.11 Add 1 mL of 10% H_2SO_4 to the other side of the outer ring. Quickly cover microdiffusion cell and gently rock/rotate to mix blood with sulfuric acid. Diffuse for approximately one hour.

10.6.12 Record results.

10.7 Calculation

10.7.1 Calculations of the carboxyhemoglobin saturation are performed by the instrument software and printed on the data. The historic calibration curve used is:

$$\frac{Abs541}{Abs555} = 0.003667(\%CO) + 0.842$$

10.7.2 The standard curve was obtained by saturating negative blood with known concentrations of HbCO and plotting the known % carboxyhemoglobin samples (10, 20, 50, 70 and 100% HbCO) versus ΔA. The curve is linear from 10-60% carboxyhemoglobin.

10.8 Quality Control and Reporting

Quantitation

10.8.1 Analyze at least one positive and one negative control with each group of case samples.

10.8.2 The negative control must be less than approximately 10% carboxyhemoglobin saturation. The low positive control must be between approximately 10% and 30% carboxyhemoglobin saturation. The high positive control must be greater than approximately 35%.

10.8.2.1 Positive controls may be tracked using a statewide spreadsheet to monitor the process performance.

10.8.3 The LOQ for the assay is 10% saturation and the ULOQ is 60% saturation. Results below the LOQ are reported as “carboxyhemoglobin none detected at approximately 10% saturation”. Results between the LOQ and ULOQ shall be reported as “carboxyhemoglobin approximately (whole number)% saturation”. Results greater than the ULOQ are reported as “carboxyhemoglobin greater than approximately 60% saturation”.

10.8.4 Samples of questionable quality may be reported as “unsuitable for analysis” or “inconclusive”. The spectra should have two distinct maxima with a distinct minimum between the maxima. A less distinct minima may indicate a sample that is less suitable for analysis.

Confirmation

10.8.5 A silver colored mirror will form in the center well of the dish in positive samples. Negative samples will appear to be unchanged (clear yellow gold color of the palladium chloride reagent). The intensity of the silver mirror will be directly proportional to the concentration of carbon monoxide in the blood.

10.8.6 Record the outcome of the reaction by using ”+” as consistent with the low or high positive controls and “-“ as consistent with the negative control.

10.8.7 Analyze at least one level of the positive controls and a negative control with each group of case samples.

10.8.8 A positive (> 10%) carboxyhemoglobin result may only be reported if it is confirmed or consistent with the palladium chloride microdiffusion result. If there are inconsistencies between the Quantitation and Confirmation results, repeat the analysis. If, after repeat analysis, inconsistencies still exist, the sample should be reported as “unsuitable for analysis” or “inconclusive.” This exception should be authorized by a toxicologist, group supervisor, or supervisor and documented in the case file with an MFR.

10.8.9 Weak positive results (> 10%) on putrefied, decomposed, or deteriorated specimens may be reported as less than a toxicologically significant carboxyhemoglobin concentration (e.g., a 12% COHb on poor quality specimen, confirmed by palladium chloride microdiffusion) may be reported as “carboxyhemoglobin less than approximately 15% saturation.” This exception should be authorized by a toxicologist, group supervisor, or supervisor and documented in the case file with an MFR.

10.9 Notes

Quantitation

Ordinarily one to three drops of whole blood per 3 ml NH_4OH should be sufficient. More than four or five drops per 3 ml may indicate that the nature of the sample is questionable. If this occurs, the sample may be reported as “unsuitable for analysis”.

Confirmation

Sulfur compounds (e.g., hydrogen sulfide from putrefied specimens) may react with $PdCl_2$. For putrefied specimens, substitute lead acetate for 10% sulfuric acid and allow to diffuse for 4 hours.

10.10 References

10.10.1 Tietz, Norbert W., Ph.D. and Fiereck, A., M.S. Annals of Clinical Laboratory Science, Vol. 3, No. 1 pp. 36-42, 1973

10.10.2 B.L. Levine, Principles of Forensic Toxicology, American Association for Clinical Chemistry, Inc., pp. 330-337, 1999.

10.10.3 Ramieri, A., Jr., Jatlow, P. and Seligson, D. New method for rapid determination of carboxyhemoglobin by use of double-wavelength spectrophotometry (ΔA Method). Clinical Chemistry, Vol. 20, No. 2, 1974.

10.10.4 van Kampen, E.J. and Klouwen, N. Tidschr. Geneeskd, 98, pp. 161-164, 1954.

10.10.5 Holmium Oxide Glass Wavelength Standards. *Journal of Research at the National Institute of Standards and Technology* Vol 112 (6): 303-306, 2007.

10.10.6 Intrinsic Wavelength Standard Absorption Bands in Holmium Oxide Solution for UV-VISible Molecular Absorption Spectrophotometry. *J Phys Chem Ref Data* Vol 34(1): 41-56, 2005.

10.10.7 Williams, L.A., Methodology for Analytical Toxicology, CRC Press, I. Sunshine, editor, Cleveland, 1975.

11 FENTANYL DERIVATIVE QUANTITATION AND CONFIRMATION BY LCMSMS

11.1 Summary

Fentanyl derivatives are extracted from biological samples using a solid phase extraction. The extracted sample is quantitated or qualitatively identified and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

11.2 Specimen Requirements

1.0 mL blood, fluid, or tissue homogenate (For fentanyl derivatives, use caution in interpretation for tissue homogenates.)

11.3 Reagents and Standards

- Quantitative drug targets and associated internal standard

Fentanyl Derivatives	
Target Compound	Internal Standard
3-Fluorofentanyl	Fentanyl-D_5
4-Methoxybutyrlfentanyl	para-Fluorobutyrylfentanyl-D_7
Acetylfentanyl	Acetylfentanyl-$^{13}C_6$
Benzodioxolefentanyl	para-Fluorobutyrylfentanyl-D_7
Butyrylfentanyl	para-Fluorobutyrylfentanyl-D_7
Carfentanil	Carfentanil-D_5
cis-3-Methylfentanyl	Carfentanil-D_5
Crotonylfentanyl	para-Fluorobutyrylfentanyl-D_7
Cyclopropylfentanyl	para-Fluorobutyrylfentanyl-D_7
Fentanyl	Fentanyl-D_5
Furanylfentanyl	Fentanyl-D_5
Methoxyacetylfentanyl	Acetylfentanyl-$^{13}C_6$
Ocfentanil	Acetylfentanyl-$^{13}C_6$
ortho-Fluorobutyrylfentanyl	para-Fluorobutyrylfentanyl-D_7
ortho-Fluorofentanyl	Fentanyl-D_5
ortho-Fluoroisobutyrylfentanyl	para-Fluorobutyrylfentanyl-D_7
para-Fluorobutyrylfentanyl	para-Fluorobutyrylfentanyl-D_7
para-Fluoroisobutyrylfentanyl	para-Fluorobutyrylfentanyl-D_7
Phenylfentanyl	para-Fluorobutyrylfentanyl-D_7
trans-3-Methylfentanyl	Carfentanil-D_5
U-49900	Fentanyl-D_5

- Qualitative drug targets and associated internal standard

Fentanyl Derivatives	
Target Compound	Internal Standard
Acrylfentanyl	Acetylfentanyl-$^{13}C_6$
alpha-Methylacetylfentanyl	Acetylfentanyl-$^{13}C_6$
alpha-Methylfentanyl	Fentanyl-D_5
beta-Hydroxythiofentanyl	Acetylfentanyl-$^{13}C_6$
Despropionylfentanyl	Fentanyl-D_5
ortho-Fluoroacrylfentanyl	Fentanyl-D_5
para-Fluoroacrylfentanyl	Fentanyl-D_5
para-Fluorofentanyl	para-Fluorobutyrylfentanyl-D_7
Tetrahydrofuranfentanyl	Fentanyl-D_5
U-47700	Fentanyl-D_5
Valerylfentanyl	para-Fluorobutyrylfentanyl-D_7

- Formic acid, eluent additive ~98%
- Type I or LCMS grade water
- Methanol, Optima (or similar) grade or higher
- Ammonium hydroxide, Optima (or similar) grade or higher
- Dichloromethane
- 2-Propanol, HPLC grade
- Sodium phosphate buffer solution concentrate (1 M, pH 6.0 e.g., Fisher)
- Hydrochloric acid, Optima (or similar) grade or higher
- Acetonitrile, Optima (or similar) grade or higher
- Acetic acid, glacial, ACS or higher grade

11.4 Solutions, Internal Standards, Calibrators and Controls

11.4.1 0.1 N Hydrochloric acid in 2-propanol: Add 8.3 mL of concentrated HCl (12 N) to 1.0 L of 2-propanol. Store at room temperature for up to one month.

11.4.2 0.1 M Phosphate Buffer, pH 6.0: Weigh out 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5.0 M potassium hydroxide while stirring and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years.

11.4.3 1 M Acetic Acid: Add 100-200 mL dH_2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH_2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH_2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

11.4.4 0.1 M Acetic Acid: Add 10 mL of 1 M acetic acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

11.4.5 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2): Mix 78 mL dichloromethane with 20 mL isopropanol. Mix well. In hood, add 2 mL ammonium hydroxide. Mix gently. PREPARE SOLUTION FRESH DAILY!

11.4.6 Mobile Phase A (H_2O with 0.01% formic acid): Add 100 µL of formic acid to 1 L of Type I or LC-MS grade H_2O. Store at room temperature for up to one month.

11.4.7 Mobile Phase B (Methanol with 0.01% formic acid): Add 100 µL of formic acid to 1 L of methanol. Store at room temperature for up to one month.

11.4.8 Working solution (0.5/1 mg/L): Pipette 50 µL of the 0.1 mg/mL stock solution (carfentanil, trans-3-methylfentanyl) into a 10.0 mL volumetric flask. Pipette 10 µL of the 0.5 mg/mL stock solution (cis-3-methylfentanyl) into the 10.0 mL volumetric flask. Pipette 10 µL of the 1.0 mg/mL stock solution (100 µL of the 0.1 mg/mL stock solution) of the remaining compounds into the 10.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

11.4.9 Working solution (0.05/0.1 mg/L): Pipette 1.0 mL of the 0.5/1 mg/L working solution into a 10.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

11.4.10 Working solution (0.005/0.01 mg/L): Pipette 1.0 mL of the 0.05/0.1 mg/L working solution into a 10.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

11.4.11 Working internal standard (0.05/0.1 mg/L): Pipette 5 µL of the 0.1 mg/mL stock solution (carfentanil-D_5) into a 10.0 mL volumetric flask. Pipette 10 µL of the 0.1 mg/mL stock solution for remaining internal standards into the 10.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

11.4.12 Controls.

11.4.12.1 Negative control blood: blood bank blood or equivalent determined not to contain target compounds.

11.4.12.2 Threshold Control: TC is prepared at one half of the LOQ, however this may be adjusted based upon the validated LOD.

11.4.12.3 An example for in-house control preparation (this is provided for convenience, this is not the required approach to controls):

Amount of 0.5/1 mg/L Control Solution (µL)	Amount of 0.05/0.1 mg/L Control Solution (µL)	Amount of 0.005/0.01 mg/L Control Solution (µL)	Final Concentration (mg/L)
-	-	40	0.0002/0.0004
-	30	-	0.0015/0.0030
15	-	-	0.0075/0.0150

11.4.12.3.1 Due to the quadratic nature of many of the targets, at least three controls, at low, medium, and high concentration, must be run across the concentration range with every batch. A high control must be run between the two highest spiked calibrator concentrations.

11.4.12.4 Commercial whole blood control (UTAK or other commercial vendor) or methanolic statewide controls (prepared by the Research Section Supervisor, Research Analyst, or designee).

11.5 Apparatus

11.5.1 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

11.5.2 Test tubes, conical bottom

11.5.3 Centrifuge capable of 2,000-3,000 rpm

11.5.4 Vortex mixer

11.5.5 GC autosampler vials with inserts

11.5.6 Solid phase extraction manifold

11.5.7 United Chemical Technologies Cleanscreen® Extraction Cartridges (ZSDAU020)

11.5.8 Evaporator/concentrator

11.5.9 Typical LCMSMS parameters

11.5.9.1 LC Parameters:

- Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm
- Guard Column: Poroshell 120 EC-C18, 2.1 x 5 mm, 2.7 µm
- Column Thermostat: 60°C
- Mobile Phase A: Water with 0.01% formic acid
- Mobile Phase B: Methanol with 0.01% formic acid
- Flow Rate: 0.7 mL/min
- Injection Volume: 20 µL

- Stop Time: 19 minutes
- Post Time: Minimum 2.00 minutes
- Gradient:

Time (minutes)	Solvent A (%)	Solvent B (%)
0.00	90.0	10.0
13.00	75.0	25.0
17.00	65.0	35.0
18.50	2.0	98.0
19.00	2.0	98.0

11.5.9.2 MSMS Parameters

- MSD Parameters:

Ionization: ESI
Polarity: Positive
Gas Temperature: 325°C
Nebulizer Pressure: 45 psi
Capillary: 3500 V
Drying Gas: 12 L/min

- Transition Ions:

Dynamic MRM Compound Settings

Compound Name	Precursor Ion	Product Ion	Approx. Ret Time	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	RRT Threshold (%)
Methoxyacetylfentanyl	353.2	**188.1**	7.6	2	115	20	7	1.5
		105				44	7	
Acetylfentanyl-$^{13}C_6$	329.4	**188.1**	7.9	2	130	20	7	
		105.1				40	7	
Acetylfentanyl	323.2	**188.1**	8.0	2	105	20	7	0.5
		105				40	7	
Despropionylfentanyl	281.2	**188.1**	8.2	2	100	12	7	2
		105.1				32	7	
beta-Hydroxythiofentanyl	359.2	**341.1**	8.2	2	100	12	7	2
		192.1				20	7	
Ocfentanil	371.2	**188.1**	8.6	2	110	20	7	2
		105				44	7	
alpha-Methylacetylfentanyl	337.2	202.1	8.9	2	110	20	7	2
		91				48	7	
Tetrahydrofuranfentanyl	379.2	**188.1**	10.7	2	100	20	7	1.5
		105.1				48	7	
Acrylfentanyl	335.2	**188.1**	10.8	2	135	20	7	1.5
		105				40	7	
para-Fluoroacrylfentanyl	353.2	**188.1**	11.0	3	125	20	7	1.5
		105				44	7	
U-47700	329.1	**284.1**	11.1	2	75	12	7	2
		172.9				36	7	
ortho-Fluoroacrylfentanyl	353.2	**188.1**	11.5	2	95	20	7	0.8
		105.1				44	7	
Fentanyl-D_5	342.2	**188.2**	11.8	2	140	25	2	

Dynamic MRM Compound Settings

Compound Name	Precursor Ion	Product Ion	Approx. Ret Time	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	RRT Threshold (%)
		105.1				50	2	
Fentanyl	337.2	**188.1**	11.9	2	110	20	7	1.5
		105				40	7	
para-Fluorofentanyl	355.2	**188.1**	12.0	4	125	20	7	2
		105				44	7	
alpha-Methylfentanyl	351.2	202.1	12.6	2	120	20	7	1.5
		91				48	7	
Furanylfentanyl	375.2	**188.1**	12.8	2	135	20	7	2
		105.1				44	7	
U-49900	357.2	**284.1**	12.8	2	110	16	7	1
		172.9				36	7	
ortho-Fluorofentanyl	355.2	**188.1**	13.1	3	135	24	7	2
		105				40	7	
Cyclopropylfentanyl	349.2	**188.1**	13.7	3	130	24	7	1
		105.1				44	7	
3-Fluorofentanyl	355.2	**206.1**	14.0	3	120	24	7	2
		299.2				16	7	
Carfentanil-D_5	400.3	**340.2**	14.0	2	115	16	7	
		113				32	7	
Carfentanil	395.2	**335.2**	14.1	2	100	16	7	0.5
		113				32	7	
trans-3-Methylfentanyl	351.2	202.1	14.3	4	95	20	7	0.5
		105				44	7	
Crotonylfentanyl	349.2	**188.1**	14.4	3	125	20	7	0.5
		105.1				44	7	
cis-3-Methylfentanyl	351.2	202.1	14.7	2	130	24	7	0.8
		105				40	7	
Butyrylfentanyl	351.2	188.1	15.5	4	130	20	7	0.5
		105				44	7	
para-Fluorobutyrylfentanyl-D_7	376.3	**188.1**	15.5	2	145	24	7	
		105.1				50	7	
ortho-Fluorobutyrylfentanyl	369.2	**188.1**	15.7	3	120	24	7	0.5
		105				44	7	
ortho-Fluoroisobutyrylfentanyl	369.2	**188.1**	15.7	3	125	24	7	0.5
		105				44	7	
para-Fluorobutyrylfentanyl	369.2	**188.1**	15.7	3	115	24	7	0.5
		105				44	7	
para-Fluoroisobutyrylfentanyl	369.2	**188.1**	15.7	3	115	24	7	0.5
		105.1				44	7	
Phenylfentanyl	385.2	**188.1**	15.7	3	150	20	7	0.5
		105				44	7	
Benzodioxolefentanyl	429.2	188.1	15.9	2	130	20	7	0.8
		149				28	7	
4-Methoxybutyrlfentanyl	381.3	**188.1**	16.5	2	125	24	7	1
		105				44	7	
Valerylfentanyl	365.3	**188.1**	17.9	2	115	24	7	
		105.1				44	7	

11.6 Procedure

11.6.1 Label appropriate clean screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

11.6.2 Prepare calibrators and controls. Calibrators and controls shall not be dried down under any circumstances (i.e., using nitrogen or heat). (Volume (µL) to deliver into the appropriately labeled screw top tubes.)

Amount of 0.5/1 mg/L Working Solution (µL)	Amount of 0.05/0.1 mg/L Working Solution (µL)	Amount of 0.005/0.01 mg/L Working Solution (µL)	Final Concentration (mg/L)
-	-	5	TC (0.000025/0.000050)
-	-	10	0.00005/0.00010
-	-	25	0.000125/0.00025
-	-	50	0.00025/0.00050
-	10	-	0.00050/0.0010
-	20	-	0.0010/0.0020
-	50	-	0.0025/0.0050
10	-	-	0.0050/0.0100
25	-	-	0.0125/0.0250

11.6.3 Pipette 1.0 mL of blank blood, calibrators, controls and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes.

11.6.4 Add 20 µL of 0.05/0.1 mg/L working internal standard solution to each tube and vortex briefly.

11.6.5 Add 4.0 mL of 0.1 M pH 6.0 phosphate buffer and 2.0 mL water to each tube and vortex briefly.

11.6.6 Centrifuge at approximately 2500 rpm for 15 minutes.

11.6.7 Solid phase extraction. Place labeled SPE cartridges in the extraction manifold. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

11.6.7.1 Add 3.0 mL of methanol to each column and aspirate.

11.6.7.2 Add 3.0 mL of water to each column and aspirate.

11.6.7.3 Add 1.0 mL of 0.1 M pH 6.0 phosphate buffer to each column and aspirate.

11.6.7.4 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges under vacuum/pressure at approximately 1-2 mL/minute flow.

11.6.7.5 Add 3.0 mL of water and aspirate at ≤ 3 inches of mercury or a low positive pressure (< ~10 psi).

11.6.7.6 Add 1.0 mL of 0.1 M acetic acid and aspirate at ≤ 3 inches of mercury or a low positive pressure (< ~10 psi).

11.6.7.7 Wash with 3.0 mL of methanol and aspirate under full vacuum/pressure for approximately 30 minutes.

11.6.7.8 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

11.6.7.9 Elute drugs by adding 3.0 mL of freshly prepared methylene chloride/2-propanol/ammonium hydroxide solution to each column. Collect eluate in conical test tubes by column aspiration or gravity drain.

11.6.8 Add 50 µL of 0.1 N HCl in 2-propanol to each tube and evaporate samples to dryness at approximately 50°C under nitrogen.

11.6.9 Reconstitute in 50 µL of 0.01% formic acid in water. (Note: Centrifugation may be necessary at this step)

11.6.10 Transfer to autosampler vials.

11.7 Quality Control and Reporting

11.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

11.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

11.7.3 The calibration models for each target are listed below:

Target	Linear/Quadratic	Weighting
3-Fluorofentanyl	Linear	Weighted (1/x)
4-Methoxybutyrylfentanyl	Quadratic	Weighted (1/x)
Acetylfentanyl	Quadratic	Weighted (1/x)
Benzodioxolefentanyl	Quadratic	Weighted (1/x)
Butyrylfentanyl	Quadratic	Weighted (1/x)
Carfentanil	Quadratic	Weighted (1/x)
cis-3-Methylfentanyl	Quadratic	Weighted (1/x)
Crotonylfentanyl	Quadratic	Weighted (1/x)
Cyclopropylfentanyl	Quadratic	Weighted (1/x)
Fentanyl	Linear	Weighted (1/x)
Furanylfentanyl	Quadratic	Weighted (1/x)
Methoxyacetylfentanyl	Quadratic	Weighted (1/x)
Ocfentanil	Quadratic	Weighted (1/x)
ortho-Fluorobutyrylfentanyl	Quadratic	Weighted (1/x)
ortho-Fluorofentanyl	Quadratic	Weighted (1/x)
ortho-Fluoroisobutyrylfentanyl	Quadratic	Weighted (1/x)
para-Fluorobutyrylfentanyl	Quadratic	Weighted (1/x)
para-Fluoroisobutyrylfentanyl	Quadratic	Weighted (1/x)
Phenylfentanyl	Quadratic	Weighted (1/x)
trans-3-Methylfentanyl	Quadratic	Weighted (1/x)
U-49900	Linear	Weighted (1/x)

11.7.4 When a target concentration is above the ULOQ, 1.0 mL of case sample shall be diluted with no more than 19.0 mL of blank matrix except for ortho-fluoroacrylfentanyl which may only be diluted with up to 1.0 mL of blank matrix.

11.7.5 For samples with volumes less than 1.0 mL, samples may be diluted up to 1/20 (0.05 mL samples with 0.95 mL blank matrix) except for ortho-fluoroacrylfentanyl which may not be diluted at these small volumes.

11.7.6 Extracted samples are stable for 4 days after reconstitution for all targets. Selected targets are stable for longer periods:

Six days: 3-fluorofentanyl, 4-methoxybutyrylfentanyl, benzodioxolefentanyl, despropionylfentanyl, fentanyl, para-fluorofentanyl, para-fluorbutyrylfentanyl, phenylfentanyl.

Seven days: para-fluoroacrylfentanyl, valerylfentanyl.

11.7.7 The threshold control is set at ½ LOQ. The validated LODs are listed below:

Limit of Detection	
Target	LOD Concentration (mg/L)
3-Fluorofentanyl	0.00005
4-Methoxybutyrylfentanyl	0.000025
Acetylfentanyl	0.000025
Acrylfentanyl	0.000025
alpha-Methylacetylfentanyl	0.000075
alpha-Methylfentanyl	0.0001
Benzodioxolefentanyl	0.0000375
beta-Hydroxythiofentanyl	0.0000375
Butyrylfentanyl	0.000025
Carfentanil	0.00005
cis-3-Methylfentanyl	0.0000125
Crotonylfentanyl	0.000025
Cyclopropylfentanyl	0.000025
Despropionylfentanyl	0.000025
Fentanyl	0.000025
Furanylfentanyl	0.0000375
Methoxyacetylfentanyl	0.000025
Ocfentanil	0.000025
ortho-Fluoroacrylfentanyl	0.000025
ortho-Fluorobutyrylfentanyl	0.000025
ortho-Fluorofentanyl	0.000025
ortho-Fluoroisobutyrylfentanyl	0.000025
para-Fluoroacrylfentanyl	0.000025
para-Fluorobutyrylfentanyl	0.000025
para-Fluorofentanyl	0.000025
para-Fluoroisobutyrylfentanyl	0.000025
Phenylfentanyl	0.000025
Tetrahydrofuranfentanyl	0.000025
trans-3-Methylfentanyl	0.00001875
U-47700	0.000075
U-49900	0.00005

11.7.8 Calibrator and control solutions may be made in larger than 10 mL volumes, if necessary. The larger volume calibrator solutions need to be prepared in calibrated glassware.

11.7.9 See Toxicology Quality Guidelines.

11.8 References

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12 BARBITURATE AND ACID DRUG QUANTITATION AND CONFIRMATION BY GC AND GC-MS

12.1 Summary

Biological samples are made acidic with monosodium phosphate buffer (~pH 5) and extracted with a mixture of hexane and ethyl acetate. The extracts may be methylated to improve chromatographic performance and injected into a GC equipped with an NPD or FID for quantitation or a GC equipped with an MSD for simultaneous quantitation/confirmation. All drugs must be confirmed by GC-MS. Drug targets may be analyzed in different combinations or separately as needed.

12.2 Specimen Requirements

1 mL of fluid(s) or 1 g of tissue(s) or comparable amounts of fluid or tissue dilutions/homogenates.

12.3 Reagents and Standards

- Butalbital, 1 mg/mL
- Pentobarbital, 1 mg/mL
- Secobarbital, 1 mg/mL
- Phenobarbital, 1 mg/mL
- Amobarbital, 1 mg/mL
- Butabarbital, 1 mg/mL
- Cyclopentobarbital (cyclopal), internal standard
- MethElute™ or other TMPAH derivatization agent (trimethylphenylammonium hydroxide), stored at room temperature
- Monosodium phosphate
- Hexane
- Ethyl acetate

12.4 Solutions, Internal Standards, Calibrators and Controls

12.4.1 1.5 M monosodium phosphate buffer: Add 103.4 grams of monosodium phosphate (NaH_2PO_4) to a 500 mL volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.

12.4.2 Hexane:ethyl acetate (50:50): Mix 500 mL hexane with 500 mL ethyl acetate. Store at room temperature for up to two years.

12.4.3 Internal Standard: Weigh 20 mg of cyclopal free acid, transfer to a 10 mL volumetric flask and qs to volume with methanol for final concentration of 2 mg/mL. Dilute 100 µL of 2 mg/mL internal standard solution with 900 µL methanol. Add 50 µL of diluted internal standard to each sample. Alternatively, mix internal standard with extraction solvent.

12.4.4 Extraction solvent for option 1 containing internal standard: Aliquot 2 mL of 2 mg/mL cyclopentobarbital (cyclopal) stock solution into a 1000 mL volumetric flask and qs to volume with extraction solvent (hexane:ethyl acetate) to yield 4 mg/L cyclopal in extraction solvent. Store at room temperature for up to two years.

12.4.5 Alternative internal standards (such as phensuximide, hexobarbital, and secobarbital) may be utilized provided they are not present in case samples.

12.4.6 Working Standard Solution A (0.1 mg/mL): Add 1.0 ml of each of the following 1 mg/mL stock solutions to a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable: butalbital and phenobarbital.

12.4.7 Working Standard Solution B (0.1 mg/mL): Add 1.0 ml of each of the following stock solutions to a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable: amobarbital, butabarbital, secobarbital, and pentobarbital.

12.4.8 Blood calibrators, standards, and controls preparation:

12.4.8.1 To prepare the following calibration curve, pipette the following volumes of working standard solution A into appropriately labeled 16 x 125 mm screw cap test tubes

- 30 mg/L Calibrator 300 µL of working standard solution A
- 20 mg/L Calibrator 200 µL of working standard solution A
- 10 mg/L Calibrator 100 µL of working standard solution A
- 5 mg/L Calibrator 50 µL of working standard solution A
- 2 mg/L Calibrator 20 µL of working standard solution A
- 1 mg/L Calibrator 10 µL of working standard solution A

- To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood.

- Add 1 mL blank blood to each tube.

12.4.8.2 Standard B contains rarely encountered drugs (amobarbital, butabarbital, secobarbital, and pentobarbital). If suspected or requested, run at least 1 standard containing working solution B for retention times. If any of the 4 drugs are present, a full calibration curve is required. These targets may be run separately or in any combination as necessary.

12.4.8.2.1 For routine analyses, pipette 100 µL of working Standard Solution B into a 16 x 125 mm labeled screw-cap test tube. Evaporate to dryness under nitrogen. Add 1 mL blank blood for a final concentration of 10 mg/L.

12.4.8.2.2 If a full calibration curve is required, pipette the following volumes of working standard solution B into appropriately labeled 16 x 125 mm screw cap test tubes

- 30 mg/L Calibrator 300 µL of working standard solution B
- 20 mg/L Calibrator 200 µL of working standard solution B
- 10 mg/L Calibrator 100 µL of working standard solution B
- 5 mg/L Calibrator 50 µL of working standard solution B
- 2 mg/L Calibrator 20 µL of working standard solution B
- 1 mg/L Calibrator 10 µL of working standard solution B

12.4.8.2.3 To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood.

12.4.8.2.4 Add 1 mL blank blood to each tube.

12.4.8.3 Standards A and B may be combined to quantitate all drugs simultaneously.

12.4.8.4 Controls

12.4.8.4.1 Negative control: Blood bank blood (or comparable) determined not to contain analytes of interest.

12.4.8.4.2 Positive control: Commercial whole blood control and/or an in-house control containing each analyte of interest from a different lot number or manufacturer than

standards, or prepared by a chemist different than the one performing the extraction.

12.5 Apparatus

12.5.1 Agilent GC-MS, manufacturer's software, compatible computer & printer, Agilent GC with NPD or FID, manufacturer's software, compatible computer & printer

12.5.2 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

12.5.3 Test tubes, glass, conical bottom

12.5.4 Centrifuge capable of 2,000 – 3,000 rpm

12.5.5 Vortex mixer

12.5.6 Evaporator/concentrator

12.5.7 GC autosampler vials and inserts

12.5.8 Test tube rotator

12.5.9 GC-NPD parameters.

12.5.9.1 Oven program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1.0 minute
- Ramp: 15°C/min
- Final Temp: 280°C
- Final Time: 4 minutes
- Run Time: 15 minutes

12.5.9.2 Inlet

- Mode: Splitless
- Temperature: 250°C
- Constant pressure: 16 psi
- Purge flow: 49.6 mL/min
- Total flow: 52.9 mL/min
- Injection volume: 1.0 µL

12.5.9.3 Detector

- Temperature: 290°C
- Hydrogen flow: 3.0. mL/min
- Air flow: 60 mL/min
- Mode: Constant column + makeup flow
- Combined flow: 20.0 mL/min
- Injection volume: 1.0 µL
- Makeup flow: On

12.5.9.4 Column: HP-1MS or HP-5MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm.

12.5.10 GC-FID parameters.

12.5.10.1 Oven program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1.0 minutes
- Ramp: 15°C/min
- Final Temp: 260°C
- Final Time: 1.5 minutes
- Run Time: 15 minutes

12.5.10.2 Inlet

- Mode: Splitless
- Temperature: 250°C
- Constant pressure: 25 psi
- Purge flow: 1.9 mL/min
- Total flow: 6.1 mL/min
- Injection volume: 1.0 µL

12.5.10.3 Detector

12.5.10.4 Column: HP-5MS or HP-1MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm.

12.5.11 GC-MS parameters.

12.5.11.1 Acquisition Mode: Scan (50 – 550 amu) or SIM

12.5.11.2 SIM ions:

butalbital	196, 195, 181
butabarbital	169, 184, 211
amobarbital	169, 184, 225
pentobarbital	169, 184, 225
secobarbital	196, 181, 237
phenobarbital	232, 117, 146
cyclopal	221, 196

12.5.11.3 Column: HP-5MS, 30 m x 0.25 mm x 0.25 µm

12.5.11.4 Detector Temperature: 280°C

12.5.11.5 Instrument conditions may be changed to permit improved performance.

12.5.11.5.1 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1 minutes
- Ramp: 15°C/min
- Final Temp: 280°C

- Final Time: 4 minutes
- Run Time: 15 minutes

12.5.11.5.2 Inlet

- Mode: Splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

12.6 Procedure

12.6.1 Label clean screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

12.6.2 Prepare calibrators and controls.

12.6.3 Pipette 1 mL of each case sample into appropriately labeled tubes.

12.6.4 Add 1 mL 1.5 M sodium phosphate buffer to each tube.

12.6.5 Add 3 mL extraction solvent (hexane:ethyl acetate) and internal standard to each tube. The extraction solvent may be premixed with internal standard.

12.6.6 Cap and rotate tubes for 30 minutes.

12.6.7 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer organic (upper) layer to clean conical bottom tubes. Discard lower layers.

12.6.8 Evaporate samples to dryness under nitrogen at 50-55°C.

12.6.9 Add 100 µL MethElute™ or TMPAH derivatization agent to each tube.

12.6.10 Transfer a small aliquot to appropriately labeled GC vials and inject 1-2 µl on GC-NPD, GC-FID or GC-MSD.

12.6.11 Save remainder of reconstituted samples for confirmation by GC-MSD (if not already confirmed).

12.7 Calculation

Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height (or area) ratios versus calibrator concentration.

12.8 Quality Control and Reporting

12.8.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

12.8.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

12.8.3 See Toxicology Quality Guidelines

12.9 References

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13 CHLORDIAZEPOXIDE QUANTITATION AND CONFIRMATION BY LCMSMS

13.1 Summary

Chlordiazepoxide is extracted from biological samples by adding sodium carbonate buffer and extracting with 1-chlorobutane. An aliquot of the extract is quantitated and confirmed by LCMSMS.

13.2 Specimen Requirements

1 mL blood, fluid or tissue homogenate.

13.3 Reagents and Standards

13.3.1 Drug targets and internal standards

Target	Internal Standard
Chlordiazepoxide	Diazepam-D_5

13.3.2 Sodium carbonate, certified ACS powder

13.3.3 1-chlorobutane, HPLC grade

13.3.4 Acetonitrile, Fisher Optima (or similar) grade or higher

13.3.5 Type I or LCMS grade water

13.3.6 Formic acid, eluent additive for LCMS

13.4 Solutions, Internal Standard, Calibrators and Controls

13.4.1 0.2 M Sodium carbonate: weigh out 10.6 g sodium carbonate, transfer to a 500 mL volumetric flask and qs to volume with dH_2O. Store at room temperature for up to 2 years.

13.4.2 Mobile Phase A (H_2O with 0.1% formic acid): add 1 mL of formic acid to 1 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

13.4.3 Mobile Phase B (Acetonitrile with 0.1% formic acid): add 1 mL of formic acid to 1 L of acetonitrile. Store at room temperature for up to one month.

13.4.4 Preparation of calibrators.

13.4.4.1 Working standard solution (0.1 mg/mL): Pipette 1.0 mL of the 1 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

13.4.4.2 Working standard solution (0.01 mg/mL): pipette 1.0 mL of the 0.1 mg/mL working standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

13.4.4.3 Working internal standard solution (0.1 mg/mL): Pipette 1.0 mL of the 1.0 mg/mL stock solution of deuterated standard into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

13.4.4.4 To prepare calibration curve, pipette the following volumes of the 0.1 mg/mL or 0.01 mg/mL working standard solution into appropriately labeled screw cap test tubes. To eliminate a

solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to obtain the final concentrations listed below.

Amount of 0.1 mg/mL stock solution (µL)	Amount of 0.01 mg/mL stock solution (µL)	Final concentration of chlordiazepoxide (mg/L)
100		10.0
80		8.0
60		6.0
50		5.0
40		4.0
20	200	2.0
10	100	1.0
-	50	0.5
	25	TC (0.25)

13.4.5 Controls

13.4.5.1 Chlordiazepoxide Control. Controls may be from an external source or prepared in-house using drugs from different manufacturers or lot numbers.

Note: Due to the quadratic nature of chlordiazepoxide, at least three controls, at low, medium and high concentrations, must be run across the concentration range in every batch. If the high calibrator is 10 mg/L, a high control must be run within 8-10 mg/L.

13.4.5.2 Negative control. Blood bank blood or equivalent determined not to contain chlordiazepoxide.

13.5 Apparatus

13.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

13.5.2 Test tubes, conical bottom

13.5.3 Centrifuge capable of 2000-3000 rpm

13.5.4 Evaporator/concentrator

13.5.5 Vortex mixer

13.5.6 GC autosampler vials with inserts

13.5.7 Typical LCMSMS parameters.

13.5.7.1 LC Parameters:

- Column: Poroshell 120 EC-C18, 2.1x75 mm, 2.7 µm particle size
- Column Thermostat: 30°C
- Mobile Phase A: H_2O with 0.1% formic acid
- Mobile Phase B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.50 mL/min
- Injection vol.: 1 µL with a minimum 20 second needle wash
- Stop Time: 5 min
- Post Run Time: Minimum 1.5 min

- Gradient:

Time (minutes)	%A	%B
0.00	80	20
4.00	5	95
4.50	5	95
5.00	80	20

13.5.7.2 Typical MS-MS parameters.

- MSD Parameters:

Ionization: ESI
Polarity: Positive
Gas temp: 350°C
Drying Gas: 10.0 L/min
Nebulizer press: 50 psi
Capillary: 4000 V
Delta EMV: 400 V

- MRM Parameters

Time Segment Number	Time Segment (minutes)	Diverter Valve
TS1	0.0-0.8	Waste
TS2	0.8-2.0	Chlordiazepoxide
TS3	2.0-3.5	Diazepam-D_5
TS4	3.5-5.0	Waste

- Transition Ions

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)
Diazepam-D5	290.1	198.1	150	7	36
		154			28
Chlordiazepoxide	300.1	**227**	105	7	22
		89.1			74

13.6 Procedure

13.6.1 Label clean screw cap tubes appropriately with calibrators, controls and case sample IDs.

13.6.2 Prepare calibrators and controls.

13.6.3 Add 1.0 mL case specimens to the appropriately labeled tubes.

13.6.4 Add 30 µL of the 0.1 mg/mL internal standard working solution to each tube and vortex.

13.6.5 Add 1 mL sodium carbonate and 6 mL 1-chlorobutane to each tube.

13.6.6 Cap and rotate tubes for 30 minutes.

13.6.7 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer organic (upper) layer to appropriately labeled tubes.

13.6.8 Evaporate samples to dryness at approximately 50°C under nitrogen.

13.6.9 Reconstitute samples in 1.0 mL methanol. Transfer to GC autosampler vials with inserts for LCMSMS analysis.

13.7 Quality Control and Reporting

13.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

13.7.2 The upper limit of quantitation (ULOQ) for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

13.7.3 The calibration model for chlordiazepoxide is weighted (1/x) quadratic. Samples with a concentration greater than 8 mg/L for chlordiazepoxide must be repeated if the high positive control is outside of acceptable limits.

13.7.4 When a target concentration is above the ULOQ, 1.0 mL of case sample shall be diluted with no more than 19.0 mL of blank matrix for a total dilution volume of 20.0 mL. Alternatively, 0.05 mL of case sample may be used for a dilution of 1/20. If less than 0.05 mL of sample is used for analysis, only qualitative results may be reported.

13.7.5 Threshold Control: The TC is spiked at 0.25 mg/L. The validated LOD for chlordiazepoxide is 0.0025 mg/L.

13.7.6 Extracted samples are stable for twenty-four hours.

13.7.7 See Toxicology Quality Guidelines.

13.8 References

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14 CARISOPRODOL AND MEPROBAMATE QUANTITATION AND CONFIRMATION BY GC AND GC-MS

14.1 Summary

Biological samples are buffered with phosphate buffer (pH 7) and extracted with a mixture of hexane and ethyl acetate. The extract is washed with hexane and reconstituted with toluene/hexane/isoamyl alcohol or n-chlorobutane. An aliquot is injected into a GC equipped with an FID detector for quantitation of carisoprodol and meprobamate. The aliquot can be subsequently injected into a GC-MS for confirmation, if necessary.

14.2 Specimen Requirements

200 µL biological fluid or comparable amount of tissue dilutions/homogenates.

14.3 Reagents and Standards

- Carisoprodol
- Meprobamate
- Cyclopal (cyclopentobarbital) or methaqualone used as internal standards
- Disodium phosphate (Na_2HPO_4)
- Monosodium phosphate (NaH_2PO_4)
- Hexanes
- Isoamyl alcohol
- Methanol
- Toluene
- Ethyl acetate
- Acetonitrile
- N-chlorobutane
- MethElute™ (TMPAH, trimethylphenylammonium hydroxide), stored at room temperature

14.4 Solutions, Internal Standard, Calibrators and Controls

14.4.1 0.1 M disodium phosphate: Weigh 14.19 g of disodium phosphate, transfer to a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.

14.4.2 0.1 M monosodium phosphate: Weigh 13.79 g monosodium phosphate and transfer to a 1 L volumetric flask. qs to volume with dH_2O. Store at room temperature for up to two years.

14.4.3 0.1 M sodium phosphate buffer (pH 7.0): Mix 500 mL 0.1 M disodium phosphate with approximately 250 mL 0.1 M monosodium phosphate. Adjust pH to 7.0 ± 0.1 with 0.1 M monosodium phosphate (lowers pH) or 0.1 M disodium phosphate (raises pH). Store at room temperature for up to two years.

14.4.4 Toluene:Hexane:Isoamyl Alcohol (THIA) (78:20:2, v:v:v): Mix 78 mL toluene, 20 mL hexane and 2 mL isoamyl alcohol. Store at room temperature for up to two years.

14.4.5 Hexane/ethyl acetate (50:50, v:v) extraction solvent: Mix 50 mL hexane with 50 mL ethyl acetate. Store at room temperature for up to two years.

14.4.6 Methanol/ dH_2O (50:50, v:v): Mix 50 mL methanol with 50 mL dH_2O. Store at room temperature for up to two years.

14.4.7 Drug stock solutions:

14.4.7.1 If 1 mg/mL commercially prepared stock solutions are not available, prepare 1 mg/mL solutions from powders. Weigh 10 mg of the free drug, transfer to a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

14.4.7.2 Working Standard (0.1 mg/mL): Pipette 100 µL of 1.0 mg/mL stock carisoprodol solution, 100 µL of 1.0 mg/mL stock meprobamate solution into a 1 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable. Alternate volumes may be used to prepare the working standard, provided the final working standard concentrations remains 0.1 mg/mL.

14.4.7.3 Internal standard solution (methylated cyclopal (cyclopentobarbital)): To 1 mL of 2 mg/mL cyclopal (in methanol), add 1 mL MethElute™. Cap and heat at 60°C for 2 hours. Let sit at room temperature overnight. Evaporate under nitrogen. Reconstitute with 10 mL methanol/ dH_2O (50:50, v:v).

14.4.7.4 Methaqualone working stock internal standard. Dilute stock 1 mg/mL 1:10 to prepare 0.1 mg/mL working stock. Methaqualone does not need to be derivatized with TMSH.

14.4.8 Blood calibrators, standards, and controls preparation:

14.4.8.1 To prepare the calibration curve, pipette the following volumes of 1 mg/mL carisoprodol and meprobamate stock solutions into appropriately labeled 13 x 100 mm screw cap test tubes (Note: 100 mg/L Calibrator may overload column, this calibrator may be removed).
Note: For options with both solutions listed, choose either the stock solutions or the working standard solution.

Calibrator Concentration (mg/L)	Volume of 1.0 mg/mL of each stock solution (µL)	Volume of 0.1 mg/mL working standard solution (µL)
100	300	
50	150	
20	60	
10 (see note)	30	300
5 (see note)	15	150
2		60

14.4.8.2 To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood.

14.4.8.3 Add 3 mL blank blood to each tube. Store at 2-8° C for up to one year. Allow standards to equilibrate for 24 hours prior to use.

14.4.8.4 Controls

14.4.8.4.1 Negative control. Blood bank blood (or comparable) determined not to contain carisoprodol or meprobamate

14.4.8.4.2 Positive control: Commercial whole blood control and/or an in-house control containing each analyte of interest from a different lot number or manufacturer than standards, or prepared by a chemist different than the one performing the extraction.

14.5 Apparatus

14.5.1 Agilent GC-MS, manufacturer's software (for confirmation, if necessary)

14.5.2 Agilent GC equipped with Flame Ionization Detector, manufacturer's software, compatible computer & printer

14.5.3 Test tubes round bottom, screw cap tubes, borosilicate glass with Teflon caps

14.5.4 Test tubes, glass centrifuge, conical bottom

14.5.5 Centrifuge capable of 2,000 – 3,000 rpm

14.5.6 Vortex mixer

14.5.7 Evaporator/concentrator

14.5.8 GC autosampler vials and inserts

14.5.9 Test tube rotator

14.5.10 GC-FID parameters.

14.5.10.1 Oven program.

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1.0 minutes
- Ramp: 20°C/min
- Final Temp: 260°C
- Final Time: 1.5 minutes
- Run Time: 15 minutes

14.5.10.2 Inlet.

- Mode: Splitless
- Temperature: 250°C
- Constant pressure: 25 psi
- Purge flow: 1.9 mL/min
- Total flow: 6.1 mL/min
- Injection volume: 1.0 µL

14.5.10.3 Detector.

- Temperature: 290°C
- Hydrogen flow: 50 mL/min
- Air flow: 450 mL/min
- Mode: Constant makeup flow
- Makeup flow: 45 mL/min

14.5.10.4 Column: HP-5MS or HP-1MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm.

14.5.11 GC-MS parameters.

14.5.11.1 Acquisition Mode: Scan (50 – 550 amu)

14.5.11.2 Column: HP-5MS or HP-1MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm

14.5.11.3 Detector Temperature: 280°C

14.5.11.4 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1 minutes
- Ramp: 10°C/min
- Final Temp: 290°C
- Final Time: 9 minutes
- Run Time: 28 minutes

14.5.11.4.1 Inlet

- Mode: Splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

14.6 Procedure

14.6.1 Label clean screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

14.6.2 Prepare calibrators and controls

14.6.3 Pipette 200 µL of each calibrator, control, negative and case samples into appropriately labeled tubes.

14.6.4 Add 30 µL methylated cyclopal or 30 µL methaqualone internal standard to each tube.

14.6.5 Add 0.5 mL sodium phosphate buffer (pH 7) to each tube.

14.6.6 Add 3 mL extract solvent (hexane/ethyl acetate) to each tube.

14.6.7 Cap and rotate tubes for 15 minutes.

14.6.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer organic upper layer to clean conical bottom centrifuge tubes. Discard lower layers.

14.6.9 Evaporate samples to dryness under nitrogen at 50-60°C.

14.6.10 Reconstitute samples with 0.2 mL acetonitrile. Vortex briefly.

14.6.11 Add 1 mL hexane to each tube. Vortex each sample for 30 seconds.

14.6.12 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

14.6.13 Aspirate (and discard) upper (hexane) layer.

14.6.14 Evaporate lower (acetonitrile) layer under nitrogen at 50-60°C.

14.6.15 Reconstitute samples with 75 µL of toluene/hexane/isoamyl alcohol solvent or n-chlorobutane and vortex briefly. The reconstitution volume may be modified as necessary to increase/decrease instrument response (e.g., use 150 µL solvent to reconstitute highest calibrator to prevent detector saturation).

14.6.16 Transfer samples to appropriately labeled GC vials and inject 1-2 µl on GC-FID.

14.6.17 Save remainder of reconstituted samples for confirmation by GC-MSD (if not already confirmed).

14.7 Calculation

Calculate the concentrations by interpolation of a linear plot of the response curve based on peak height (or area) ratios versus calibrator concentration.

14.8 Quality Control and Reporting

14.8.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte. The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

14.8.2 See Toxicology Quality Guidelines

14.9 References

14.9.1 In-house development, T England.

15 GHB, GBL, and 1,4-BUTANEDIOL QUANTITATION AND CONFIRMATION BY LCMSMS

15.1 Summary

Gamma-hydroxybutyrate (GHB), gamma-butyrolactone (GBL), and 1,4-Butanediol are extracted from biological samples using an acidic methanol precipitation. An aliquot of the extract is quantitated and confirmed by LCMSMS. GHB and 1,4-butanediol are quantitative analyses and GBL is qualitative. Drug targets may be analyzed in different combinations or separately as needed.

15.2 Specimen Requirements

0.2 mL of blood, fluid or tissue homogenate.

Note: For quantitative analysis of urine, matrix matched calibrators and controls shall be prepared.

15.3 Reagents and Standards

- Drug targets and associated internal standard

Targets	Internal Standards
GHB	GHB-D_6
GBL	
1,4-Butanediol	

- Formic acid, eluent additive ~98%
- Type I or LCMS grade water
- Methanol, Optima (or similar) grade or higher

15.4 Solutions, Internal Standard, Calibrators and Controls

15.4.1 Mobile Phase A (H_2O with 0.1% formic acid): add 1.0 mL of formic acid to 1 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

15.4.2 Mobile Phase B (Methanol with 0.1% formic acid): add 1.0 mL of formic acid to 1 L of methanol (Optima (or similar) grade or higher). Store at room temperature for up to one month.

15.4.3 1,4-Butanediol Stock Solution (10.17 mg/mL): Pipette 10.0 µL of 1,4-butanediol standard into 990 µL of methanol in an autosampler vial. For traceability purposes, a calibrated mechanical pipette is required for the delivery of the above volumes.

15.4.4 Working Standard Solution (0.2 mg/mL): Pipette 1.223 mL of the 1.0 mg/mL GHB sodium salt stock solution*, 98.25 µL of the 10.17 mg/mL 1,4-butanediol stock solution, 100 µL of the 10 mg/mL GBL stock solution, and 5 µL of formic acid into a 5.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

*The volume delivered accounts for the salt form of the GHB solution. The larger volume requires the opening of two 1 mL ampules.

15.4.5 Working Internal Standard (0.2 mg/mL) Pipette 2.42 mL of 1.0 mg/mL stock solution and 10 µL of formic acid into a 10.0 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

15.4.6 Controls

15.4.6.1 Negative control blood: blank blood or blank urine determined not to contain target compounds.

15.4.6.2 Due to the quadratic nature of many of the targets, at least three controls, at low, medium, and high concentration, shall be run across the concentration range with every batch. Suggested control levels are listed in the procedure below.

15.4.6.3 Commercial whole blood control (UTAK or other commercial vendor) or methanolic statewide controls (prepared by the research analyst or designee).

15.4.6.4 Threshold Control: TC is to be spiked at 15 mg/L but may be adjusted based upon validated LOD.

15.5 Apparatus

15.5.1 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

15.5.2 Centrifuge capable of 2,000-3,000 rpm

15.5.3 Vortex mixer

15.5.4 GC autosampler vials and inserts

15.5.5 Typical LCMSMS parameters.

15.5.5.1 LC Parameters:

- Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm particle
- Column Thermostat: 30.0 °C
- Mobile Phase A: H_2O with 0.1% formic acid
- Mobile Phase B: Methanol with 0.1% formic acid
- Starting Flow Rate: 0.2 mL/min
- Injection Volume: 5 µL with a minimum 20 second needle wash
- Stop Time: 8.00 minutes
- Post Time: Minimum 3.00 minutes
- Gradient:

Time (minutes)	Solvent A (%)	Solvent B (%)	Flow Rate (mL/min)
0.00	98.0	2.0	0.2
5.00	94.0	6.0	0.4
6.50	5.0	95.0	0.4
7.50	5.0	95.0	0.4
8.00	98.0	2.0	0.2

15.5.5.2 Typical MSMS parameters

- MSD Parameters:

Ionization: ESI
Polarity: Positive
Gas Temperature: 350 °C
Nebulizer Pressure: 40 psi
Capillary: 4000 V
Drying Gas: 10 L/min

- Transition Ions: (Ions in bold are the quantitation ion transition)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
GHB	105.1	**87.0**	33	1	7
		45.1		17	
GHB-D_6	111.1	**93.0**	52	1	7
		49.1		17	
GBL	87.1	**45.1**	56	13	7
		43.1		9	
1,4-Butanediol	91.1	**73.1**	25	1	7
		55.1		9	

15.6 Procedure

15.6.1 Label appropriate clean screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs.

15.6.2 Prepare calibrators and controls. Due to the volatility of compounds, calibrators and controls shall not be dried down under any circumstances (i.e., using nitrogen or heat) prior to the addition of blank blood.

Calibrators	
Amount of 0.2 mg/mL Standard Solution A (µL)	Final Concentration (mg/L)
30	30
60	60
100	100
140	140
180	180
220	220
260	260
300	300

Controls	
Amount of 0.2 mg/mL Standard Solution A (µL)	Final Concentration (mg/L)
80	80
150*	150
250	250

*This control level will be used for control charting.

15.6.3 Pipette 0.2 mL of blank blood, calibrators, controls and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes.

15.6.4 Add 100 µL of 0.2 mg/L working internal standard solution to each tube.

15.6.5 Add appropriate amount of 0.1% formic acid in methanol to each tube for a total volume of 1.0 mL for calibrators and controls. Add 900 µL of 0.1% formic acid in methanol to case samples.

Final Concentration (mg/L)	Amount of Acidic Methanol (µL)
Calibrators	
30	870
60	840
100	800
140	760
180	720

220	680
260	640
300	600
Controls	
80	820
150	750
250	650

15.6.6 Vortex for approximately 30 seconds. Ensure that the blood in the bottom of the tube mixes thoroughly with the methanol.

15.6.7 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation.

15.6.8 Transfer 10 µL of topmost layer into test tubes and dilute with 100 µL of 0.1% formic acid in water.

15.6.9 Vortex briefly.

15.6.10 Transfer to autosampler vials.

15.7 Quality Control and Reporting

15.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

15.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

15.7.3 Negative QCs and matrix blanks may contain GHB due to naturally occurring levels. Refer to Negative QC criteria in ¶ 2.4 for acceptance criteria.

15.7.4 Urine can only be reported quantitatively if a matrix-matched calibration is utilized.

15.7.5 Dilution

15.7.5.1 All blood samples may be diluted up to 1/20 dilution (0.2 mL sample diluted with 3.8 mL of blank matrix).

15.7.5.2 Urine samples may be diluted up to 1/20 dilution (0.2 mL sample diluted with 3.8 mL of blank matrix) for GHB and GBL. 1,4-butanediol may only be diluted up to 1/5 dilution (0.2 mL sample diluted with 0.8 mL blank matrix).

15.7.6 The calibration models for the targets:

Blood Calibration		
Target	Linear/Quadratic	Weighting
GHB	Linear	Weighted (1/x)
1,4-Butanediol	Quadratic	Weighted (1/x)
GBL	Linear	Weighted (1/x)

Urine Calibration		
Target	Linear/Quadratic	Weighting
GHB	Linear	Weighted (1/x)
1,4-Butanediol	Linear	Weighted (1/x)
GBL	Quadratic	Weighted (1/x)

15.7.7 Extracted samples are stable for 24 hours after reconstitution.

15.7.8 Threshold control: TC is prepared at 15 mg/L and the validated LODs are:

Limit of Detection	
Blood Calibration	Concentration (mg/L)
GHB	10
1,4-Butanediol	10
GBL	15
Urine Calibration	Concentration (mg/L)
GHB	10
1,4-Butanediol	10
GBL	10

15.8 References

15.8.1 Crapps, P. and Wagner, R. GHB, GBL, and 1,4-butanediol quantitation and confirmation by LCMSMS validation. Virginia Department of Forensic Science. **2019**.

15.8.2 Crapps, P. and Wagner, R. GHB, GBL, and 1,4-butanediol quantitation and confirmation by LCMSMS method development. Virginia Department of Forensic Science. **2018**.

15.8.3 Johansen, S.S., Windberg, C.N. Simultaneous determination of beta-hydroxybutyrate (GHB) and its analogues (GBL, 1,4-BD, GVL) in whole blood by liquid chromatography coupled to tandem mass spectrometry. *Journal of Analytical Toxicology.* **2011**, 35, 8-14.

15.8.4 Busardo, F.P., Jones, A.W. GHB pharmacology and toxicology: acute intoxication, concentrations in blood and urine in forensic cases and treatment of the withdrawal syndrome. *Current Neuropharmacology.* **2015**, 13, 47-70.

15.8.5 Scientific Working Group for Forensic Toxicology (SWGTOX) Standard practices for method validation in forensic toxicology. *JAT* **2013**, 37, 452-474.

16 VALPROIC ACID QUANTITATION AND CONFIRMATION BY GC-MS

16.1 Summary

Biological samples are slightly acidified with monosodium phosphate buffer (~pH 5) and extracted with toluene/hexane/isoamyl alcohol (THIA) or chloroform. An aliquot is injected into a GC-MS for quantitation and confirmation.

16.2 Specimen Requirements

1 mL biological fluid or comparable amount of tissue dilutions/homogenates.

16.3 Reagents and Standards

- Phensuximide
- Valproic acid
- Monosodium phosphate (NaH_2PO_4)•H_20
- Toluene
- Hexane
- Isoamyl Alcohol
- Methanol
- Chloroform

16.4 Solutions, Internal Standard, Calibrators and Controls

16.4.1 1.5 M monosodium phosphate: Weigh 103.4 g monosodium phosphate (NaH_2PO_4•H_20) and transfer to a 500 mL volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.

16.4.2 Toluene:Hexane:Isoamyl Alcohol (THIA) (78:20:2, v:v:v): Mix 78 mL toluene, 20 mL hexane and 2 mL isoamyl alcohol. Store at room temperature for up to 2 years.

16.4.3 1 mg/mL valproic acid stock solution. Weigh 10 mg valproic acid, transfer to a 10 mL volumetric flask and qs to volume with methanol. Alternatively, 1 mg/mL reference standards from approved manufacturers/vendors may be used.

16.4.4 1 mg/mL phensuximide internal standard solution. Weigh 10 mg phensuximide, transfer to a 10 mL volumetric flask and qs to volume with methanol.

16.4.5 Calibrators and controls

16.4.5.1 To prepare the calibration curve, pipette the following volumes of 1 mg/mL valproic acid into appropriately labeled 13 x 100 mm screw cap test tubes. To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to achieve final concentration.

- 400 mg/L Calibrator — 400 µl valproic acid (1 mg/mL)
- 200 mg/L Calibrator — 200 µl valproic acid (1 mg/mL)
- 100 mg/L Calibrator — 100 µL valproic acid (1 mg/mL)
- 50 mg/L Calibrator — 50 µL valproic acid (1 mg/mL)
- 25 mg/L Calibrator — 25 µL valproic acid (1 mg/mL)

16.4.5.2 Negative control. Blood bank blood (or comparable) determined not to contain valproic acid or phensuximide.

16.4.5.3 Positive control. In-house control containing valproic acid spiked at concentration similar to case specimens.

16.5 Apparatus

16.5.1 Agilent GC-MS, manufacturer's software

16.5.2 Test tubes, glass centrifuge, conical bottom

16.5.3 Centrifuge capable of 2000 – 3000 rpm

16.5.4 Vortex mixer

16.5.5 GC autosampler vials and inserts

16.5.6 GC-MS parameters.

16.5.6.1 Acquisition Mode: SIM

16.5.6.2 SIM ions: valproic acid 73, 102, 115
phensuximide 104, 189, 78

16.5.6.3 Column: HP-5MS or HP-1MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm

16.5.6.4 Detector Temperature: 280°C

16.5.6.5 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 50°C
- Initial time: 1 minutes
- Ramp: 15°C/min
- Final Temp: 280°C
- Final Time: 1 minute
- Run Time: 15 minutes

Inlet

- Mode: Splitless/Split (suggested split ratio 75:1)
- Temperature: 260°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

16.6 Procedure

16.6.1 Label clean screw cap tubes accordingly, negative and positive control and case sample IDs.

16.6.2 Prepare calibrators and controls.

16.6.3 Pipette 1 mL of each case sample into appropriately labeled tubes. Note: since this procedure is used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case. One will serve as a screen and the second as a confirmation.

16.6.4 Add 10 µL of 1 mg/mL phensuximide internal standard to each tube for a final concentration of 10 mg/L.

16.6.5 Add 1 mL monosodium phosphate buffer to each tube and vortex briefly.

16.6.6 Add 1 mL THIA or chloroform to each tube.

16.6.7 Cap tubes and rotate for 30 minutes.

16.6.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. If using THIA, remove a portion of upper organic layer to appropriately labeled GC vials. If using chloroform, the organic layer will be the bottom layer. Therefore when using chloroform, remove the upper aqueous layer, break any plug, and transfer a portion of the bottom organic layer to appropriately labeled GC vials. Note: samples may be diluted to prevent saturation of the detector.

16.6.9 Inject 1 µl of each sample onto GC-MS.

16.7 Calculation

Drug concentrations are calculated by linear regression analysis using the instrument software based on peak height (or area) ratios versus calibrator concentration.

16.8 Quality Control and Reporting

16.8.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte. The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

16.8.2 If the same specimen is analyzed in duplicate (for screening and confirmation) and both results are quantitative, the results should be averaged prior to reporting.

16.8.3 See Toxicology Quality Guidelines

16.9 References

16.9.1 I. Sunshine. Methodology for Analytical Toxicology. CRC Press, 1982.

16.9.2 Carol O'Neal, Amy Jango, Lucy Sale and Dwight Flammia, in-house development.

17 RETAINED FOR FUTURE USE

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18 QUETIAPINE QUANTITATION AND CONFIRMATION BY GC-MS

18.1 Summary

Quetiapine is extracted from biological fluids and tissues using solid phase or liquid-liquid extraction followed by derivatization with BSTFA and instrumental analysis by GC-MS using selected ion monitoring.

18.2 Specimen Requirements

2 mL whole blood, urine, bile, gastric contents, other fluids or tissue homogenates.

18.3 Reagents and Standards

- Ammonium hydroxide
- Glacial Acetic Acid
- Potassium Hydroxide
- Potassium Phosphate
- Ethyl Acetate
- Methanol
- Acetonitrile
- Dichloromethane
- Isopropyl alcohol
- Hexane
- Toluene
- Isoamyl alcohol
- Potassium or sodium phosphate buffer solution concentrate (1 M, pH 6.0, e.g., Fisher)
- Sodium phosphate, monobasic ($NaH_2PO_4 \bullet H_20$)
- Sodium phosphate, dibasic (Na_2HPO_4)
- Sodium tetraborate decahydrate
- Sodium hydroxide
- Sodium hydrogen carbonate
- Potassium carbonate
- Sulfuric acid
- BSTFA containing 1% TMCS, stored at 2-8°C
- Quetiapine, 1 mg/mL
- Methapyrilene, 1 mg/mL

18.4 Solutions, Internal Standards, Calibrators and Controls

18.4.1 Solutions for UCT CleanScreen® SPE Extraction

18.4.1.1 When using UCT CleanScreen® SPE Extraction columns, either sodium or potassium phosphate buffer may be used. However, the same buffer (sodium or potassium) must be used throughout the duration of the procedure.

18.4.1.1.1 0.1 M Potassium Phosphate Buffer, pH 6.0. Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M potassium hydroxide while stirring and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years. (Note: potential exothermic reaction, exercise caution when making this solution)

18.4.1.1.2 5 M Potassium hydroxide: Add 28.05 g potassium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be

prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

OR

18.4.1.1.3 0.1 M Sodium Phosphate Buffer, pH 6.0. Weigh 1.70g Na_2HPO_4 and 12.14g $NaH_2PO_4 \cdot H_2O$ and transfer to a 1 L volumetric flask containing approximately 800 mL dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M sodium hydroxide and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years.

18.4.1.1.4 5 M Sodium Hydroxide: Add 20.0 g of sodium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

18.4.1.2 1.0 M Acetic Acid. Add 100-200 mL dH2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH2O and qs to volume with dH2O. Store at room temperature for up to two years.

18.4.1.3 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2). Mix 78 mL dichloromethane with 20 mL isopropanol. Mix well. In hood, add 2 mL ammonium hydroxide. Mix gently. PREPARE SOLUTION FRESH DAILY!

18.4.2 Solutions for liquid/liquid base extraction

18.4.2.1 Saturated borate buffer solution. Add sodium tetraborate decahydrate to dH_2O until no more dissolves after shaking vigorously. Store at room temperature for up to two years.

18.4.2.2 Toluene:Hexane:Isoamyl Alcohol (THIA) extraction solvent (78:20:2), v:v:v: Mix toluene (780 mL), hexane (200 mL), and isoamyl alcohol (20 mL). Store at room temperature for up to two years.

18.4.2.3 Sodium Hydrogen Carbonate/Potassium Carbonate (dry 3:2 w/w) Mix 300 g $NaHCO_3$ with 200 g K_2CO_3. Store at room temperature for up to two years.

18.4.2.4 0.5 N Sulfuric Acid: Add 13.8 mL concentrated sulfuric acid to a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to two years.

18.4.3 Internal Standard

Working internal standard solution (0.01 mg/mL methapyrilene): Pipette 100 µL of the 1 mg/mL methapyrilene stock solution into a 10 mL volumetric flask and qs to volume with methanol.

18.4.4 Calibrators

18.4.4.1 Working standard solution (0.02 mg/mL quetiapine): Pipette 100 µL of the 1 mg/mL quetiapine standard stock solution into a 5 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

18.4.4.2 To prepare the calibration curve, pipette the following volumes of working standard solution into 16 x 125 mm tubes. To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood. Add 2 mL blank blood to each tube to obtain the final concentrations listed below:

Final Concentration of quetiapine (mg/L)	µL of quetiapine solution (0.02 mg/mL)
0.10	10
0.20	20
0.40	40
1.0	100
2.0	200
5.0	500

18.4.5 Controls

18.4.5.1 Control may be from an external source or prepared in-house using drugs from different manufacturers, lot numbers or prepared by a chemist different than the individual performing the extraction.

18.4.5.2 Negative control. Blood bank blood or equivalent determined not to contain quetiapine.

18.5 Apparatus

18.5.1 Agilent GC-MS, manufacturer's software, compatible computer & printer

18.5.2 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

18.5.3 Test tubes, round bottom tubes, borosilicate glass

18.5.4 Test tubes, glass centrifuge, conical bottom

18.5.5 Test tubes, round bottom, screw cap tubes, borosilicate glass

18.5.6 Centrifuge capable of 2,000 – 3,000 rpm

18.5.7 Cleanscreen® Extraction Cartridges (ZSDAU020) from United Chemical Technologies (200 mg columns)

18.5.8 Solid phase extraction manifold

18.5.9 Vortex mixer

18.5.10 Evaporator/concentrator

18.5.11 GC autosampler vials and inserts

18.5.12 Test tube rotator

18.5.13 GC-MS parameters.

18.5.13.1 Acquisition Mode: SIM

Quetiapine: 210, 239, 322

Methapyrilene: 58, 97

18.5.13.2 Column: HP-5MS or HP-1MS (or equivalent columns from alternative manufacturers) 30 m x 0.25 mm x 0.25 µm

18.5.13.3 Detector Temperature: 280°C

18.5.13.4 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 140°C
- Initial time: 0 minutes
- Ramp: 30°C/min
- Final Temp: 300°C
- Final Time: 7 minutes
- Run Time: 12.5 minutes

18.5.13.5 Inlet

- Mode: Pulsed splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

18.6 Procedure

18.6.1 Extraction: CleanScreen SPE Columns.

18.6.1.1 Label clean screw cap tubes accordingly.

18.6.1.2 Pipette 2 mL of blank blood, calibrators, controls and case sample bloods, fluids or tissue homogenates into appropriately labeled tubes. Note: since this procedure is used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case. One will serve as a screen and the second as a confirmation (unless quetiapine has already been confirmed using the base screen procedure).

18.6.1.3 Pipette 100 µL internal standard working solution into all tubes for a final concentration of 0.5 mg/L methapyrilene. Vortex briefly.

18.6.1.4 Add 4 mL deionized water to each tube. Mix, vortex briefly and let stand for 5 minutes.

18.6.1.5 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer supernatant to clean tubes and discard the tube with the remaining pellet.

18.6.1.6 Add 2 mL of pH 6 phosphate buffer, mix and vortex. As necessary adjust the pH to 5.5 to 6.5 with additional 0.1 M phosphate buffer. Note: larger volumes of water or phosphate buffer may be used to further dilute some specimens prior to SPE analysis.

18.6.1.7 Solid phase extraction. Place SPE cartridges in the extraction manifold. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

18.6.1.7.1 Add 3 mL hexane to each column and aspirate.

18.6.1.7.2 Add 3 mL methanol to each column and aspirate.

18.6.1.7.3 Add 3 mL dH_2O and aspirate.

18.6.1.7.4 Add 1 mL of 0.1 M pH 6.0 phosphate buffer and aspirate.

18.6.1.7.5 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges under vacuum/pressure at approximately 1-2 mL/ minute flow.

18.6.1.7.6 Add 3 mL dH_2O and aspirate at ≤ 3inches of mercury or at a low positive pressure (< ~10 psi).

18.6.1.7.7 Repeat the dH_2O wash.

18.6.1.7.8 Wash with 2.0 mL 1.0 M acetic acid and aspirate.

18.6.1.7.9 Add 3 mL methanol and aspirate under full vacuum/pressure for at least 5 minutes.

18.6.1.7.10 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

18.6.1.7.11 Elute quetiapine by adding 3 mL of freshly prepared methylene chloride/isopropanol/ammonium hydroxide solution to each column. Collect eluate in conical test tubes by column aspiration or gravity drain.

18.6.1.8 Evaporate eluates to dryness at approximately 50-60°C under nitrogen.

18.6.1.9 Add 50 µL BSTFA and 50 µL ethyl acetate to the extracts. Heat at 90°C for 30 minutes.

18.6.1.10 Transfer to autosampler vials for analysis by GC-MS.

18.6.2 Extraction: Basic LLE

18.6.2.1 Label clean screw cap tubes accordingly.

18.6.2.2 Pipette 2 mL of blank blood, calibrators, controls and case sample bloods, fluids or tissue homogenates into appropriately labeled tubes. Note: since this procedure is used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case. One will serve as a screen and the second as a confirmation (unless quetiapine has already been confirmed using the base screen procedure).

18.6.2.3 Pipette 100 µL internal standard working solution into all tubes for a final concentration of 0.5 mg/L methapyrilene. Vortex briefly.

18.6.2.4 Add 2 mL of saturated borate buffer to each tube.

18.6.2.5 Add 4 mL of toluene/hexane/isoamyl alcohol extraction solvent to each tube.

18.6.2.6 Rotate tubes for 20 minutes.

18.6.2.7 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

18.6.2.8 Transfer the top (organic) layer to appropriately labeled screw-cap test tubes. Discard lower (aqueous) layer.

18.6.2.9 Add 2 mL of 0.5 N sulfuric acid to tubes. Cap and rotate 20 minutes. Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation.

18.6.2.10 Aspirate off top (organic) layer and discard.

18.6.2.11 Adjust aqueous layer to a basic pH by slowly adding solid 3:2 $NaHCO_3/K_2CO_3$ buffer until effervescence ceases. Then add approximately 0.3 g excess $NaHCO_3/K_2CO_3$ buffer to saturate the aqueous layer.

18.6.2.12 Add 200 µL of toluene/hexane/isoamyl alcohol extraction solvent to each tube, cap tubes and vortex for 10-15 seconds. Centrifuge tubes at approximately 2500 rpm for 15 minutes to achieve separation.

18.6.2.13 Transfer approximately 200 µL of top (organic) layer into conical bottom vials. Evaporate to dryness at approximately 55°C under nitrogen.

18.6.2.14 Add 50 µL BSTFA and 50 µL ethyl acetate to the extracts. Heat at 90°C for 30 minutes.

18.6.2.15 Transfer to autosampler vials for analysis by GC-MS.

18.7 Calculation

Quantitation. Prepare a response curve of area (height) of analyte to area (height) of internal standard ratio versus calibrator concentration. Calculate the analyte concentration by interpolation of the linear plot.

18.8 Quality Control and Reporting

18.8.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte. The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

18.8.2 If the same specimen is analyzed in duplicate (for screening and confirmation) and both results are quantitative, the results should be averaged prior to reporting.

18.8.3 See Toxicology Quality Guidelines.

18.9 References

18.9.1 D Anderson and K Fritz. Quetiapine (Seroquel) Concentrations in Seven Postmortem Cases. J. Anal. Toxicol. 2000; May/June (24): 300-304.

18.9.2 D Flammia, T Valouch and S Venuti. Tissue Distribution of Quetiapine in 20 Cases in Virginia. J. Anal. Toxicol. 2006, May (30): 287-292.

19 HYDROXYZINE QUANTITATION AND CONFIRMATION BY GC-MS

19.1 Summary

Hydroxyzine is extracted from biological fluids or tissues using solid phase extraction (SPE), derivatized with BSTFA and analyzed by GC-MS in SIM mode.

19.2 Specimen Requirements

- 2 mL whole blood, urine, bile, gastric contents, other fluids or tissue homogenates

19.3 Reagents and Standards

- Ammonium hydroxide
- Glacial Acetic Acid
- Potassium Hydroxide
- Potassium Phosphate
- Ethyl Acetate
- Methanol
- Acetonitrile
- Hexane
- Dichloromethane
- Isopropyl alcohol
- Potassium or sodium phosphate buffer solution concentrate (1 M, pH 6.0, e.g., Fisher)
- Sodium phosphate, monobasic ($NaH_2PO_4 \bullet H_20$)
- Sodium phosphate, dibasic (Na_2HPO_4)
- Anhydrous sodium sulfate
- BSTFA with 1% TMCS, stored at 2-8°C
- Hydroxyzine, 1 mg/mL
- Methapyrilene, 1 mg/mL

19.4 Solutions, Internal Standards, Calibrators and Controls

19.4.1 Solutions for UCT CleanScreen® SPE Extraction

19.4.1.1 When using UCT CleanScreen® SPE Extraction columns, either sodium or potassium phosphate buffer may be used. However, the same buffer (sodium or potassium) must be used throughout the duration of the procedure.

19.4.1.1.1 0.1 M Potassium Phosphate Buffer, pH 6.0. Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M potassium hydroxide while stirring and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Dilute one volume potassium phosphate buffer solution concentrate with nine volumes of dH_2O. Store at room temperature for up to two years. (Note: potential exothermic reaction, exercise caution when making this solution)

19.4.1.1.2 5 M Potassium hydroxide: Add 28.05 g potassium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

OR

19.4.1.1.3 0.1 M Sodium Phosphate Buffer, pH 6.0. Weigh 1.70g Na_2HPO_4 and 12.14g $NaH_2PO_4 \cdot H_2O$ and transfer to a 1 L volumetric flask containing approximately 800 mL dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5 M sodium hydroxide and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Dilute one volume sodium phosphate buffer solution concentrate with nine volumes of dH_2O. Store at room temperature for up to two years.

19.4.1.1.4 5 M Sodium Hydroxide: Add 20.0 g of sodium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

19.4.1.2 1.0 M Acetic Acid. Add 100-200 mL dH2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH2O and qs to volume with dH2O. Store at room temperature for up to two years.

19.4.1.3 Ethyl acetate/Hexane, 50:50 v/v. Mix 500 mL ethyl acetate with 500 mL hexane. Store at room temperature for up to two years.

19.4.1.4 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2). Mix 78 mL dichloromethane with 20 mL isopropanol. Mix well. In hood, add 2 mL ammonium hydroxide. Mix gently. PREPARE SOLUTION FRESH DAILY!

19.4.2 Reconstitution solvent (50:50 hexane:ethyl acetate): Mix 50 mL hexane with 50 mL ethyl acetate. Add enough anhydrous sodium sulfate until no more dissolves after shaking vigorously. Store at room temperature for up to two years.

19.4.3 Internal Standard

Working internal standard solution (0.01 mg/mL methapyrilene or other suitable IS): Pipette 100 µL of the 1 mg/mL stock solution of methapyrilene into a 10 mL volumetric flask and qs to volume with methanol.

19.4.4 Preparation of calibrators.

19.4.4.1 Working hydroxyzine standard solution (0.02 mg/mL). Pipette 100 µL of 1 mg/mL hydroxyzine standard into a 5 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

19.4.4.2 To prepare the calibration curve, pipette the following volumes of the 0.02 mg/mL working hydroxyzine standard solution into appropriately labeled 16 x 125 mm screw cap test tubes.

To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood. Add 2 mL blank blood to obtain the final concentrations listed below.

Amount of 0.02 mg/mL hydroxyzine solution (µL)	Final concentration of hydroxyzine (mg/L)
400	4.0
300	3.0
200	2.0
100	1.0
40	0.4

20	0.2
10	0.1

19.4.5 Hydroxyzine Control. Control may be from an external source or prepared in-house using drugs from different manufacturers, lot numbers or prepared by a chemist different than the individual performing the extraction.

19.4.6 Negative control. Blood bank blood or equivalent determined not to contain hydroxyzine.

19.5 Apparatus

19.5.1 Agilent GC-MS, manufacturer's software, compatible computer & printer

19.5.2 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

19.5.3 Test tubes, round bottom tubes, borosilicate glass

19.5.4 Test tubes, glass centrifuge, conical bottom

19.5.5 Test tubes, round bottom, screw cap tubes, borosilicate glass

19.5.6 Centrifuge capable of 2,000 – 3,000 rpm

19.5.7 Cleanscreen® Extraction Cartridges (ZSDAU020) from United Chemical Technologies (200 mg columns)

19.5.8 Solid phase extraction manifold

19.5.9 Vortex mixer

19.5.10 Evaporator/concentrator

19.5.11 Test tube rotator

19.5.12 GC autosampler vials and inserts

19.5.13 GC-MS parameters.

19.5.13.1 Acquisition Mode: SIM

Hydroxyzine: 201, 165, 299

Methapyrilene: 58, 97

19.5.13.2 Column: HP-1MS or HP-5MS (or equivalent columns from alternative manufacturers), 30 m x 0.25 mm x 0.25 µm

19.5.13.3 Detector Temperature: 280°C

19.5.13.4 Oven Program

- Equilibration time: 0.50 minutes
- Initial temp: 110°C
- Initial time: 1 minute
- Ramp: 10°C /min
- Final Temp: 290°C
- Final Time: 9 minutes
- Run Time: 28 minutes

19.5.13.5 Inlet

- Mode: Splitless
- Temperature: 270°C
- Injection volume: 1.0 µL
- Purge Time: ON at 1.0 minute

19.6 Procedure

19.6.1 Extraction, CleanScreen SPE Columns.

19.6.1.1 Label clean screw cap tubes accordingly.

19.6.1.2 Pipette 2 mL of corresponding negative and positive control bloods and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes. Note: since this procedure is used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case. One will serve as a screen and the second as a confirmation.

19.6.1.3 Add 100 µL working internal standard solution to all tubes for a final concentration of 0.5 mg/L. Vortex briefly.

19.6.1.4 Add 6.0 mL deionized water to each tube. Mix, vortex briefly and let stand for 5 minutes.

19.6.1.5 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer supernatant to clean tubes and discard the tube with the remaining pellet.

19.6.1.6 Add 2.0 mL of pH 6 phosphate buffer, mix and vortex. As necessary adjust the pH to 5.5 to 6.5 with additional 0.1 M phosphate buffer. Note: larger volumes of water or phosphate buffer may be used to further dilute some specimens prior to SPE analysis.

19.6.1.7 Solid phase extraction. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

19.6.1.7.1 Add 3 mL hexane to each column and aspirate on vacuum manifold

19.6.1.7.2 Add 3 mL methanol to each column and aspirate on vacuum manifold.

19.6.1.7.3 Add 3 mL dH_2O and aspirate.

19.6.1.7.4 Add 1 mL of 0.1 M pH 6.0 phosphate buffer and aspirate

19.6.1.7.5 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges under vacuum/pressure at approximately 1-2 mL/ minute flow.

19.6.1.7.6 Add 3 mL dH_2O and aspirate at ≤ 3inches of mercury or at a low positive pressure (< ~10 psi).

19.6.1.7.7 Repeat the dH_2O wash a second time.

19.6.1.7.8 Wash with 2.0 mL 1.0 M acetic acid and aspirate.

19.6.1.7.9 Add 3 mL methanol, aspirate under full vacuum/pressure for at least 2 minutes

19.6.1.7.10 Add 2 mL hexane to each column. Dry columns at ≥ 10 inches of mercury or a high positive pressure (~50 psi) for five minutes.

19.6.1.7.11 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

19.6.1.7.12 Elute hydroxyzine by adding 3 mL of freshly prepared methylene chloride/isopropanol/ammonium hydroxide solution to each column. Collect eluate in conical test tubes by column aspiration or gravity drain.

19.6.1.7.13 Elute at 1 - 2 mL/minute (no vacuum) and collect eluates.

19.6.1.8 Evaporate eluates to dryness under nitrogen at 50-60°C.

19.6.1.9 Add 50 µL BSTFA and 50 µL ethyl acetate to the extracts. Heat at 60°C for 30 minutes.

19.6.1.10 Transfer to GC autosampler vials for analysis by GC-MS.

19.7 Calculation

Quantitation. Prepare a response curve of area (height) of analyte to area (height) of internal standard ratio versus calibrator concentration. Calculate the analyte concentration by interpolation of the linear plot.

19.8 Quality Control and Reporting

19.8.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte. The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

19.8.2 If the same specimen is analyzed in duplicate (for screening and confirmation) and both results are quantitative, the results should be averaged prior to reporting.

19.8.3 See Toxicology Quality Guidelines SOP for quality control and reporting.

19.9 References

19.9.1 T. Soriano, C. Jurado, M. Menendez and M. Repetto, “Improved Solid-Phase Extraction Method for Systematic Toxicological Analysis in Biological Fluids.” J. Anal. Toxicol. 2001; March (25): 137-143.

19.9.2 W.H. Anderson and D.C. Fuller, “A Simplified Procedure for the Isolation, Characterization, and Identification of Weak Acid and Neutral Drugs from Whole Blood.” J. Anal. Toxicol. 1987, Sep/Oct (11): 198-204.

20 VOLATILE SCREEN AND CONFIRMATION BY HEADSPACE GC AND GC-MS

20.1 Summary

An aliquot of specimen is diluted semi-automatically with an internal standard (IS) solution (n-propanol) into a glass vial, sealed, and placed in a heated automatic sampler. The concentration of volatiles in a dilute aqueous biological sample is directly proportional to its concentration in the gas phase or headspace. A portion of the resultant headspace vapor above the liquid is automatically injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID). Volatiles are identified by retention time and, whenever possible, confirmed by headspace GC-MS.

20.2 Specimen Requirements

Approximately 2 mL of biological fluid(s) or 2 gm tissue. Alternative specimens include canisters/containers of huffing agent or headspace vials containing various tissues (e.g., lung tissue) or air samples (e.g., tracheal air).

20.3 Reagents and Standards

- Reference standards of volatiles or gases
- N-propanol

20.4 Solutions, Internal Standard, Calibrators and Controls

20.4.1 0.10% stock solution: To prepare a mixture of volatiles of interest, use density to calculate the volumes of each volatile necessary to prepare a 0.10% stock solution. For example, to create a 0.10% stock solution of toluene, ethyl acetate and chloroform, pipette the following volumes into a 100 mL volumetric flask and qs to volume with dH_2O:

Volatile	Density	Volume
Toluene	0.86	116 µL
Ethyl Acetate	0.90	110 µL
Chloroform	1.47	68 µL

Note: Due to the insolubility of toluene in water, an alternative preparation may be followed in a similar fashion to 20.4.3.1. The headspace vial will be flushed with nitrogen, capped, and sealed. A gas-tight syringe will be used to add 1 mL of toluene to the sealed vial. The vial will be heated at ~50ºC for ~ 3 minutes. Serial dilutions may be prepared following 20.4.3.1.3. Internal standard (0.03% v/v n-propanol) will be added to the vials containing the final dilutions using a gas-tight syringe. This procedure may be followed for other analytes that are immiscible in water.

20.4.2 Preparation of 0.004% standard solution: Pipette 400 µL of the 0.10% stock solution into a 10 mL volumetric flask and qs to volume with dH_2O. This 0.004% standard is used as the limit of detection and the response of the reported analyte in the case specimen must be greater than that of the analyte in the 0.004% standard.

20.4.3 For analytes that are in the gas phase at room temperature (e.g., 1,1 difluoroethane), the following are options for the preparation of a qualitative standard:

20.4.3.1 Capture of primary standard in a headspace vial.

20.4.3.1.1 In a fume hood, flush a 20 mL headspace vial with the analyte (usually in the form of a pressurized cylinder or can) for several seconds.

20.4.3.1.2 While continuing to flush the vial, cap vial with septa and hold down until crimped with aluminum cap.

20.4.3.1.3 Generally, the stock primary standard is of such high concentration that it must be diluted in order to avoid overloading the GC column and/or detector. Serial dilutions of the primary standard may be made into nitrogen purged headspace vials using gastight syringes to transfer aliquots of the headspace from vial to vial.

20.4.3.1.4 The appropriate dilutions for an analyte will have to be determined via trial and error. The analyte peak should not be so large as to cause a shift in retention time of the internal standard.

20.4.3.1.5 The final dilution of the standard should be into a vial containing internal standard so that the relative retention times of the analytes may be determined and used to identify the unknowns in case specimens.

20.4.3.2 Capture of a semi-quantitative primary standard in a methanolic solution.

20.4.3.2.1 Fill a 2 mL volumetric flask to the mark with methanol.

20.4.3.2.2 Weigh flask and tare balance.

20.4.3.2.3 Slowly bubble analyte gas into flask.

20.4.3.2.4 Weigh flask to determine approximate concentration (mg gas/2 mL methanol).

20.4.3.2.5 Dilute standard to approximately 0.004% w/v for the limit of detection standard.

20.4.4 0.03% (v/v) n-propanol internal standard solution. Pipette 300 µL n-propanol into a 1 L volumetric flask and qs to volume with dH_2O. Store at room temperature for up to one year.

20.4.5 Negative blood control: Blood bank blood or equivalent previously determined not to contain volatiles.

20.5 Apparatus

20.5.1 Agilent headspace GC equipped with flame ionization detector, manufacturer's software, compatible computer and printer

20.5.2 Agilent GC-MS, manufacturer's software, compatible computer and printer

20.5.3 Glass 20 mL (23 x 75 mm) headspace vials with Teflon septa and aluminum seals

20.5.4 Vial seal crimper

20.5.5 Hamilton Microlab Diluter or equivalent

20.5.6 Gastight Hamilton syringes

20.5.7 Headspace GC-FID parameters: The following conditions are recommended starting parameters. Modify the parameters as necessary to achieve optimum separation and detection of analytes. Print out the instrument method and optimum conditions and store with each batch analysis.

20.5.7.1 Note: For approximate retention times of volatiles, see Restek Application Note #59548 on GC Analysis of Commonly Abused Inhalants in Blood Using Rtx BAC1 and Rtx BAC2 Columns. Column. Restek Rtx®-BAC 1 or BAC 2 capillary column.

20.5.7.2 Instrument parameters.

- Column Restek BAC1 or BAC2 or Agilent DB-ALC1 and Agilent DB-ALC2 capillary columns, or Agilent DB-BAC1 UI and DB-BAC2 UI capillary columns
- Oven 40ºC
- Ramp 1 5°C/min to 60°C
- Ramp 2 5°C/min to 80°C, 1 min hold
- Injector 200ºC
- Detector (FID) 250°C
- Hydrogen flow 30 mL/min
- Air flow 400 mL/min
- Make-up flow 20 ml/min
- Make-up gas Helium
- Inlet Split
- Split ratio 1:1
- Split flow 14.2 mL/min
- Total flow 30.6 mL/min
- Pressure 8.9 psi constant pressure mode

20.5.8 Headspace GC-FID Parameters. The following conditions are recommended starting parameters. Modify the parameters as necessary to achieve optimum operation. Print out the optimum conditions and save them with each batch analysis.

- Incubation Temp 70ºC
- Sample Valve 85ºC
- Transfer Line 95ºC
- GC Cycle 10.0 min
- Sample Equilibration 3.0 min
- Vial Pressurization 0.91 min
- Loop Fill 0.20 min (or 500 µL volume)
- Loop Equilibration 0.05 min
- Sample Inject 1.00 min
- Oven Stabilization 1.0 min
- Agitation Low
- Extractions 1
- Puncture Mode Single

20.5.9 Headspace GC-MS parameters. The following conditions are recommended starting parameters. Modify the parameters as necessary to achieve optimum operation. Print out the optimum conditions and save them with each batch analysis. GCMS column may differ from the HSGC columns listed above and may include HP-5MS or HP-1MS. The use of other column types shall be approved by the Toxicology Program Manager.

- Injection Manual
- Split Mode 10:1 split
- Split flow 12 mL/min
- Injector Temp 200ºC
- Pressure 9.1 psi
- Total flow 15.9 mL/min
- Flow 1.2 ml/min (40 cm/sec)
- Column HP-5MS, 30 m x 0.25 mm ID x 0.25 µm film
- He Flow 1.2 ml/min (40 cm/sec)
- Oven 40°C initial, 0.3 minute hold
- Ramp 10°C/min to 100°C

- Equilibration time 0.50 min
- Scan mass range 45-100 amu
- EM voltage 200
- Threshold 150

20.6 Procedure

20.6.1 Headspace GC-FID Analysis

20.6.1.1 Prepare 0.004% volatile standard or qualitative standard.

20.6.1.2 Pipette 0.2 mL of the volatile standard or qualitative standard, negative control and case specimens into appropriately labeled clean headspace vials. Add 450 µL 0.03% n-propanol to each headspace vial.

20.6.1.2.1 Note: If sample is already in headspace vial (tracheal air or lung), add 450 µL 0.03% n-propanol to the headspace vial using a needle and syringe through the septa/seal. If the submitted sample is to be used for both HS-FID and GCMS (see below), add 1 mL of 0.03% n-propanol to the headspace vial using a needle and syringe through the septa/seal.

20.6.1.2.2 Note: If analyzing weighed tissue specimens, place approximately 0.5 gm weighed tissue in the headspace vial and add 450 µL 0.03% n-propanol to each headspace vial.

20.6.1.3 Stopper each headspace vial with a Teflon seal, as needed. Vortex or manually shake each vial for several seconds and place in the sample rack.

20.6.1.4 Seal all headspace vials by crimping the aluminum rings over the Teflon seals.

20.6.1.5 Load headspace vials in the headspace auto sampler.

20.6.2 Headspace GC-MS Confirmation

20.6.2.1 Prepare 0.01% volatile standard or qualitative standard.

20.6.2.2 Pipette 1 mL of the volatile standard or qualitative standard, negative control and case specimens into appropriately labeled clean headspace vials. Add 1 mL 0.03% n-propanol to each headspace vial.

20.6.2.2.1 Note: If sample is already in headspace vial (tracheal air or lung), add 1 mL 0.03% n-propanol to the headspace vial using a needle and syringe through the septa/seal.

20.6.2.2.2 Note: If analyzing weighed tissue specimens, place approximately 2 gm weighed tissue in the headspace vial and add 1 mL 0.03% n-propanol to each headspace vial.

20.6.2.3 Stopper each headspace vial with a Teflon seal, as needed. Vortex or manually shake each vial for several seconds and place in the sample rack.

20.6.2.4 Seal all headspace vials by crimping the aluminum rings over the Teflon seals.

20.6.2.5 Heat each headspace vial at 80°C for 10 minutes in oven.

20.6.2.6 Use disposable 1 mL gas tight syringe to withdraw 1 mL headspace from each vial and manually inject directly into GC-MS. Note: start MSD 3 seconds prior to injection.

20.6.2.7 Compare specimen unknown mass spectra to known volatile mass spectra.

20.7 Quality Control and Reporting

20.7.1 Volatiles are identified on headspace GC-FID based on relative retention times compared to the volatile standard. Identification is performed by instrument software. Relative retention times should be within ± 2% of the relative retention time obtained from the volatile standard.

20.7.2 Volatiles must be confirmed by headspace GC-MS by comparison of the retention time and mass spectra of the unknown sample to that the retention and mass spectra of a known volatile standard.

20.7.3 Volatiles are reported as “present” only.

20.7.4 Exceptions to these guidelines must be justified with supporting analytical documentation, authorized by the Program Manager and documented in the case file with an MFR.

20.8 References

20.8.1 Restek Application Note #59548. GC Analysis of Commonly Abused Inhalants in Blood Using Rtx BAC1 and Rtx BAC2 Columns.

20.8.2 L. C. Nickolls, "A Modified Cavett Method for the Determination of Alcohol in Body Fluids," Nov. 1960, Analyst, Vol. 85, pp 840-942.

20.8.3 B. Kolb, "Head Space Analysis by Means of the Automated Gas Chromatograph F-40 Mulitfract", Bodenseewerk Perkin-Elmer and Co., Technical Manual #15E.

20.8.4 K. M. Dubowski, "Manual for Analysis of Ethanol in Biological Liquids," Department of Transportation Report No. DOT TSC NHTSA-76-4, Jan 1977.

20.8.5 G. Machata, "Determination of Alcohol in Blood by Gas Chromatographic Head Space Analysis," Clin Chem. Newsletter, 4(1972), 29.

20.8.6 B.L. Levine, Principles of Forensic Toxicology, American Association for Clinical Chemistry, Inc., 1999, p. 180.

20.8.7 Randall Edwards, in-house development.

21 HEAVY METALS BY REINSCH TEST

21.1 Summary

Rapid presumptive tests are simple colorimetric tests that may be performed directly on blood, urine, gastric contents or liver with little or no previous sample preparation. When run with negative and positive controls, these tests are sensitive enough to detect overdoses; however since these tests are presumptive only, confirmation should be performed on positive findings.

21.2 Heavy Metal Screen (Reinsch Test)

21.2.1 Principle: certain heavy metals can be quickly and easily identified when ingested in acute toxic doses using the classical Reinsch Test. The Reinsch test identifies arsenic, antimony, bismuth and mercury. The test is based on the ability of metallic copper, in the presence of strong acid, to reduce selected heavy metals to their elemental form (e.g., arsenic is deposited on the copper as a visible dull black film):

$$3Cu^{0} + 2As^{+3} + HCl \rightarrow 3Cu^{+2} + 2As^{0}$$

21.2.2 Reagents and controls

21.2.2.1 Concentrated hydrochloric acid

21.2.2.2 Copper spiral (#20 gauge) or foil strip. Wind copper around a glass rod or pencil. Clean the copper by dunking in concentrated nitric acid for a few seconds, then immediately immerse in water. The copper should be bright and shiny.

21.2.2.3 Arsenic reference solution, 1 mg/mL. Dissolve 0.132 g of arsenic trioxide in 1.0 mL of 10 N sodium hydroxide and add 50 mL dH_2O. Neutralize the solution with concentrated HCl, then qs to 100 mL with dH_2O.

21.2.3 Procedure (urine, gastric contents or liver are preferred specimens)

21.2.3.1 Place clean copper spirals into separate 100 mL beakers or 125 mL Erlenmeyer flasks labeled for negative and positive controls and unknown(s).

21.2.3.2 Place 20 mL urine, approximately 10-15 g minced tissue in 20 mL dH_2O, or an aliquot of gastric contents dissolved in 20 mL dH_2O into a labeled beaker. Place 20 mL negative control urine in two separate beakers. Spike one of them with 40 µL of 1 mg/mL arsenic reference solution (final concentration, 2 mg/L).

21.2.3.3 Carefully add 4 mL concentrated HCl to each beaker.

21.2.3.4 In a hood, heat the solutions to a gentle boil for approximately 1 hour. Add 10% HCl as necessary to maintain the original volume.

21.2.3.5 After 1 hour, remove the copper coils and gently rinse with dH_2O. Compare the negative control and positive control to the unknown for the presence of a gray to black deposit.

21.2.4 Interpretation

21.2.4.1 If the positive and negative control work and the copper coils in the unknown samples are still bright in appearance, then the test can be reported as per section 2.7.

21.2.4.2 If the positive and negative controls work and the copper coils in the unknown samples become gray, black or silvery, then the result is a presumptive positive for the presence of

heavy metals. Since DFS does not have the capability to confirm positive results, the following statement may be reported on the Certificate of Analysis:

21.2.4.2.1 Test results indicate a presumptive positive for the presence of heavy metals (antimony, arsenic, bismuth and mercury) via the Reinsch Test. The samples should be sent to another laboratory for the definitive identification, confirmation and quantitation of heavy metals.

21.3 References

21.3.1 I., Sunshine, Methodology for Analytical Toxicology, CRC Press, Cleveland, OH, 1975.

21.3.2 N. W. Tietz, Fundamentals of Clinical Chemistry, W.B. Saunders, Philadelphia, PA, 1976.

21.3.3 E.C.G. Clarke, Clark's Isolation and Identification of Drugs, The Pharmaceutical Press, London, UK, 1986.

22 NSAID QUANTITATION AND CONFIRMATION BY LCMSMS

22.1 Summary

NSAIDs (nonsteroidal anti-inflammatory drugs) are extracted from biological samples using a liquid-liquid extraction. An aliquot of the extract is quantitated and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

22.2 Specimen Requirements

0.1 mL blood, fluid or tissue homogenate (post-centrifugation homogenate sample)

Note: For analysis of tissue homogenate, centrifuge homogenate at approximately 2500 rpm for 15 minutes prior to aliquotting supernatant for analysis.

22.3 Reagents and Standards

22.3.1 Drug targets and internal standards

Target	Internal Standard
Acetaminophen	Acetaminophen-D_4
Salicylic acid	Salicylic Acid-D_4
Naproxen	Naproxen-D_3
Ibuprofen	Ibuprofen-D_3

22.3.2 Acetic Acid, glacial, eluent additive for LC-MS

22.3.3 Type I or LC-MS grade water (dH_2O)

22.3.4 Sodium acetate, ACS grade (or similar)

22.3.5 n-Hexane, Fisher Optima grade (or similar) or higher

22.3.6 Ethyl acetate, Fisher Optima grade (or similar) or higher

22.3.7 Acetonitrile, Fisher Optima grade (or similar) or higher

22.4 Solutions, Internal Standard, Calibrators and Controls

22.4.1 Sodium Acetate Buffer - weigh out 8.0 g sodium acetate, transfer to a 1 L volumetric flask and dissolve in approximately 800 mL deionized water. Adjust the pH to 4.5 with glacial acetic acid and qs to volume with deionized water. Store at room temperature for up to one month.

22.4.2 Hexane/Ethyl acetate (50:50, v:v) - Mix 100 mL hexane with 100 mL ethyl acetate. Store at room temperature for up to 2 years.

22.4.3 Mobile Phase A (H_2O with 0.04 % acetic acid): add 400 µL of acetic acid to 1.0 L of dH_2O. Store at room temperature for up to one month.

22.4.4 Mobile Phase B (Acetonitrile): 1.0 L of acetonitrile. Store at room temperature.

22.4.5 Water/Acetonitrile (50:50): Mix 50 mL dH_2O with 50 mL acetonitrile. Store at room temperature for up to 2 years. Note: reconstitution solvent which may be utilized from other methods (e.g., cannabinoid quantitation reconstitution solvent).

22.4.6 Preparation of internal standard.

22.4.6.1 **High Dynamic Range (10 mg/L-400 mg/L):** Working internal standard solution (0.2/0.1 mg/mL): Pipette 100 µL of 10.0 mg/mL or 1.0 mL of 1.0 mg/mL Naproxen-D_3 stock standard solution into a 10 mL volumetric flask. For all other deuterated standards, pipette 200 µL of 10.0 mg/mL or 2.0 mL of 1.0 mg/mL stock solution into the same 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

22.4.6.2 **Low Dynamic Range (1.0 mg/L-10 mg/L):** Working internal standard solution (10.0/5.0 mg/L): Pipette 10 µL of 10.0 mg/mL or 100 µL of 1.0 mg/mL Naproxen-D_3 stock standard solution into a 20 mL volumetric flask. For all other deuterated standards, pipette 20 µL of 10.0 mg/mL or 200 µL of 1.0 mg/mL stock solution into the same 20 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

22.4.7 Preparation of calibrators

22.4.7.1 **High Dynamic Range (10 mg/L-400 mg/L):** Working standard solution (0.2 mg/mL): Pipette 2.0 mL of the 1.0 mg/mL or 200 µL of a 10 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

22.4.7.2 **High Dynamic Range (10 mg/L-400 mg/L):** Working standard solution (0.02 mg/mL): Pipette 1.0 mL of the 0.2 mg/mL working standard solution into a 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

22.4.7.3 **Low Dynamic Range (1.0 mg/L-10 mg/L):** Working standard solution (10.0 mg/L): Pipette 100 µL of the 1.0 mg/mL or 10 µL of a 10 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

22.4.7.4 To prepare the calibration curve, pipette the following volumes of the 0.2 mg/mL, 0.02 mg/mL, or 10.0 mg/L working standard solutions into appropriately labeled screw cap test tubes. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood. Add 0.1 mL blank blood to obtain the final concentrations listed below.

High Dynamic Range (10 mg/L-400 mg/L):

Amount of 0.2 mg/mL stock solution (µL)	Amount of 0.02 mg/mL stock solution (µL)	Final concentration of NSAID's (mg/L)
200	-	400
150	-	300
100	-	200
50	-	100
-	250	50
-	125	25
-	50	10

Low Dynamic Range (1.0 mg/L-10 mg/L):

Amount of 10.0 mg/L stock solution (µL)	Final concentration of NSAID's (mg/L)
100	10
85	8.5
70	7.0
55	5.5
40	4.0
25	2.5
10	1.0

22.4.8 Controls

22.4.8.1 NSAID Controls. Controls may be from an external source or prepared in-house using drugs from different manufacturers or lot numbers.

22.4.8.2 Control levels for charting.

22.4.8.2.1 High Dynamic Range: 80 mg/L

22.4.8.2.2 Low Dynamic Range: 5.0 mg/L

22.4.8.3 Suggested control level preparation instructions.

22.4.8.3.1 High Dynamic Range:

Amount of 0.2 mg/mL stock solution (µL)	Amount of 0.02 mg/mL stock solution (µL)	Final concentration of NSAID's (mg/L)
175	-	350
100	-	200
40	-	80
-	200	40

22.4.8.3.2 Low Dynamic Range:

Amount of 10.0 mg/L stock solution (µL)	Final concentration of NSAID's (mg/L)
80	8.0
65	6.5
50	5.0
30	3.0

22.4.8.4 Negative control. Blood bank blood or equivalent determined not to contain NSAIDs or other targets.

22.4.8.5 Threshold Controls: Threshold controls are to be prepared at 5 mg/L (HDR) and 0.5 mg/L (LDR) but may be adjusted based on validated LOD.

22.5 Apparatus

22.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

22.5.2 Test tubes, conical bottom, borosilicate glass

22.5.3 Centrifuge capable of 3000 rpm

22.5.4 Evaporator/concentrator

22.5.5 Vortex mixer

22.5.6 Rotator

22.5.7 GC autosampler vials with inserts

22.5.8 LCMSMS parameters.

22.5.8.1 LC Parameters:

- Column: Poroshell 120 SB-C18, 2.1 x 50 mm, 2.7 µm particle size
- Guard Column: Poroshell 120 SB-C18, 2.1 x 5 mm, 2.7 µm particle size
- Column Thermostat: 60 °C
- Solvent A: H_2O with 0.04% acetic acid
- Solvent B: Acetonitrile
- Initial Flow Rate: 0.70 mL/min
- Injection vol.: 1.0 µL with a minimum 20 second needle wash
- Stop time: 15 min
- Gradient:

Initial	2% B
3.0 minutes	35% B
6.0 minutes	98% B
14.0 minutes	98% B
15.0 minutes	2% B
Post time minimum 1.5 minutes	

22.5.8.2 MS-MS parameters.

- MSD Parameters:

Ionization:	ESI
Polarity:	Positive/Negative
Gas temp:	350 °C
Drying Gas:	10.0 L/min
Nebulizer press:	40 psi
Capillary	4000 V/4000 V
Delta EMV	400 V/0 V

- Transition Ions (Quantitative transitions are bold font):

Compound Name	Precursor Ion	Product Ion	Approx. Ret Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	Polarity
Acetaminophen	152	**110**	0.7	115	12	5	Positive
		65			32		
Acetaminophen-D4	156	**114**	0.7	115	12	5	Positive
		69			32		
Salicylic Acid	137	**93**	1.7	80	12	7	Negative
		65.1			32		
Salicylic Acid-D4	141.05	**97**	1.7	85	16	7	Negative
		69.1			32		
Naproxen	229.1	170	3.8	65	8	3	Negative
		169			24		
Naproxen-D3	232.1	**173**	3.8	70	8	3	Negative
		171			24		
Ibuprofen	205.1	205.1	4.5	75	0	3	Negative
		161.1			0		
Ibuprofen-D3	208.1	208.1	4.5	70	0	3	Negative
		164.1			0		

22.6 Procedure

22.6.1 Label clean screw cap round-bottom centrifuge tubes appropriately with calibrators, controls and case sample IDs.

22.6.2 Prepare calibrators and controls. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood.

22.6.3 Add 0.1 mL case specimens/blank blood to the appropriately labeled tubes.

22.6.4 **High Dynamic Range (10 mg/L-400 mg/L):** Add 50 µL of the 0.2/0.1 mg/mL working internal standard solution and vortex briefly.

Low Dynamic Range (1.0 mg/L-10 mg/L): Add 50 µL of the 10.0/5.0 mg/L working internal standard solution and vortex briefly.

22.6.5 Add 0.5 mL of sodium acetate buffer to each tube and vortex for 15-30 seconds.

22.6.6 Add 2.0 mL of 50:50 hexane/ethyl acetate to each tube DO NOT VORTEX.

22.6.7 Cap all tubes and rotate them for 30 minutes.

22.6.8 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation.

22.6.9 Transfer supernatant into conical bottom tubes and evaporate to dryness at approximately 60°C under nitrogen.

22.6.10 **High Dynamic Range (10 mg/L-400 mg/L)**: Reconstitute in 400 µL water:acetonitrile (50:50).

Low Dynamic Range (1.0 mg/L-10 mg/L): Reconstitute in 50 µL water:acetonitrile (50:50). (Note: Centrifugation may be necessary at this step.)

22.6.11 Transfer to autosampler vials.

22.7 Quality Control and Reporting

22.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

22.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

22.7.3 When a target concentration is above the ULOQ, 100 µL of case sample shall be diluted with no more than 1.9 mL of blank matrix for a total dilution volume of 2.0 mL for the high dynamic range. For the low dynamic range, 100 µL of case sample shall be diluted with no more than 0.9 mL of blank matrix for a total dilution volume of 1.0 mL.

22.7.4 The calibration model for all targets is weighted (1/x) linear with the exception of acetaminophen when analyzing at the high dynamic range which is weighted (1/x) quadratic. Samples with a concentration greater than the second highest calibrator concentration for a target with a quadratic fit must be repeated if the high positive control is outside of acceptable limits.

22.7.5 Threshold Controls: TC is prepared at 5 mg/L (HDR) and 0.5 mg/L (LDR) and the validated LODs are 2.0 mg/L (HDR) and 0.2 mg/L (LDR).

22.7.6 Extracted samples are stable for up to 24 hours after reconstitution for the low dynamic range. Extracted samples are stable for up to 7 days for the high dynamic range with the exception of salicylic acid which is stable for up to 24 hours after reconstitution.

22.7.7 See Toxicology Quality Guidelines.

22.8 References

22.8.1 Crisp, M. and Wagner, R. Virginia Department of Forensic Science in-house method development. **2018**.

22.8.2 Virginia Department of Forensic Science Toxicology Procedures Manual. **2018**.

22.8.3 Skibinski, R., Komsta, L. The stability and degradation kinetics of acetylsalicylic acid in different organic solutions revisited-an UHPLC-ESI-QTOF spectrometry study. *Current Issues in Pharmacy and Medical Science.* **2016**, 29, 39-41.

22.8.4 Ferrer, I., Thurman, E.M. EPA Method 1694: Agilent's 6410A LC/MS/MS solution for pharmaceuticals and person care products in water, soil, sediment, and biosolids by HPLC/MS/MS. *Agilent Technologies Application Note.* **2008**.

22.8.5 Telving, R., Hasselstrom, J.B., Andreasen, M.F. Targeted toxicological screening for acidic, neutral and basic substances in postmortem and antemortem whole blood using simple protein precipitation and UPLC-HR-TOF-MS. *Forensic Science International.* **2016**. 266, 453-461.

22.8.6 Gicquel, T., Aubert, J., Lepage, S., Fromenty, B., Morel, I. Quantitative analysis of acetaminophen and it primary metabolites in small plasma volumes by liquid chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology.* **2013**. 37, 110-116.

23 ETHANOL CONTENT OF ALCOHOLIC BEVERAGES BY HEADSPACE GC

The Virginia Alcoholic Beverage Control (ABC) Authority regulates the sale of alcoholic beverages and enforces the alcoholic beverage control laws within the Commonwealth of Virginia.

The DFS Toxicology Lab tests beverages submitted by law enforcement agencies. Most of these cases involve the investigation of minors in possession of alcohol, open intoxicants in vehicles and illegal sale/distribution of alcohol. These types of cases require the analysis of alcohol content. Any beverage containing greater than or equal to 0.5% ethanol is defined as an alcoholic beverage (Code of Virginia § 4.1-100).

23.1 Summary

An aliquot of sample is diluted semi-automatically with an internal standard (IS) solution (n-propanol) into a glass vial, sealed, and placed in a heated automatic sampler. The concentration of ethanol in a dilute alcoholic beverage is directly proportional to its concentration in the gas phase or headspace. A portion of the resultant headspace vapor above the liquid is automatically injected into a gas chromatograph (GC) equipped with two flame ionization detectors (FID). Ethanol is identified by retention time and its concentration is calculated by comparison to similarly treated aqueous calibrators by using peak heights or areas. Positive cases are confirmed in a second run.

23.2 Specimen Requirements

Approximately 0.5 mL liquid

23.3 Reagents and Standards

- Ethanol-water solution (Nominal Mass Fraction 95.6%) NIST Standard Reference Material (SRM) (95.6% Mass Fraction = 96.5% v/v) (Note: once opened, sampling of this vial shall occur within two minutes to ensure traceability)
- N-propanol
- Reference standard ethanol solutions (e.g., National Institute of Standards and Technology (NIST) or NIST traceable)

23.4 Solutions, Internal Standard, Calibrators and Controls

23.4.1 All prepared calibrators and positive controls shall be stored at 2-8ºC for up to one year.

23.4.2 Internal Standard: 0.5% v/v n-propanol internal standard. Pipette 5 mL n-propanol into a 1 L volumetric flask and qs to volume with dH_2O.

23.4.3 Calibrator Preparation: Once opened, the NIST ethanol SRM maintains traceability if sampled within two minutes – ensure the aliquotting of the SRM in the preparation of calibrator solutions occurs with this time limitation. The date/time of opening and sampling of the ampoule shall be recorded on the multi-component standard preparation form in the notes section.

23.4.3.1 0.5% v/v ethanol standard. Pipette 51.8 µL ethanol SRM into a 10 mL calibrated volumetric flask and qs to volume with dH_2O.

23.4.3.2 5% v/v ethanol standard. Pipette 518 µL ethanol SRM into a 10 mL calibrated volumetric flask and qs to volume with dH_2O.

23.4.3.3 25% v/v ethanol standard. Pipette 2.59 mL ethanol SRM into a 10 mL calibrated volumetric flask and qs to volume with dH_2O.

23.4.3.4 50% v/v ethanol standard. Pipette 5.18 mL ethanol SRM into a 10 mL calibrated volumetric flask and qs to volume with dH_2O.

23.4.4 Controls

23.4.4.1 NIST ethanol solutions (2.5%, 7.5% and 31.25% v/v ethanol). All control levels will be used for control tracking purposes.

23.4.4.1.1 After opening the CRM manufacturer's ampoule, transfer all contents to an airtight container for storage in the appropriate conditions for up to one year.

23.4.4.2 Negative control (dH_2O)

23.5 Apparatus

23.5.1 Gas chromatograph with data system, dual columns, two flame ionization detectors and a headspace autosampler

23.5.2 Columns. Restek Rtx-BAC1 and Rtx-BAC2, or Agilent DB-ALC1 and Agilent DB-ALC2 columns, or Agilent DB-BAC1 UI and DB-BAC2 UI columns

23.5.3 Glass 20 mL (23 x 75 mm) headspace vials with butyl septa and metal seals

23.5.4 Vial seal crimper

23.5.5 Hamilton Microlab Diluter or equivalent

23.5.6 Test tubes or sample cups

23.5.7 Headspace GC-FID parameters.

23.5.7.1 GC parameters

- Oven 50ºC Isothermal
- Injector 200ºC
- Detector (FID) 300ºC
- Hydrogen flow 30 mL/min
- Air flow 400 mL/min
- Make-up flow 20 mL/min
- Make-up gas helium
- Inlet Split
- Split ratio 20:1
- Split flow 674 mL/min
- Total flow 690 mL/min
- Pressure 8 psi constant pressure mode

23.5.7.2 Headspace autosampler parameters

- Incubation temp 70ºC
- Sample valve 80ºC
- Transfer line 90ºC
- Incubation time 240 seconds
- Syringe temp 80ºC
- Agitator speed 500 rpm
- Fill speed 1000 µL/sec
- Injection speed 1000 µL/sec
- Syringe 2.5 mL headspace

23.6 Procedure

Calibrators, controls, and case samples are prepared and analyzed singly. If a case sample is positive, it will be realiquotted and confirmed on a second run. Negative case samples may be reported with the quantitative results from the initial run. Case samples shall be maintained in a refrigerator. Refer to the Quality Control section for batch analysis requirements.

23.6.1 Pour approximately 0.2 mL of calibrator, control or sample into a clean test tube. For cases with small or limited volume, pouring off the samples is not required.

23.6.2 Place the diluter delivery tip into the specimen, making sure its tip is below the surface of the sample. Activate the diluter. At this point, the diluter draws 0.05 mL of sample into its delivery tube.

23.6.3 Withdraw the tip and wipe it with a Kimwipe/tissue paper, as necessary between sampling and dispensing.

23.6.4 Direct the delivery tip into the appropriately labeled headspace vial and activate the diluter. The diluter will dispense the sample and 0.950 mL of the n-propanol IS solution into the vial.

23.6.5 Flush the diluter as necessary by activating the diluter one or more times or rinsing with dH_2O, depending on the viscosity or other nature of the sample. Dispense washings into a waste beaker.

23.6.6 Stopper the headspace vial with the butyl septa.

23.6.7 Repeat previous six steps for all calibrators, controls and case samples.

23.6.8 Seal all headspace vials by crimping the metal rings over the butyl septa.

23.6.9 Load headspace vials into the headspace auto sampler.

23.7 Calculation

23.7.1 Ethanol is identified based on relative retention time compared to calibrators for both columns. Identification is performed by instrument software. Retention times for both ethanol and internal standard should be within ± 2% of the retention time obtained from the average of the calibrators.

23.7.2 Concentration is calculated automatically by the software based on linear regression of the 4 point calibration curve based on peak area or peak height measurement. The data from the RTX-BAC1, Agilent DB-ALC1, or Agilent DB-BAC1 UI column is utilized for quantitative results.

23.8 Quality Control and Reporting

23.8.1 Batch sample analysis. Headspace ethanol analysis is performed as a batch analysis. At a minimum, analyze one control after every 10 injections.

23.8.2 Coefficient of determination (r^2). The r^2 value for the linear regression curve must be 0.995 or greater. If not, the instrument must be recalibrated or other appropriate measures taken. The coefficient of determination is automatically printed on the calibration table and curves (and a copy should be included with the batch data file).

23.8.3 Carryover. The negative control (dH_2O) is used to check for carryover and is run immediately following the high 50 %v/v ethanol calibrator. An acceptable negative control must contain less than 0.1% ethanol %v/v.

23.8.3.1 If the negative control is unacceptable, prepare a fresh negative dH2O control and reanalyze immediately after the high calibrator. If, after reanalysis, ethanol is present in the negative control greater than 0.1% v/v, perform instrument maintenance to correct the problem.

23.8.4 Calibration: The acceptable tolerance for ethanol calibrators is ±5% of the target concentration. The calibration model to be used is linear with linear weighting.

23.8.5 Positive controls. The acceptable tolerance for ethanol controls is ±5% of target concentration. Case samples shall be bracketed by acceptable positive ethanol controls. If one control fails, repeat all positive case samples not bracketed between acceptable controls. If more than one control fails, all positive samples in the batch must be repeated. Negative results may be reported. Document corrective actions and exceptions on the ABC QC Worksheet. In general, corrective actions for failed controls may include repeating the batch, recalibrating the instrument, opening new controls or making new calibrators.

23.8.6 Vial Verification. After the completion of a batch, the identity of each vial in the headspace sampler is verified with the sequence table and the alcohol worksheet. Vial verification is performed by an analyst other than the operator and is documented by initials and date on the instrument sequence table (or equivalent).

23.8.7 Case samples are analyzed in replicates and the mean of the replicates is rounded to two (2) significant figures (e.g., 5.4% v/v). Replicate results must agree within ±10% of the mean. If the samples are outside of 10%, reanalyze another replicate and check for agreement between the replicates. An exception to exclude one or more replicates from the mean may be made if the value(s) causes the mean and the acceptance range to be unacceptable. There may also be a need to exclude a value where the replicates have two sets of data that are acceptable (e.g., replicates 1 & 3 are acceptable and replicates 2 & 3 are acceptable). The examiner may choose the set of replicates with the smallest difference between the values or, report the results as present with the permission of the customer. Document the exception in the case file.

23.8.8 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve. The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve.

23.9 References

23.9.1 B. Kolb, "Head Space Analysis by Means of the Automated Gas Chromatograph F-40 Mulitfract", Bodenseewerk Perkin-Elmer and Co., Technical Manual #15E.

23.9.2 K. M. Dubowski, "Manual for Analysis of Ethanol in Biological Liquids," Department of Transportation Report No. DOT TSC NHTSA-76-4, Jan 1977.

24 CANNABINOID QUANTITATION AND CONFIRMATION BY LCMSMS

24.1 Summary

Δ9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy- Δ9-tetrahydrocannabinol (THC-COOH, Carboxy-THC) are extracted from biological samples by adding acetic acid and hexane:ethyl acetate (9:1). An aliquot of the extract is quantitated and confirmed by LCMSMS. Although not routine, 11-hydroxy- Δ9-tetrahydrocannabinol (OH-THC) may also be quantitated and confirmed via this method. Drug targets may be analyzed in different combinations or separately as needed.

Note: Upon verification of the method in Chapter 33 Cannabinoid Quantitation and Confirmation using Supported Liquid Extraction by LCMSMS, this method (Ch. 24) will be retained to only be used if the method in Ch. 33 is taken offline or unavailable (e.g., supply issues).

24.2 Specimen Requirements

1 mL blood, fluid or tissue homogenate

24.3 Reagents and Standards

- Drug targets and internal standards

Targets	Internal Standards
Δ9-tetrahydrocannabinol (THC)	Δ9-tetrahydrocannabinol-D3
11-nor-9-carboxy- Δ9-tetrahydrocannabinol (Carboxy-THC, THC-COOH)	11-nor-9-carboxy- Δ9-tetrahydrocannabinol-D3
11-hydroxy- Δ9-tetrahydrocannabinol (OH-THC), if performed	11-hydroxy- Δ9-tetrahydrocannabinol-D3, if performed

- Acetic acid, glacial, ACS or higher grade
- n-Hexane, Fisher Optima (or similar) grade or higher
- Ethyl acetate, Fisher Optima (or similar) grade or higher
- HPLC grade water/dH2O
- Acetonitrile, Fisher Optima (or similar) grade or higher
- Formic acid, eluent additive for LC-MS

24.4 Solutions, Internal Standard, Calibrators and Controls

24.4.1 10% Acetic Acid: add 50 mL of glacial acetic acid into a 500 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to 2 years.

24.4.2 Hexane:Ethyl Acetate (9:1): Add 900 mL hexane into a 1 L graduated cylinder. Add 100 mL of ethyl acetate. Store at room temperature for up to 2 years.

24.4.3 Acetonitrile:Water (50:50): Mix 50 mL acetonitrile with 50 mL HPLC grade water. Store at room temperature for up to 2 years.

24.4.4 Mobile Phase A (H_2O with 0.1% formic acid): add 1 mL of formic acid to 1 L of dH_2O. Store at room temperature for up to one month.

24.4.5 Mobile Phase B (Acetonitrile with 0.1% formic acid): add 1 mL of formic acid to 1 L of acetonitrile. Store at room temperature for up to one month.

24.4.6 Preparation of calibrators

24.4.6.1 Working internal standard solution (1 µg/mL): Pipette 100 µL of the 0.1 mg/mL (or 10 µL of

1.0 mg/mL) stock solution of deuterated standards into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

24.4.6.2 Working internal standard solution (0.1 µg/mL): Pipette 1 mL of the 1 µg/mL working internal standard solution of deuterated standards into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

24.4.6.3 Working standard solution (1/5 µg/mL): Pipette 10 µL/50 µL (25 µL/125 µL) of the 1.0 mg/mL stock solution standards (THC, OH-THC/Carboxy-THC) into a 10 mL (25 mL) volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable. Only prepare in 25 mL volumetric flask if a calibrated volumetric flask is available.

24.4.6.4 Working standard solution (0.1/0.5 µg/mL): Pipette 1 mL of the 1/5 µg/mL working standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

24.4.6.5 To prepare the calibration curve, pipette the following volumes of the 1/5 or the 0.1/0.5 µg/mL working cannabinoid standard solution into appropriately labeled 16 x 125 mm screw cap test tubes. Calibrators and controls shall not be dried down under any circumstances (i.e using nitrogen or heat). Add 1 mL blank blood to obtain the final concentrations listed below.

Amount of 1/5 µg/mL stock solution (µL)	Amount of 0.1/0.5 µg/mL stock solution (µL)	Final concentration of cannabinoids (mg/L)
100		0.100/0.500
50		0.050/0.250
25		0.025/0.125
	100	0.010/0.050
	50	0.005/0.025
	25	0.0025/0.0125
	10	0.001/0.005

24.4.7 Controls

24.4.7.1 Cannabinoid Control. Run control from an external source (e.g., UTAK), if available. In-house controls should be prepared using standards from different manufacturers or lot numbers.

24.4.7.2 Negative control. Blood bank blood or equivalent determined not to contain cannabinoids.

24.5 Apparatus

24.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

24.5.2 Test tubes, conical bottom

24.5.3 Centrifuge capable of 2000-3000 rpm

24.5.4 Evaporator/concentrator

24.5.5 Vortex mixer

24.5.6 GC autosampler vials with inserts

24.5.7 Typical LCMSMS parameters.

24.5.7.1 LC Parameters

- Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size
- Column Thermostat: 40°C
- Solvent A: H_2O with 0.1% formic acid
- Solvent B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.50 mL/min
- Injection vol.: 10 µL with a minimum 5 second needle wash
- Stop time: 10.5 min
- Gradient:

Initial	40% B
1 minutes	40% B
7 minutes	95% B
10 minutes	95% B
10.5 minutes	40% B

Post time minimum 2 minutes

24.5.7.2 Typical MS-MS parameters.

- MSD Parameters:

Ionization:	ESI
Polarity:	positive
Gas temp:	350°C
Drying Gas:	10.0 L/min
Nebulizer press:	40 psi
Capillary:	4000 V
Delta EMV:	400 V

- Transition Ions (bold formatting indicates quantitation transition)
  - Time Segments (TS)
  - TS1 – 0-4 minutes (To Waste)
  - TS2 – 4-6.15 minutes (Carboxy-THC-D3, Carboxy-THC, OH-THC-D3, OH-THC)
  - TS3 – 6.15-8 minutes (THC-D3, THC)
  - TS4- 8-10.5 minutes (To Waste)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)
THC-COOH-D3	348.2	**330.2**	50	125	7	12
		302.2				16
THC-COOH	345.2	**299.2**	50	125	7	16
		193.1				24
OH-THC-D3	334	**316**	50	120	7	8
		196				20
OH-THC	331	**313**	50	105	7	8
		193				20
THC-D3	318.2	**196.1**	100	120	7	20
		123				32
THC	315.2	**193.1**	100	120	7	20
		123				32

24.6 Procedure

24.6.1 Note: Urine specimens may be hydrolyzed to remove glucuronide conjugates prior to extraction using the following hydrolysis procedure. A glucuronide positive control should be used to ensure the hydrolysis was effective.

24.6.1.1 Alkaline hydrolysis

Add 40 µL of 10 N NaOH to 1 mL of each urine specimen. The pH should be greater than 10. Cap, vortex and heat at 60°C for 20 minutes. After cooling, add approximately 25 µL of glacial acetic acid or as necessary to neutralize pH.

24.6.2 Prepare calibrators and controls.

24.6.3 Add 1.0 mL case specimens/blank blood to the appropriately labeled tubes.

24.6.4 Add 100 µL of the 0.1 µg/mL internal standard working solution to each tube.

24.6.5 Add 2 mL of water and vortex briefly.

24.6.6 Add 800 µL of 10% acetic acid and vortex.

24.6.7 Add 8.0 mL of 9:1 hexane:ethyl acetate solution.

24.6.8 Cap and rotate tubes for 30 minutes

24.6.9 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation.

24.6.10 Transfer organic (upper) layer to appropriately labeled tubes.

24.6.11 Evaporate samples to dryness at approximately 40-50°C under nitrogen.

24.6.12 Reconstitute samples in 100 µL of 50:50 acetonitrile:water.

24.6.13 Centrifuge at approximately 2800 rpm for 15 minutes.

24.6.14 Transfer to GC autosampler vials with inserts for LCMSMS analysis.

24.7 Quality Control and Reporting

24.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

24.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

24.7.3 The calibration model for all targets, except THC-COOH when quantitated by height, is linear weighted 1/x. THC and its associated internal standard shall be quantitated by height. THC-COOH and OH-THC and their associated internal standards may be quantitated by area or height.

24.7.3.1 If OH-THC is quantitated by height, the calibration model is linear 1/x. If THC-COOH is quantitated by height, the calibration model is quadratic 1/x.

24.7.4 When a target concentration is above the upper limit of quantitation, 1.0 mL of case sample shall be diluted with no more than 3.0 mL of blank matrix for a total dilution volume of 4.0 mL. Alternatively, 0.5 mL of case sample may be used for a dilution of 1/2. If less than 0.5 mL of sample is used for analysis, qualitative results may be reported.

24.7.5 Extracted samples are stable for four days after reconstitution.

24.7.6 This method was validated using the delta-9-tetrahydrocannabinol (Δ9-THC) for reporting tetrahydrocannabinol (THC). However, two known isomers of tetrahydrocannabinol have been indicated to co-elute with Δ9-THC and may cause interference with the identification of THC if present in mixtures with Δ9-THC. The potential impact of these isomers has been evaluated and addressed in the approach to cannabinoid data evaluation to avoid potential misidentification.

The two known isomers are:

Delta-8-tetrahydrocannabinol (Δ8-THC) – Traditionally a phytocannabinoid that is present in low concentrations in cannabis which can be extracted and concentrated.

Delta-9,11-tetrahydrocannabinol (exo-THC) – A synthetic tetrahydrocannabinol isomer that can be a by-product formed during the synthesis of trans-delta-9-tetrahydrocannabinol for pharmaceutical preparations (e.g., Marinol or Dronabinol).

24.7.7 Until new methodology is developed, validated, and approved, cannabinoid batches shall be reviewed by either supervisory staff or designees.

24.7.8 See Toxicology Quality Guidelines

24.8 Notes

Additional guidance for cannabinoid data review and evaluation

24.8.1 Ensure that the expected retention time line is added to all chromatograms for ease of data analysis.

24.8.2 Data review of cannabinoids should follow the below guidance.

24.8.2.1 Do the results meet the QA/QC criteria for reporting positive results established in the Toxicology PM Ch. 2 and 24?

24.8.2.2 Perform a visual analysis of the chromatography and the transition ratios. Is there a noticeable shoulder, double peak, retention time shift, etc. for either THC or THC-COOH?

24.8.2.3 Using Compounds-at-a-Glance, is there a noticeable change to the peak shape?

24.8.2.4 If there are no notable abnormalities in the chromatography using the tools listed above, the quantitative results shall be reported using peak height for THC. Any notable abnormalities will be evaluated on a case-by-case basis.

24.9 References

24.9.1 J.S. Hudson, J.W. Hutchings, P. Friel, in-house development.

24.9.2 Alabama DFS Cannabinoids Method

24.9.3 D.M. Schwope, K.B. Scheidweiler, M.A. Huestis. Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography-tandem mass spectrometry. Analytical Bioanalytical Chemistry. 401(4):1273-1283 (2011)

25 BENZODIAZEPINES, ZOLPIDEM, ZOPICLONE AND ZALEPLON QUANTITATION AND CONFIRMATION BY LCMSMS

Summary

Benzodiazepines, zolpidem, zopiclone, and zaleplon are extracted from biological samples by adding sodium carbonate buffer and extracting with 1-chlorobutane. An aliquot of the extract is quantitated and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

Alprazolam, clonazepam, lorazepam, diazepam, nordiazepam, oxazepam, temazepam, and zolpidem are quantitative targets within this method.

7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxymidazolam, α-hydroxytriazolam, flunitrazepam, flurazepam, N-desalkylflurazepam, midazolam, phenazepam, etizolam, triazolam, zopiclone, and zaleplon are qualitative targets that may be quantitated upon toxicologist review.

Flubromazolam, flubromazepam, pyrazolam, flualprazolam, α-hydroxyalprazolam, 8-aminoclonazolam, bromazepam, clonazolam, and bromazolam are analyzed qualitatively only.

Chlordiazepoxide is analyzed qualitatively only with this method but can be quantitated using the Chlordiazepoxide method.

25.2 Specimen Requirements

1 mL blood, fluid or tissue homogenate

25.3 Reagents and Standards

- Drug targets and associated internal standards.

Targets	Internal Standards
7-aminoclonazepam	7-aminoclonazepam-D_4
7-aminoflunitrazepam	7-aminoclonazepam-D_4
Zopiclone	Zopiclone-D_4
Zolpidem	Zolpidem-D_6
Zaleplon	Zolpidem-D_6
Chlordiazepoxide	Diazepam-D_5
Flurazepam	Diazepam-D_5
Nordiazepam	Diazepam-D_5
N-desalkylflurazepam	Diazepam-D_5
Phenazepam	Diazepam-D_5
Diazepam	Diazepam-D_5
α-hydroxyalprazolam	α-hydroxyalprazolam-D_5
α-hydroxymidazolam	α-hydroxyalprazolam-D_5
α-hydroxytriazolam	α-hydroxyalprazolam-D_5
Midazolam	Alprazolam-D_5
Alprazolam	Alprazolam-D_5
Triazolam	Alprazolam-D_5
Oxazepam	Oxazepam-D_5
Lorazepam	Oxazepam-D_5
Clonazepam	Clonazepam-D_4
Flunitrazepam	Clonazepam-D_4
Temazepam	Temazepam-D_5

Etizolam	Diazepam-D_5
Flubromazolam	Alprazolam-D_5
Flubromazepam	Clonazepam-D_4
Flualprazolam	Alprazolam-D_5
Pyrazolam	Alprazolam-D_5 or a-hydroxyalprazolam-D_5
8-aminoclonazolam	Alprazolam-D_5
Bromazepam	Alprazolam-D_5
Clonazolam	Alprazolam-D_5
Bromazolam	Alprazolam-D_5

- Sodium carbonate, certified ACS powder
- 1-chlorobutane, HPLC grade
- Acetonitrile, Fisher Optima (or similar) grade or higher
- Type I or LC-MS grade water
- Formic Acid, eluent additive for LC-MS

25.4 Solutions, Internal Standard, Calibrators and Controls

25.4.1 0.2 M Sodium Carbonate: weigh out 10.6 g sodium carbonate, transfer to a 500 mL volumetric flask and qs to volume with dH_2O. Store at room temperature for up to 2 years.

25.4.2 Mobile Phase A (H_2O with 0.1% formic acid): add 1 mL of formic acid to 1 L of Type I or LC-MS grade H_2O. Store at room temperature for up to one month.

25.4.3 Mobile Phase B (Acetonitrile with 0.1% formic acid): add 1 mL of formic acid to 1 L of acetonitrile. Store at room temperature for up to one month.

25.4.4 Drug stock solutions:

If 1 mg/mL commercially prepared stock solutions are not available, prepare 1 mg/mL solutions from powders. Weigh 10 mg of the free drug, transfer to a 10 mL volumetric flask and qs to volume with methanol. Note: If using the salt form, determine the amount of the salt needed to equal 10 mg of the free drug, and weigh this amount. Stock solutions are stored capped in a freezer and are stable for up to 2 years or manufacturer's specifications, whichever is earlier.

25.4.5 Preparation of calibrators. Note: calibrator solutions may be prepared as a mix or as separate solutions.

25.4.5.1 Working standard solution A (0.01 mg/mL): This standard solution is used to prepare calibrators of frequently quantitated targets. Pipette 100 µL of the 1 mg/mL (or 1 mL of 0.1 mg/mL) stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable. Targets include: alprazolam, clonazepam, diazepam, lorazepam, nordiazepam, oxazepam, temazepam, and zolpidem. Other targets may be spiked into working standard solution A (e.g., midazolam for screening).

25.4.5.2 Working standard solution A (0.001 mg/mL): Pipette 1.0 mL of the 0.01 mg/mL working standard solution A into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

25.4.5.3 Working standard solution B (0.01 mg/mL): This standard solution is used to prepare calibrators of infrequently quantitated targets. Pipette 100 µL of the 1 mg/mL (or 1 mL of 0.1 mg/mL) stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable. Targets may include any compounds that have been validated for quantitation. Solutions for zopiclone should be prepared fresh for each quantitative analysis.

25.4.5.4 Working standard solution B (0.001 mg/mL): Pipette 1.0 mL of the 0.01 mg/mL working standard solution B into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

25.4.5.5 Working standard solution C (0.01 mg/mL): This standard solution is used in the method to act as a threshold control for qualitative targets. Pipette 100 µL of the 1 mg/mL (or 1 mL of 0.1 mg/mL) stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable. Targets may be analyzed in different combinations or individually as needed.

25.4.5.6 Working standard solution C (0.001 mg/mL): Pipette 1.0 mL of the 0.01 mg/mL working standard solution C into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

25.4.5.7 Stock internal standard solution (0.01 mg/mL): Pipette 100 µL of the 1 mg/mL (or 1 mL of 0.1 mg/mL) stock solution of deuterated standards into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

25.4.5.8 Working internal standard solution (0.001 mg/mL): Pipette 1.0 mL of the 0.01 mg/mL stock internal standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

25.4.5.9 To prepare calibration curve A or B, pipette the following volumes of the 0.01 mg/mL and 0.001 mg/mL working standard solution A or B into appropriately labeled 16 x 125 mm screw cap test tubes. To eliminate a solvent effect, calibrators and controls may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to obtain the final concentrations listed below.

Calibrators shall be divided into three calibrator solutions. Calibrator working standard A will be spiked quantitatively for every analysis. In addition, calibrator working standard C will be spiked and analyzed at high and low (threshold) concentrations. In the event that a target from working standard C is positive, a separate extraction may be spiked using an appropriate working standard (the separate extraction is not necessary if the appropriate calibrator solutions are utilized concurrently).

Amount of 0.01 mg/mL stock solution (µL)	Amount of 0.001 mg/mL stock solution (µL)	Final concentration of benzodiazepines (mg/L)
200		2.0
100		1.0
50		0.5
	100	0.1
	50	0.05
	20	0.02
	10	0.01

Threshold Control (TC) Standard C is prepared by pipetting 5 µL of 0.001 mg/mL working standard solution C for a threshold control. This may be adjusted based upon validated LOD for targets. A higher concentration control may be spiked as well.

25.4.6 Controls

25.4.6.1 Benzodiazepine/ZZZ Controls. Controls may be from an external source or prepared in-house using drugs from different manufacturers or lot numbers.

25.4.6.1.1 Due to the quadratic nature of many of the targets, at least three controls, at low medium and high concentrations, must be run across the concentration range in every batch. If the high calibrator is 2 mg/L, a high control between 1 and 2 mg/L must be run.

25.4.6.2 Negative control. Blood bank blood or equivalent determined not to contain benzodiazepines or other targets.

25.5 Apparatus

25.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

25.5.2 Test tubes, conical bottom

25.5.3 Centrifuge capable of 2000-3000 rpm

25.5.4 Evaporator/concentrator

25.5.5 Vortex mixer

25.5.6 GC autosampler vials with inserts

25.5.7 Typical LCMSMS parameters.

25.5.7.1 LC Parameters

- Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size
- Column Thermostat: 35°C
- Mobile Phase A: H_2O with 0.1% formic acid
- Mobile Phase B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.50 mL/min
- Injection vol.: 3 µL with a minimum 5 second needle wash
- Stop time: 11 min
- Gradient:

Initial	10% B
4 minutes	30% B
8 minutes	40% B
8.5 minutes	95% B
10.5 minutes	95% B
11 minutes	10% B
Post Time	Minimum 1.5 min

25.5.7.2 Typical MS-MS parameters.

- MSD Parameters:

Ionization:	ESI
Polarity:	positive
Gas temp:	350°C
Drying Gas:	10.0 L/min
Nebulizer press:	50 psi
Capillary:	4000 V
Delta EMV:	400 V

- Transition Ions (bold formatting indicates the quantitation/target transition)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)	Approx. RT (min)
7-aminoclonazepam-D4	290.1	**121.1**	142	7	30	1.8
		77.1			66	
7-aminoclonazepam	286.1	**121.1**	147	7	34	1.9
		77.1			62	
Zopiclone-D4	393.1	**245**	72	7	12	2.3
		217			32	
Zopiclone	389.1	**245**	82	7	13	2.3
		217			33	
7-aminoflunitrazepam	284.1	**135.1**	147	7	26	2.4
		77.1			74	
8-aminoclonazolam	324.1	296	41	7	29	3.1
		220.1			50	
Zolpidem-D6	314.2	263.1	168	7	24	3.1
		235.1			36	
Zolpidem	308.2	263.1	160	7	24	3.2
		235.1			36	
Chlordiazepoxide	300.1	**227**	105	7	22	3.4
		89.1			74	
Pyrazolam	354	206	147	7	45	3.8
		167			45	
Bromazepam	316.1	209.1	50	7	29	4.1
		182.1			41	
α-hydroxymidazolam	342.1	**324.1**	148	7	21	4.2
		168.1			45	
Midazolam	326.1	**291.1**	194	7	29	4.4
		249.1			41	
Flurazepam	388.2	317.1	158	7	17	4.5
		315			21	
Zaleplon	306.1	264.1	130	7	20	5
		236.1			24	
α-hydroxyalprazolam-D5	330.1	**302.1**	147	7	26	5.3
		210.1			50	
α-hydroxyalprazolam	325.1	**216.1**	148	7	42	5.3
		205.1			50	
Clonazolam	354.1	**308.1**	145	7	28	5.3
		177			76	
α-hydroxytriazolam	359.1	239	148	7	46	5.4
		176			26	
Oxazepam-D5	292.1	274.1	120	7	8	5.5
		246.1			20	
Oxazepam	287.1	269	110	7	8	5.5
		241			20	
Flualprazolam	327.1	299	168	7	18	5.7
		223			48	
Clonazepam-D4	320.1	**274.1**	135	7	24	5.7
		218.1			40	
Clonazepam	316.1	**270**	142	7	22	5.8

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)	Approx. RT (min)
		214			46	
Nordiazepam	271.1	165	153	7	26	5.8
		140			30	
Lorazepam	321	**303**	120	7	8	5.8
		229	115		28	
Alprazolam-D5	314.1	**286.1**	148	7	26	5.9
		210.1			42	
Alprazolam	309.1	**205.1**	143	7	46	6
		151.1			74	
Flubromazolam	371	342.9	168	7	30	6
		292			26	
Triazolam	343	315	170	7	28	6.2
		308			24	
N-desalkylflurazepam	289.1	226.1	140	7	28	6.2
		140			28	
Bromazolam	353	**325**	50	7	33	6.2
		205.1			50	
Flunitrazepam	314.1	**268.1**	153	7	25	6.2
		239.1			37	
Temazepam-D5	306.1	288.1	106	7	10	6.5
		260.1			22	
Temazepam	301.1	283	116	7	10	6.5
		255.1			18	
Flubromazepam	333	**226**	148	7	30	6.6
		104			70	
Etizolam	343.1	**314**	140	7	24	6.6
		289			20	
Phenazepam	351	**206**	140	7	36	7.1
		179			50	
Diazepam-D5	290.1	198.1	150	7	36	7.2
		154			28	
Diazepam	285.1	**193.1**	130	7	32	7.4
		91.1			52	

25.6 Procedure

25.6.1 Note: Urine specimens may be hydrolyzed to remove glucuronide conjugates prior to extraction using one of the following hydrolysis procedures. A glucuronide positive control should be used to ensure the hydrolysis was effective.

25.6.1.1 Enzyme hydrolysis

Add 5000 Fishman units of β-glucuronidase to each mL of urine. Perform hydrolysis as recommended by the supplier based on the source of β-glucoronidase (e.g., 5000 F units/mL Patella vulgata in 100 mM acetate buffer (pH 5.0) hydrolyzed at 65°C for 3 hours).

25.6.1.2 Alkaline hydrolysis

Add 80 µL of 10 N NaOH to 2 mL of each urine specimen. The pH should be greater than 10. Cap, vortex and heat at 60°C for 20 minutes. After cooling, add 50 µL of glacial acetic acid and acetate buffer as necessary to neutralize pH.

25.6.2 Label clean screw cap tubes appropriately with calibrators, controls and case sample IDs.

25.6.3 Prepare calibrators and controls.

25.6.4 Add 1.0 mL case specimens to the appropriately labeled tubes.

25.6.5 Add 100 µL of the 0.001 mg/mL internal standard working solution to each tube and vortex.

25.6.6 Add 1 mL sodium carbonate and 6 mL 1-chlorobutane to each tube.

25.6.7 Cap and rotate tubes for 30 minutes.

25.6.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. Transfer organic (upper) layer to appropriately labeled tubes.

25.6.9 Evaporate samples to dryness at approximately 50°C under nitrogen.

25.6.10 Reconstitute samples in 200 µL methanol. Transfer to GC autosampler vials with inserts for LCMSMS analysis.

25.7 Quality Control and Reporting

25.7.1 For quantitated targets, the LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

25.7.2 For quantitated targets, the upper limit of quantitation (ULOQ) for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

25.7.3 Qualitative targets (i.e., 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxymidazolam, α-hydroxytriazolam, N-desalkylflurazepam, chlordiazepoxide, flunitrazepam, flurazepam, midazolam, phenazepam, etizolam, triazolam, flubromazolam and flubromazepam) are compared to the threshold control prepared from working standard solution C (or other labeled standard solution).

If present, N-desalkylflurazepam, midazolam, phenazepam, etizolam, and triazolam may be quantitatively assessed using a calibration curve prepared from working standard solution B. Chlordiazepoxide may be quantitatively assessed (see ¶ 15).

25.7.4 The calibration model for benzodiazepines is weighted (1/x) quadratic whereas the calibration model for zolpidem, zopiclone and zaleplon is weighted (1/x) linear. Samples with a concentration greater than 1 mg/L for a target with a quadratic fit must be repeated if the high positive control is outside of acceptable limits.

25.7.5 When a target concentration is above the upper limit of quantitation, 1.0 mL of case sample shall be diluted with no more than 3.0 mL of blank matrix for a total dilution volume of 4.0 mL. Alternatively, 0.5 mL of case sample may be used for a dilution of 1/2. If less than 0.5 mL of sample is used for analysis, only qualitative results may be reported.

25.7.6 Threshold Control: Threshold control is prepared at 0.005 mg/L however this may be adjusted to the validated LOD for each target. Validated LOD for each target is:

0.005 mg/L: 7-aminoclonazepam, α-hydroxyalprazolam, α-hydroxytriazolam, lorazepam, flualprazolam, pyrazolam, 8-aminoclonazolam, bromazepam, clonazolam, bromazolam

0.0025 mg/L: 7-aminoflunitrazepam, N-desalkylflurazepam, alprazolam, clonazepam, diazepam, etizolam, nordiazepam, oxazepam, phenazepam, zaleplon

0.001 mg/L: α-hydroxymidazolam, chlordiazepoxide, flunitrazepam, flurazepam, midazolam, temazepam, triazolam, zolpidem, zopiclone

25.7.7 Chlordiazepoxide: If a case sample has a relative response greater than the TC but lower than the qualitative control, this may be reported as "Present, less than 0.50 mg/L." If the case sample relative response is greater than the qualitative control, the sample may be quantitated with the Chlordiazepoxide Quantitation and Confirmation by LCMSMS method.

25.7.8 Due to the known instability of zopiclone in alkaline solutions, cases with poor zopiclone IS recovery do not require reinjection and/or reanalysis unless indicated by case history and/or the detection of transitions consistent with zopiclone during analysis.

25.7.9 Extracted samples are stable for seven days with the exception of diazepam and triazolam which are only stable for six days after reconstitution. Etizolam is considered stable for three days after extraction.

25.7.10 See Toxicology Quality Guidelines.

25.8 References

25.8.1 J.S. Hudson, J.W. Hutchings, P. Friel, in-house development

25.8.2 Remane D, Meyer MR, Wissenbach DK and Maurer HH. "Ultra high performance liquid chromatographic-tandem mass spectrometric multi-analyte procedure for target screening and quantification in human blood plasma: validation and application for 31 neuroleptics, 28 benzodiazepines, and Z-drugs", *Anal Bioanal Chem*., 2011, 401(4), pp. 1341-52.

25.8.3 R.L. Wagner, Validation of Etizolam Quantitation and Confirmation by Liquid-Liquid Extraction Using LCMSMS, 2021.

25.8.4 K. Meinweiser, Qualitative Validation for Pyrazolam, 2019.

25.8.5 C. Harris and T. Wright, Qualitative Flualprazolam Validation, 2019.

25.8.6 K. Schneider, Flubromazolam Validation, 2015.

25.8.7 K. Schneider, Qualitative Validation for Flubromazepam, 2016.

25.8.8 R. Wagner, A. Siddiqi, Validation of Etizolam Quantitation and Confirmation by Liquid-Liquid Extraction Using LCMSMS, 2021.

25.8.9 A. Jango, Qualitative Validation of 8-Aminoclonazolam, Bromazepam, Clonazolam, and Bromazolam, 2022.

26 AMPHETAMINES, PHENTERMINE AND DESIGNER STIMULANTS QUANTITATION AND CONFIRMATION BY LCMSMS

26.1 Summary

Biological samples are made basic with trisodium phosphate buffer and extracted with 6 mL of n-butylchloride. The organic layer is dried down and reconstituted in mobile phase and injected into the LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

26.2 Specimen Requirements

1 mL of whole blood, bodily fluids, or tissue homogenate

26.3 Reagents and Standards

- Drug targets and associated internal standards. Note: drugs may be analyzed in combinations or separately.

Targets	Internal Standards
Methcathinone	Mephedrone-D_3
Pseudoephedrine	Pseudoephedrine-D_3
Methylone	Methylone-D_3
Amphetamine	Amphetamine-D_{11}
Methamphetamine	Methamphetamine-D_{11}
MDA	MDA-D_5
Methedrone	Mephedrone-D_3
MDMA	MDMA-D_5
Phentermine	Methamphetamine-D_{11}
Mephedrone	Mephedrone-D_3
Alpha-PVP	Mephedrone-D_3
MDPV	Mephedrone-D_3
Bupropion	Mephedrone-D_3
Ethylone*	Methylone-D_3
Ephedrine*	Pseudoephedrine-D_3

*Ephedrine and ethylone may be qualitatively analyzed only.

- Trisodium phosphate (Na_3PO_4), ACS powder
- 1-chlorobutane, HPLC grade
- Hydrochloric acid, Optima (or similar) grade or higher
- Isopropanol, HPLC grade
- Formic Acid, eluent additive ~98%
- Type I or LC-MS grade water
- Methanol, HPLC grade or higher
- Acetonitrile, Optima (or similar) grade or higher

26.4 Solutions, Internal Standards, Calibrators and Controls

26.4.1 0.2% Hydrochloric acid in isopropanol: add 1 mL of concentrated HCl (12 N) into 500 mL of isopropanol. Store at room temperature for up to one month.

26.4.2 Mobile Phase A (H_2O with 0.1% formic acid): add 1 mL of formic acid to 1 L of Type I or LC-MS grade H_2O. Store at room temperature for up to one month.

26.4.3 Mobile Phase B (Acetonitrile with 0.1% formic acid): add 1 mL of formic acid to 1 L of acetonitrile. Store at room temperature for up to one month.

26.4.4 Saturated trisodium phosphate buffer. Add trisodium phosphate to dH_2O until no more dissolves after vigorous shaking. Store at room temperature for up to 2 years.

26.4.5 Working Solution A (10 µg/mL): Add 100 µL of 1.0 mg/mL standard to a 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

26.4.6 Working Solution B (1.0 µg/mL): Pipette 1.0 mL Working Solution A into a 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

26.4.7 Working Internal Standard (1 µg/mL): Pipette 10 µL of 1.0 mg/mL (or 100 µL of 0.1 mg/mL) internal standard into 10 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

26.4.8 To prepare the calibration curve, add the following volumes of standard solution into 16 x 125 mm screw cap test tubes. Calibrators and controls shall not be dried down under any circumstances (i.e., using nitrogen or heat). Add 1 mL blank blood to each tube to obtain the final concentrations listed below.

Amount of 10 µg/mL stock solution (µL)	Amount of 1.0 µg/mL stock solution (µL)	Final concentration (mg/L)
200		2.00
100		1.00
50		0.50
25		0.25
	100	0.10
	50	0.05
	20	0.02
	10	0.01
	5	TC (0.005)

26.4.9 Controls

26.4.9.1 Negative control blood: blood bank blood or equivalent determined not to contain target compounds.

26.4.9.2 In-house control is prepared from a different lot number or different manufacturer of standard.

26.4.9.2.1 Due to the quadratic nature of many of the targets, at least three controls, at low, medium and high concentrations, must be run across the concentration range in every batch. A high control must be run within 1 – 2 mg/L

26.5 Apparatus

26.5.1 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

26.5.2 Test tubes, conical bottom

26.5.3 Centrifuge capable of 2,000 – 3,000 rpm

26.5.4 Vortex mixer

26.5.5 Test tube rotator

26.5.6 GC autosampler vials and inserts

26.5.7 LC Parameters

- Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size
- Column Thermostat: 50 °C
- Mobile Phase A: H_2O with 0.1% formic acid
- Mobile Phase B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.5 mL/min
- Injection vol.: 2 µL with a minimum 6 second needle wash
- Stop time: 9 min
- Gradient:

Initial	2% B
2 minutes	5% B
4 minutes	10% B
6 minutes	30% B
7 minutes	90% B
8.5 minutes	90% B
9 minutes	2% B
Post time	Minimum 1 minute

26.5.8 Typical MS-MS parameters.

- MSD Parameters:

Ionization:	ESI
Polarity:	positive
Gas temp:	350 °C
Drying Gas:	10.0 L/min
Nebulizer pressure:	45 psi
Capillary:	3000 V
Delta EMV:	200 V

- Transition Ions (Note: Methamphetamine and phentermine may be detected in each other's MRM windows. Retention times below are indicative of elution order of these compounds, ensure that the correct peak is chosen for analysis. Phentermine elutes later than methamphetamine.)

Compound	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**Fragmentor (V)**	**Cell Accelerator (V)**	**Collision Energy (V)**	**Approx. RT (min)**
Ephedrine	166.1	**148.1**	81	7	5	2.3
		133.1	80		21	
Methcathinone	164.2	**146**	85	7	10	2.4
		130			34	
Pseudoephedrine-D3	169.1	**151.1**	80	7	8	2.7
		115			28	
Pseudoephedrine	166.1	**148.1**	81	7	5	2.8
		133.1	80		21	
Methylone	208.2	190	80	7	14	3
		132			26	
Amphetamine-D11	147.2	130.1	75	7	4	3.2
		98.1			16	
Amphetamine	136.1	119.1	75	7	4	3.4
		91.1			16	
Methylone-D3	211.2	**163**	85	7	13	3.4
		135			29	
MDA-D5	185.1	**168.1**	80	7	8	3.5
		110.1			24	
MDA	180.1	**163.1**	75	7	4	3.6
		105.1			24	

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)	Approx. RT (min)
Methamphetamine-D11	161.2	127.1	85	7	8	3.7
		97.1			20	
Ethylone	222.1	**174**	110	7	18	3.7
		146			28	
Methamphetamine	150.1	119.1	90	7	8	3.8
		91.1			20	
Methedrone	194.2	**176**	90	7	8	3.8
		161			20	
MDMA-D5	199.1	**165.1**	90	7	8	4
		107.1			24	
MDMA	194.1	**163.1**	90	7	8	4.1
		105.1			24	
Mephedrone	178.3	**160**	85	7	10	4.7
		144			30	
Mephedrone-D3	181.3	**163**	90	7	9	4.8
		148			21	
Phentermine	150.1	**91.1**	70	7	21	4.8
		65.1			45	
α-PVP	232.2	126.1	115	7	28	6.5
		91			24	
MDPV	276.3	135	130	7	25	6.7
		126			25	
Bupropion	240	**184**	80	7	5	7
		166			10	

26.6 Procedure

26.6.1 Label clean screw cap tubes appropriately with calibrators, controls, and case sample IDs.

26.6.2 Prepare calibrators and controls.

26.6.3 Add 1 mL of case specimens to the appropriately labeled tubes. Note: since this procedure may be used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case as appropriate. One will serve as a screen and the second as a confirmation.

26.6.4 Add 100 µL of 1 µg/mL internal standard working solution to each tube and vortex.

26.6.5 Add 2 mL saturated trisodium phosphate buffer to each tube. Vortex briefly.

26.6.6 Add 6 mL 1-chlorobutane to each tube.

26.6.7 Cap and rotate for 15 minutes at slow speed.

26.6.8 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. If emulsion or suspension forms, knock it down with a wooden stick and centrifuge again.

26.6.9 Transfer upper layer (1-chlorobutane) to clean, screw-cap tube.

26.6.10 Add 100 µL of 0.2% HCl in isopropanol to each tube and evaporate samples to dryness at approximately 40°C.

26.6.11 Reconstitute in 200 µL of 0.1% formic acid in water.

26.6.12 Inject into LCMSMS

26.7 Quality Control and Reporting

26.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

26.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

26.7.3 The following dilution factors may be utilized for this analysis:

Targets that can be diluted up to 1/20 (0.05 mL sample and 0.95 mL blank matrix or 1.0 mL sample and 19.0 mL blank matrix)
Methcathinone
Pseuodephedrine
Methylone
Amphetamine
Methamphetamine
MDA
Methedrone
MDMA
Phentermine
Mephedrone
α-PVP

Targets that can be diluted up to 1/5 (0.2 mL sample and 0.8 mL blank matrix or 1.0 mL sample and 4.0 mL blank matrix)
Bupropion

Targets that can be diluted up to 1/2 (0.5 mL sample and 0.5 mL blank matrix or 1.0 mL sample and 1.0 mL blank matrix)
MDPV

26.7.4 The calibration model for all targets is weighted (1/x) quadratic except methcathinone, pseudoephedrine, methylone, and mephedrone which is weighted (1/x) linear. Samples with a concentration greater than 1 mg/L for a target with a quadratic fit must be repeated if the high positive control is outside of acceptable limits.

26.7.5 Threshold Control: Threshold control is prepared at 0.005 mg/L however this may be adjusted to the validated LOD for each target. Phentermine's validated LOD is 0.01 mg/L however it will be set administratively at 0.005 mg/L and evaluated with each batch. Validated LOD for other targets:

0.005 mg/L: methcathinone, pseudoephedrine, amphetamine, methamphetamine

0.0025 mg/L: methylone, α-PVP, ephedrine

0.00125 mg/L: MDA, methedrone, MDMA, mephedrone, MDPV, bupropion

26.7.6 Extracted samples are stable for seven days with the exception of MDA (five days) and methcathinone (three days) after reconstitution.

26.7.7 See Toxicology Quality Guidelines

26.7.8 Pseudoephedrine and ephedrine can be known precursors to the production of methamphetamine therefore pseudoephedrine and ephedrine may be reported from one analysis if methamphetamine is present. If pseudoephedrine and/or ephedrine is present without methamphetamine, it needs to be analyzed and detected twice to be reported.

26.8 References

26.8.1 “Amphetamine and Methamphetamine" by Wayne Harrington, Methodology of Analytical Toxicology by Irving Sunshine (Ed), CRC Press, 1975.

26.8.2 R.L. Wagner, J.S. Hudson, J.W. Hutchings, and P. Friel, in-house development.

26.8.3 R.L. Wagner, Validation of Ephedrine Confirmation by Liquid-Liquid Extraction Using LCMSMS, 2022.

26.8.4 K. Schneider, Ethylone validation, 2015.

27 ANTI-EPILEPTIC DRUGS QUANTITATION AND CONFIRMATION BY LCMSMS

27.1 Summary

Anti-epileptic drugs (AEDs) are extracted from biological samples using a methanol precipitation. An aliquot of the extract is quantitated and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

27.2 Specimen Requirements

0.2 mL blood, fluid or tissue homogenate

27.3 Reagents and Standards

- Drug targets and associated internal standards

Target	Internal Standard
Gabapentin	Gabapentin-D_{10}
Levetiracetam	Levetiracetam-D_6
Lamotrigine	Levetiracetam –D_6
Zonisamide	Topiramate-D_{12}
Licarbazepine	Carbamazepine-D_{10}
Oxcarbazepine	Carbamazepine-D_{10}
Topiramate	Topiramate-D_{12}
Carbamazepine	Carbamazepine-D_{10}
Phenytoin	Phenytoin-D_{10}
Pregabalin	Gabapentin-D_{10}
Lacosamide	Gabapentin-D_{10}

- Methanol, HPLC grade or higher
- Acetonitrile, Fisher Optima (or similar) grade or higher
- Formic Acid, eluent additive for LC-MS
- Type I or LC-MS grade water
- Ammonium Acetate

27.4 Solutions, Internal Standard, Calibrators and Controls

27.4.1 Mobile Phase A (H_2O with 5 mM Ammonium Acetate): add approximately 0.385 grams of ammonium acetate to 1.0 L of dH_2O. Store at room temperature for up to one month.

27.4.2 Mobile Phase B (Acetonitrile with 0.1% formic acid): add 1.0 mL of formic acid to 1.0 L of acetonitrile. Store at room temperature for up to one month.

27.4.3 Preparation of calibrators.

27.4.3.1 Working internal standard solution (10 µg/mL): Pipette 1.0 mL of the 0.1 mg/mL (or 100 µL of 1.0 mg/mL) stock solution of deuterated standards into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

27.4.3.2 Working standard solution (100 µg/mL): Pipette 1.0 mL of the 1.0 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

27.4.3.2.1 Alternative: (50 µg/mL): Pipette 500 µL of the 1.0 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

27.4.3.3 Working standard solution (10 µg/mL): Pipette 1.0 mL of the 100 µg/mL working standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

27.4.3.3.1 Alternative: (5 µg/mL): Pipette 1.0mL of the 50 µg/mL working standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

27.4.3.4 To prepare the calibration curve, pipette the following volumes of the 100 µg/mL and 10 µg/mL (50 mg/mL and 5 ug/mL, in parentheses in table) working standard solutions into appropriately labeled 16 x 100 mm or 16 x 125 mm screw cap test tubes. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood. Add 0.2 mL blank blood to obtain the final concentrations listed below.

Amount of 100 µg/mL (50 µg/mL) stock solution (µL)	Amount of 10 µg/mL (5 µg/mL) stock solution (µL)	Final concentration of AEDs (mg/L)
80 (160)		40
60 (120)		30
40 (80)		20
20 (40)		10
	100 (200)	5
	50 (100)	2.5
	20 (40)	1
	10 (20)	TC (0.5)

27.4.4 **Controls**

27.4.4.1 AED Controls. Controls may be from an external source or prepared in-house using standards from different manufacturers or lot numbers.

27.4.4.1.1 Due to the quadratic nature of many of the targets, at least three controls, at low medium and high concentrations, must be run across the concentration range in every batch. If the high calibrator is 40 mg/L, a high control must be run within 30 – 40 mg/L.

27.4.4.2 Negative control. Blood bank blood or equivalent determined not to contain AEDs or other targets.

27.5 Apparatus

27.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

27.5.2 Centrifuge capable of 2000-3000 rpm

27.5.3 Evaporator/concentrator

27.5.4 Vortex mixer

27.5.5 GC autosampler vials with inserts

27.5.6 LC Parameters

- Column: Poroshell 120 EC-C18, 3.0 x 100 mm, 2.7 µm particle size
- Column Thermostat: 40 °C
- Mobile Phase A: H_2O with 5 mM Ammonium Acetate

- Mobile Phase B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.50 mL/min
- Injection vol.: 5.0 µL with a minimum 5 second needle wash
- Stop time: 11 min
- Gradient:

Initial	10% B
1 minutes	20% B
8 minutes	60% B
9.5 minutes	95% B
10 minutes	95% B
11 minutes	10% B
Post time	Minimum 2 minutes

27.5.7 MS-MS parameters.

- MSD Parameters:

Ionization:	ESI
Polarity:	Positive
Gas temp:	350 °C
Drying Gas:	10.0 L/min
Nebulizer press:	40 psi
Capillary	4000 V
Delta EMV	300 V

- Transition Ions (bold formatting indicates the quantitation transition)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)	Approx. RT (min)
Gabapentin-D10	182.2	**164.2**	100	7	12	1.8
		147.2			16	
Gabapentin	172	**154**	80	7	15	1.8
		95			25	
Pregabalin	160.1	**142.1**	75	7	4	1.9
		55.1			20	
Levetiracetam-D6	177.1	160.1	70	7	0	2
		132.1			12	
Levetiracetam	171	**126**	90	7	10	2
		69			25	
Lamotrigine	256	**159**	100	7	20	3.5
		145			25	
Zonisamide	213	132	70	7	20	3.5
		77			30	
Lacosamide	251.1	**108**	80	7	4	3.7
		91			16	
Licarbazepine	255	**237.1**	75	7	4	4
		194.1			20	
Oxcarbazepine	253.1	236	105	7	8	4.8
		180			28	
Topiramate-D12	369.2	**270.1**	75	7	8	5
		190			12	
Topiramate	357.1	**264.1**	85	7	8	5
		184.1			16	
Carbamazepine-D10	247.2	**204.1**	120	7	20	5.5
		202.1			40	
Carbamazepine	237	**194**	135	7	16	5.5
		179			36	

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Cell Accelerator (V)	Collision Energy (V)	Approx. RT (min)
Phenytoin-D10	263.2	**192.1**	85	7	16	5.6
		109.1			40	
Phenytoin	253.1	**182**	85	7	16	5.6
		104			32	

27.6 Procedure

27.6.1 Label clean screw cap centrifuge tubes appropriately with calibrators, controls and case sample IDs.

27.6.2 Prepare calibrators and controls. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen prior to the addition of blank blood.

27.6.3 Add 0.2 mL case specimens/blank blood to the appropriately labeled tubes. Note: since this procedure is used for screening and confirmation, it is recommended to analyze two different aliquots (or tissues) with each case as appropriate. One will serve as a screen and the second as a confirmation.

27.6.4 Add 100 µL of the 10 µg/mL internal standard working solution to each tube.

27.6.5 Vortex briefly.

27.6.6 Add 1.0 mL of methanol to each tube and vortex for 15-30 seconds. Ensure that the blood in the bottom of the tube mixes thoroughly with the methanol.

27.6.7 Centrifuge the tubes for 15 minutes at approximately 2800 rpm.

27.6.8 Transfer approximately 150 µL of the upper methanol phase into labeled autosampler vials. Avoid transferring any of the precipitate into the autosampler vial.

27.7 Quality Control and Reporting

27.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

27.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

27.7.3 Dilutions of case samples with target concentrations greater than the ULOQ. Dilutions are listed as 1/2 which is 0.2 mL of case sample diluted with 0.2 mL of blank matrix, 1/5 which is 0.2 mL of case sample diluted with 0.8 mL of blank matrix, 1/10 which is 0.2 mL of case sample diluted with 1.8 mL of blank matrix, 1/20 which is 0.2mL of case sample diluted with 3.8mL of blank matrix. After dilution, 0.2 mL of diluted sample is used for extraction.

The following targets can be diluted by a factor of 1/20
Lacosamide
Pregabalin

The following targets can be diluted by a factor of 1/10
Gabapentin
Levetiracetam
Licarbazepine
Topiramate
Carbamazepine

The following targets can be diluted by a factor of 1/5
Lamotrigine

The following targets can be diluted by a factor of ½
Zonisamide
Oxcarbazepine
Phenytoin

27.7.4 The calibration model for all targets is weighted (1/x) quadratic except for phenytoin and levetiracetam which is weighted (1/x) linear. Samples with a concentration greater than 30 mg/L for a target with a quadratic fit must be repeated if the high positive control is outside of acceptable limits.

27.7.5 Threshold Control is prepared at 0.5 mg/L for all targets however the validated LOD for all targets is 0.125 mg/L.

27.7.6 Extracted samples are stable for seven days with the exception of phenytoin (four days), oxcarbazepine metabolite (five days), and carbamazepine (six days) following reconstitution.

27.7.7 See Toxicology Quality Guidelines

27.8 References

27.8.1 R.L. Wagner, J.S. Hudson, J.W. Hutchings, P. Friel, in-house development.

27.8.2 Daniel Dietmann, Uta Juergas, Bernhard J. Steinhoff, Lea Bonnington, Juergen Wendt; *Simultaneous Analysis of Newer Antiepiliptic Drugs by Rapid Resolution LC/Triple Quadrupole Mass Spectrometry*, ASMS 2008; Agilent Technologies.

27.8.3 R.L. Wagner, Addition of Pregabalin and Lacosamide to the Existing Anti-Epileptic Drugs Quantitation and Confirmation by LCMSMS Method.

28 OPIOID, COCAINE, BENZOYLECGONINE AND COCAETHYLENE QUANTITATION AND CONFIRMATION BY LCMSMS

28.1 Summary

Opioids, cocaine, cocaethylene, and benzoylecgonine are extracted from biological samples using an acetonitrile precipitation or solid phase extraction. An aliquot of the extract is quantitated and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

Fentanyl derivatives are extracted using the above stated process and are qualitatively analyzed by LCMSMS using a different acquisition method.

Xylazine and medetomidine are extracted from biological samples using acetonitrile precipitation (Option 1 LLE) then quantitated and confirmed by LCMSMS.

Method title may be abbreviated as: OpiCoc, opicoc, Opi-Coc, opi-coc, Opicoc (or similar)

28.2 Specimen Requirements

1.0 mL of blood, fluid or tissue homogenate (For fentanyl derivatives, use caution in interpretation for tissue homogenates due to potential ionization suppression for targets and internal standard.) Xylazine and medetomidine can be analyzed quantitatively in blood and qualitatively in blood and/or urine.

28.3 Reagents and Standards

- Drug targets and associated internal standards (OpiCoc)

Targets	Internal Standards
Morphine	Morphine-D_3
Oxymorphone	Oxymorphone-D_3
Hydromorphone	Hydromorphone-D_3
Codeine	Codeine-D_3
Oxycodone	Oxycodone-D_3
6-Acetylmorphine	6-Acetylmorphine-D_3
Hydrocodone	Hydrocodone-D_3
Benzoylecgonine	Benzoylecgonine-D_3
Tramadol	Tramadol-^{13}C-D_3
Cocaine	Cocaine-D_3
Meperidine	Meperidine-D_4
Acetyl Fentanyl	Fentanyl-D_5
Cocaethylene	Cocaine-D_3
Fentanyl	Fentanyl-D_5
Methadone	Methadone-D_3
Xylazine	Xylazine-D_6
Dexmedetomidine*	Medetomidine-^{13}C,D_3

*Dexmedetomidine is the reference standard used for medetomidine. Dexmedetomidine and levomedetomidine co-elute therefore confirmed results are reported as “medetomidine.”

- Fentanyl derivative targets and associated internal standards

Targets	Internal Standards
3-Fluorofentanyl	Fentanyl-D_5
4-Methoxybutyrylfentanyl	
Acetylfentanyl	
Acrylfentanyl	

Targets	Internal Standards
alpha-Methylacetylfentanyl	
alpha-Methylfentanyl	
Benzodioxolefentanyl	
beta-Hydroxythiofentanyl	
Butyrylfentanyl	
Carfentanil	
cis-3-Methylfentanyl	
Cyclopropylfentanyl	
Despropionylfentanyl	
Fentanyl	
Furanylfentanyl	
meta-Fluorobutyrylfentanyl	
meta-Fluorofentanyl	
meta-Fluoroisobutyrylfentanyl	
Methoxyacetylfentanyl	
Ocfentanil	
ortho-Fluoroacrylfentanyl	
ortho-Fluorobutyrylfentanyl	
ortho-Fluorofentanyl	
ortho-Fluoroisobutyrylfentanyl	
para-Fluoroacrylfentanyl	
para-Fluorobutyrylfentanyl	
para-Fluorofentanyl	
para-Fluoroisobutyrylfentanyl	
Phenylfentanyl	
Tetrahydrofuranfentanyl	
trans-3-Methylfentanyl	
U-47700	
U-49900	
Valerylfentanyl	

- LCMS grade ammonium formate
- Formic acid, eluent additive ~98%
- Hydrochloric acid, concentrated
- Monopotassium phosphate
- Potassium hydroxide
- Glacial acetic acid
- Dichloromethane
- Isopropanol
- Ammonium hydroxide
- Hexane
- Type I or LCMS grade water
- Acetonitrile, Optima (or similar) grade or higher
- Methanol, Optima (or similar) grade or higher

28.4 Solutions, Internal Standard, Calibrators and Controls

28.4.1 Mobile Phase A (H_2O with 0.01% formic acid and 6 mM ammonium formate): add 100 µL of formic acid and approximately 0.385 grams of ammonium formate to 1 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

28.4.2 Mobile Phase B

28.4.2.1 OpiCoc: (Acetonitrile with 0.01% formic acid): add 100 µL of formic acid to 1 L of acetonitrile (Optima (or similar) grade or higher). Store at room temperature for up to one month.

28.4.2.2 Fentanyl Derivatives: (Methanol with 0.01% formic acid): add 100 µL of formic acid to 1 L of methanol (Optima (or similar) grade or higher). Store are room temperature for up to one month.

28.4.3 0.1 M Phosphate Buffer, pH 6.0. Weigh out 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the above solution to 6.0 by the addition of 5.0 M potassium hydroxide while stirring and qs to volume with dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to two years.

28.4.4 1 M Acetic Acid. Add 100-200 mL dH2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH_2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH_2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

28.4.5 0.5 M Acetic Acid. Add 50 mL of 1 M acetic acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

28.4.6 0.1 N HCl in isopropanol: Add 4.1 mL of concentrated HCl into 500 mL of isopropanol. Store at room temperature for up to one month.

28.4.7 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2). Mix 78 mL dichloromethane with 20 mL isopropanol. Mix well. In hood, add 2 mL ammonium hydroxide. Mix gently. PREPARE SOLUTION FRESH DAILY!

28.4.7.1 If preparing for use on the automated liquid handling system, prepare the minimum necessary volume (3 mL x # of samples) plus 56 mL (eluate dead volume).

28.4.8 Working Calibrator 1-A (1.0/10.0 mg/L): Pipette **10.0 µL** of the 1.0 mg/mL stock solution **(oxymorphone, hydromorphone, 6-acetyl morphine, acetyl fentanyl and fentanyl)** into a 10.0 mL volumetric flask. Pipette **100.0 µL** of the 1.0 mg/mL stock solution **(benzoylecgonine, meperidine, tramadol, and methadone)** into the same 10.0 mL volumetric flask and qs to volume with acetonitrile. An alternative preparation of acetylfentanyl: pipette 200.0 µL of the 50 µg/mL stock solution into a 10.0 mL volumetric flask.

28.4.8.1 Xylazine and dexmedetomidine (may be added to Working Calibrator 1-A or analyzed alone): Pipette 10.0 µL of the 1.0 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with acetonitrile.

28.4.9 Working Calibrator 1-B (0.1/1.0 mg/L): Pipette 1.0 mL of 1.0/10.0 mg/L working calibrator 1-A into 10.0 mL volumetric flask and qs to volume with acetonitrile.

28.4.9.1 Xylazine and dexmedetomidine may be added into Working Calibrator 1-B or analyzed alone.

28.4.10 Working Calibrator 2-A (10.0 mg/L): Pipette **100.0 µL** of 1.0 mg/mL stock solution **(morphine, codeine, oxycodone, hydrocodone, cocaethylene, and cocaine)** into a 10.0 mL volumetric flask and qs to volume with acetonitrile.

28.4.11 Working Calibrator 2-B (1.0 mg/L): Pipette 1.0 mL of 10.0 mg/L working calibrator 2-A into 10.0 mL volumetric flask and qs to volume with acetonitrile.

28.4.12 Working Qualitative Mix A (0.5/1.0 mg/L): Pipette 5 µL of 1.0 mg/mL or 50 µL of 0.1 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with methanol (trans-3-methylfentanyl, cis-3-methylfentatnyl, carfentanil). Pipette 10 µL of 1.0 mg/mL or 100 µL of 0.1 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with methanol (all other fentanyl analogs).

28.4.13 Working Qualitative Mix B (0.005/0.01 mg/L): Pipette 100 µL of 0.5/1.0 mg/L working qualitative mix A into 10.0 mL volumetric flask and qs to volume with methanol.

28.4.14 Working Internal Standard (1.0/2.5 mg/L): Pipette 10.0 µL of the 1.0 mg/mL stock solution into a 10.0 mL volumetric flask (hydromorphone-D_3, 6-monacetylmorphine-D_3, oxymorphone-D_3, and fentanyl-D_5). Pipette 25.0 µL of the 1.0 mg/mL stock solution into the same 10.0 mL volumetric flask and qs to volume with acetonitrile (morphine-D_3, codeine-D_3, hydrocodone-D_3, oxycodone-D_3, benzoylecgonine-D_3, cocaine-D_3, tramadol-^{13}C-D_3, methadone-D_3, and meperidine-D_4).

28.4.14.1 Xylazine-D_6 and Medetomidine-^{13}C,D_3 (may be added to Working Internal Standard or analyzed alone): Pipette 10.0 µL of the 1.0 mg/mL (or 100.0 µL of 100 µg/mL) stock solution into a 10.0 mL volumetric flask and qs to volume with acetonitrile.

28.4.15 Controls

28.4.15.1 Negative control blood: blood bank blood or equivalent determined not to contain target compounds.

28.4.15.2 Threshold Control: TC is prepared at one half of the LOQ (for OpiCoc) however this may be adjusted based upon validated LOD. The TC for fentanyl derivatives is listed in the procedure below.

28.4.15.3 An example for in-house control preparation (this is provided for convenience, this is not the only required approach to controls): create mixes much like Calibrator 1-A, Cal 1-B, Cal 2-A, and Cal 2-B for the same targets and label them as Control 1-A, Ctrl 1-B, Ctrl 2-A, and Ctrl 2-B. These can then be used to spike the following control levels:

Control Concentration (mg/L)	Ctrl 1-A (µL)	Ctrl 1-B (µL)	Ctrl 2-A (µL)	Ctrl 2-B (µL)
0.003/0.03/0.03	-	30	-	30
0.01/0.1/0.1	-	100	-	100
0.15/1.5/0.9	150	-	90	

28.4.15.3.1 Due to the quadratic nature of many of the targets, at least three controls, at low, medium, and high concentration, must be run across the concentration range with every batch. A high control must be run between the two highest spiked calibrator concentrations (e.g 0.15/1.5/0.9 mg/L control level in the above table).

28.4.15.4 Commercial whole blood control (UTAK or other commercial vendor) or methanolic statewide controls (prepared by the research analyst or designee), if available.

28.4.15.5 Note: Calibrator and control reference materials may have limited availability at the stated concentrations above due to listings on DEA Schedules. Alternative concentrations of the reference materials may be used to prepare calibrators and controls with the final concentrations of the targets being the same as listed above.

28.5 Apparatus

28.5.1 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

28.5.2 Test tubes, conical bottom

28.5.3 Centrifuge capable of 2,000-3,000 rpm

28.5.4 Vortex mixer

28.5.5 GC autosampler vials and inserts and snap caps (if using SPE by ALH) (Note for Hamilton: The vials should be the ones with the inserts manufactured into them. The vials with inserts placed into them may cause issues with the robotic delivery of samples.)

28.5.6 UCT CleanScreen SPE Columns ZSDAU020

28.5.7 Hamilton MicroLab STAR System and all included equipment for SPE (Note: dimensions for tubes, storage plates, and troughs need to be the same size as the products noted below as the system is programmed for those sizes).

- 16 x 125 mm sample tubes (e.g., VWR Item No. 53283-804)
- 48 well storage plates (e.g., Agilent Item No. 201238-100)
- 13 x 100 mm Borosilicate collection tubes (e.g., VWR Item No. 47729-572)
- 400 mL trough (e.g., Eppendorf Item No. 5075751364)
- 300 mL trough (e.g, Hamilton Robotics Item No. 5666901)
- Sav-It rubber stoppers (e.g, Fisherbrand Item No. 02-707-13)
- ASV snap caps (e.g., DWK Item No. 24277604)
- 1000 µL conductive tips (Hamilton Item No. 235904)
- 5000 µL conductive tips (Hamilton Item No. 184020)
- 1000 µL wide bore 3.2 mm orifice conductive tips (Hamilton Item No. 235444)
- 300 µL conductive tips (Hamilton Item No. 235875)

28.5.8 Opioid, cocaine, benzoylecgonine, and cocaethylene quantitative method parameters

28.5.8.1 LC Parameters:

- Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm particle size
- Column Thermostat: 60.0 °C
- Mobile Phase A: H_2O with 0.01% formic acid and 6 mM ammonium formate
- Mobile Phase B: Acetonitrile with 0.01% formic acid
- Flow Rate: 0.5 mL/min
- Injection Volume: 6 µL with a minimum 20 second needle wash
- Stop Time: 9.00 minutes
- Post Time: Minimum 2.00 minutes
- Gradient:

Time (minutes)	Solvent A	Solvent B
0.00	98.0	2.0
3.00	85.0	15.0
5.50	35.0	65.0
7.00	5.0	95.0
8.00	5.0	95.0
9.00	98.0	2.0

28.5.8.2 Typical MSMS parameters

- MSD Parameters:

  Ionization: ESI
  Polarity: Positive
  Gas Temperature: 325 °C
  Nebulizer Pressure: 45 psi
  Capillary: 3500 V
  Drying Gas: 12 L/min
  Delta EMV: 400

- Transition Ions: (Bold font indicates the quantitation transition. Note: Morphine and hydromorphone may be detected in each other's MRM windows. Codeine and hydrocodone may be detected in each other's MRM windows. Retention times below are indicative of elution order of these compounds, ensure that the correct peak is chosen for analysis.)

Dynamic MRM Compound Settings						
Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	Approx. Retention Time (min)
Morphine	286.2	165.1 **152.1**	148	46 70	7	1.765
Morphine-D_3	289.2	165.1 **152.1**	128	42 74	7	1.784
Oxymorphone-D_3	305.2	**287.2** 230.1	121	14 26	7	1.979
Oxymorphone	302.1	**284.1** 227.1	121	14 26	7	1.989
Hydromorphone	286.2	**185.1** 157.1	153	30 46	2	2.242
Hydromorphone-D_3	289.2	**185.1** 157.1	148	30 46	2	2.252
Codeine-D_3	303.2	165.1 **152.0**	143	42 74	7	3.080
Codeine	300.2	165.1 **152.0**	137	46 74	7	3.090
Oxycodone-D_3	319.2	**301.2** 244.1	126	14 26	7	3.428
Oxycodone	316.2	**298.1** 241.1	121	14 26	7	3.454
6-Acetylmorphine-D_3	331.2	**165.1** 152.1	148	46 78	2	3.568
6-Acetylmorphine	328.2	**165.1** 152.1	138	42 78	2	3.570
Hydrocodone-D_3	303.2	**199.1** 128.0	133	30 70	7	3.690
Hydrocodone	300.2	**199.1** 128.0	137	30 70	7	3.699
Benzoylecgonine-D_3	293.1	105.0 **77.0**	133	25 45	7	4.134
Benzoylecgonine	290.1	**168.1** 77.0	118	10 45	7	4.143
Xylazine	221.1	164 **90**	145	24 20	7	4.4
Xylazine-D_6	227.1	170 **90**	140	24 20	7	4.5
Medetomidine-$^{13}C,D_3$	205.16	**99** 72.1	110	16 36	7	4.6
Tramadol-^{13}C-D_3	269.2	**269.2** 58.1	105	0 16	2 7	4.700
Tramadol	264.1	**264.1** 58.1	119 180	10 10	7	4.703
Cocaine-D_3	307.2	85.1 **77.0**	133	25 45	7	4.878
Cocaine	304.2	**182.1** 77.0	138	5 45	7	4.879

Dynamic MRM Compound Settings						
Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	Approx. Retention Time (min)
Meperidine-D_4	252.2	**224.2**	140	15	7	4.906
		178.1		15		
Meperidine	248.2	**220.0**	140	15	7	4.907
		174.1		15		
Dexmedetomidine	201.1	**95**	110	24	7	5
		68.1		40		
Acetyl Fentanyl	323.0	**188.0**	135	20	7	5.094
		105.0		36		
Cocaethylene	318.2	**196.1**	123	5	7	5.170
		82.1		18		
Fentanyl-D_5	342.2	**188.2**	140	25	7	5.368
		105.1		50		
Fentanyl	337.2	**188.2**	140	25	7	5.378
		105.1		45		
Methadone	310.2	**223.0**	112	9	7	5.912
		105.0		10		
Methadone-D_3	313.2	**268.1**	118	12	7	5.916
		105.0		28		

28.5.9 Fentanyl derivative qualitative method parameters

28.5.9.1 LC Parameters:

- Column: Poroshell 120 EC-C18, 3.0 x 50 mm, 2.7 µm particle size
- Column Thermostat: 60.0 °C
- Mobile Phase A: H_2O with 0.01% formic acid and 6 mM ammonium formate
- Mobile Phase B: Methanol with 0.01% formic acid
- Flow Rate: 0.7 mL/min
- Injection Volume: 20 µL with a minimum 20 second needle wash
- Stop Time: 17.00 minutes
- Post Time: 2.00 minutes
- Gradient:

Time (minutes)	Mobile Phase A	Mobile Phase B
0.00	85.0	15.0
7.00	75.0	25.0
13.00	65.0	35.0
16.00	25.0	75.0
17.00	2.0	98.0

28.5.9.2 MSMS Parameters

- MSD Parameters:

  Ionization: ESI
  Polarity: Positive
  Gas Temperature: 325 °C
  Nebulizer Pressure: 45 psi
  Capillary: 3500 V
  Drying Gas 12 L/min
  Delta EMV: 400

- Transition Ions: (Note: Target transitions are in bold font, qualifier transition is in normal font.)

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Approx. Retention Time (min)	Delta Retention Time (min)
Methoxyacetylfentanyl	353.2	**188**	**150**	**20**	**2**	**5.7**	**3**
		105		40			
Acetylfentanyl	323	188	135	20	2	6.1	3
		105		**36**			
beta-Hydroxythiofentanyl	359.2	**341.2**	**135**	**12**	**2**	**6.4**	**3**
		192.1		20			
Ocfentanil	371.2	**188.1**	**135**	**20**	**7**	**6.5**	**3**
		105.1		40			
alpha-Methylacetylfentanyl	337.2	202.1	125	20	2	6.8	3
		91.1		**48**			
Despropionylfentanyl	281.2	188	102	18	2	6.8	3
		105		**30**			
Tetrahydrofuranfentanyl	379.2	**188**	**132**	**22**	**2**	**8.1**	**3**
		105		46			
Acrylfentanyl	335.2	188	134	22	2	8.5	3
		105		**38**			
para-Fluoroacrylfentanyl	353.2	188.1	135	20	7	8.8	3
		105.1		**40**			
Fentanyl-D_5	342.2	**188.2**	**140**	**25**	**2**	**8.9**	**3**
		105.1		50			
ortho-Fluoroacrylfentanyl	353.2	188.1	130	20	7	9	3
		105.1		**44**			
U-47700	329.1	**283.9**	**100**	**14**	**2**	**9.2**	**3**
		172.8		34			
Fentanyl	337.2	188.2	140	25	2	9.3	3
		105.1		**45**			
Para/meta-Fluorofentanyl	355.2	188.1	155	24	2	9.6	3
		105.1		**44**			
alpha-Methylfentanyl	351.2	202.2	115	20	2	9.9	3
		91.1		**50**			
Furanylfentanyl	375.2	**188**	**136**	**26**	**2**	**9.9**	**3**
		105		42			
ortho-Fluorofentanyl	355.2	188.1	115	24	2	10.3	3
		105.1		**44**			
Cyclopropylfentanyl	349.2	188.1	120	24	2	10.7	3
		105.1		**50**			
U-49900	357.2	**284.1**	**110**	**16**	**7**	**10.9**	**3**

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Approx. Retention Time (min)	Delta Retention Time (min)
		172.9		36			
Carfentanil	395.2	**335**	**112**	**18**	**2**	**11.5**	**3**
		113		34			
cis-3-Methylfentanyl	351.3	202	142	22	2	11.7	3
		105		**46**			
trans-3-Methylfentanyl	351.3	202	136	22	2	11.7	3
		105		**42**			
Butyrylfentanyl	351.3	188	138	22	2	12.4	3
		105		**42**			
Para/meta-Fluoroisobutyrylfentanyl	369.2	**188**	**140**	**22**	**2**	**12.4**	**3**
		105		46			
Phenylfentanyl	385.2	188	150	20	2	12.6	3
		105		**44**			
3-Fluorofentanyl	355.2	186.1	120	20	2	12.7	3
		206.1		**24**			
Benzodioxolefentanyl	429.2	188	140	24	2	12.7	3
		149		**28**			
Para/meta-Fluorobutyrylfentanyl	369.2	**188.1**	**135**	**24**	**2**	**12.7**	**3**
		105.1		48			
ortho-Fluoroisobutyrylfentanyl	369.2	188.1	115	24	7	13	3
		105.1		**44**			
ortho-Fluorobutyrylfentanyl	369.2	188.1	145	24	7	13.4	3
		105.1		**44**			
4-Methoxybutyrylfentanyl	381.3	188.1	165	24	2	13.5	3
		105.1		**50**			
Valerylfentanyl	365.3	188	146	22	2	14.6	3
		105		**46**			

28.5.9.3 Data Analysis Parameters (Note: Retention times listed are for elution order reference and may vary by system.)

Target	Approximate Retention Time	RRT Acceptance (%)
Methoxyacetylfentanyl	5.7	1
Acetylfentanyl	6.1	1
beta-Hydroxythiofentanyl	6.4	0.8
Ocfentanil	6.5	0.8
alpha-Methylacetylfentanyl	6.8	1
Despropionylfentanyl	6.8	2
Tetrahydrofuranfentanyl	8.1	0.5
Acrylfentanyl	8.5	0.5

Target	Approximate Retention Time	RRT Acceptance (%)
para-Fluoroacrylfentanyl	8.8	0.5
ortho-Fluoroacrylfentanyl	9	0.5
U-47700	9.2	0.8
Fentanyl	9.3	0.5
para/meta-Fluorofentanyl	9.6	0.5
alpha-Methylfentanyl	9.9	0.5
Furanylfentanyl	9.9	1
ortho-Fluorofentanyl	10.3	0.8
Cyclopropylfentanyl	10.7	0.8
U-49900	10.9	1.5
Carfentanil	11.5	1
trans-3-Methylfentanyl	11.7	1
cis-3-Methylfentanyl	11.7	1
Butyrylfentanyl	12.4	1
para/meta-Fluoroisobutyrylfentanyl	12.4	1
Phenylfentanyl	12.6	1.5
3-Fluorofentanyl	12.7	1.5
Benzodioxolefentanyl	12.7	1.5
para/meta-Fluorobutyrylfentanyl	12.7	1
ortho-Fluoroisobutyrylfentanyl	13	1
ortho-Fluorobutyrylfentanyl	13.4	1
4-Methoxybutyrylfentanyl	13.5	1.5
Valerylfentanyl	14.6	2

28.6 Procedure

Note: Extracts from this method may be analyzed for OpiCoc targets and fentanyl derivatives individually or simultaneously.

Note: When preparing samples for automated extraction using Hamilton STAR, ensure a smooth aliquot when pipetting samples (blank blood, case sample blood, liver, etc.) with the use of mechanical pipettes. If a smooth aliquot is not obtained, the sample may be poured into a secondary vessel and pipetting should be tried again.

28.6.1 Note: Urine specimens may be hydrolyzed to remove glucuronide conjugates prior to extraction using one of the following hydrolysis procedures. A glucuronide positive control should be used to ensure the hydrolysis was effective.

28.6.1.1 Enzyme hydrolysis**:** Add 5000 Fishman units of β-glucuronidase to each mL of urine. Perform hydrolysis as recommended by the supplier based on the source of β-glucoronidase (e.g., 5000 F units/mL Patella vulgata in 100 mM acetate buffer (pH 5.0) hydrolyzed at 65°C for 3 hours).

28.6.2 Label appropriate clean screw cap tubes accordingly, negative, calibrators, control(s) and case sample IDs

28.6.3 Prepare calibrators and controls. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood. (Volume (µL) to deliver into the appropriately labeled screw top tubes)

Cal 1-A 1.0/10.0 mg/L (µL)	Cal 1-B 0.1/1.0 mg/L (µL)	Cal 2-A 10.0 mg/L (uL)	Cal 2-B 1.0 mg/L (µL)	Final Concentration Calibrator 1 Targets (mg/L)	Final Concentration Calibrator 2 Targets (mg/L)
-	10	-	10	0.001/0.01	0.01
-	20	-	20	0.002/0.02	0.02
-	50	-	50	0.005/0.05	0.05
-	100	-	100	0.01/0.1	0.1
25	-	20	-	0.025/0.25	0.2
75	-	40	-	0.075/0.75	0.4
100	-	80	-	0.1/1.0	0.8
200	-	100	-	0.2/2.0	1

Calibrator 1 Targets:

1.0 mg/L and 0.1 mg/L Oxymorphone, Hydromorphone, 6-acetylmorphine, Acetyl Fentanyl, Fentanyl, Xylazine, Dexmedetomidine

10.0 mg/L and 1.0 mg/L Benzoylecgonine, Meperidine, Tramadol, Methadone

Calibrator 2 Targets:

10.0 mg/L and 1.0 mg/L Morphine, Codeine, Oxycodone, Hydrocodone, Cocaethylene, Cocaine

Qualitative Fentanyl Analog Control Preparation

A low threshold control, high carryover control, and negative control shall be assessed with each fentanyl analog qualitative analysis

Control Level	Qualitative Mix A Volume of 0.5/1.0 mg/L (µL)	Qualitative Mix B Volume of 0.005/0.01 mg/L (µL)	Final Concentration Qualitative Control (mg/L)
Low Threshold Control	-	50	0.00025/0.0005
High Carryover Control	100	-	0.05/0.1

28.6.4 Pipette 1.0 mL of blank blood, calibrators, controls and case sample bloods, fluids or tissue homogenates in appropriately labeled tubes

28.6.5 Add 20 µL 1.0/2.5 mg/L working internal standard solution to each tube

28.6.6 Vortex briefly

28.6.7 Option 1 - LLE

28.6.7.1 Add 2.0 mL of acetonitrile to each tube and vortex for 15-30 seconds. Ensure that the blood in the bottom of the tube mixes thoroughly with the acetonitrile

28.6.7.2 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation

28.6.7.3 Freeze samples for approximately 30 minutes at approximately -20 ºC (Note: upon removal from freezer there should be three distinct layers)

28.6.7.4 Transfer topmost layer into conical bottom tubes and evaporate to dryness at approximately 50-60 °C under nitrogen

28.6.7.5 Reconstitute in 200 µL 0.01% formic acid and 6 mM ammonium formate in water. (Note: Centrifugation may be necessary at this step)

28.6.7.6 Transfer to autosampler vials

28.6.8 Option 2 – SPE (Note: This procedure may be automated utilizing the approved automated sample preparation system (e.g., Hamilton MicroLab STAR). Xylazine and dexmedetomidine are not validated for the SPE method.)

28.6.8.1 Add 2 mL dH_20 to each tube.

28.6.8.2 Add 1 mL of pH 6 0.1 M phosphate buffer. Vortex briefly and let stand for at least 5 minutes.

28.6.8.3 Centrifuge at approximately 2500 rpm for 15 minutes to achieve separation. (Note: For tissue homogenates: following centrifugation, separate the liquid portion from the rest of the samples and load only the liquid portion onto the SPE column during the sample loading step.)

28.6.8.4 Solid phase extraction – The following steps (through the transfer to the autosampler vials) may be performed manually or with the automated sample preparation system. With the automated process, there may be additional steps that require the user to interact with the system. Follow the prompts from the automated sample preparation system.

28.6.8.4.1 Note: For the manual process, place labeled SPE cartridges in the extraction manifold. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

28.6.8.4.2 Add 3 mL hexane to each column and aspirate.

28.6.8.4.3 Add 3 mL methanol to each column and aspirate.

28.6.8.4.4 Add 3 mL dH_2O and aspirate.

28.6.8.4.5 Add 1 mL of 0.1 M pH 6.0 phosphate buffer and aspirate.

28.6.8.4.6 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with ~ 1-2 mL/ minute flow.

28.6.8.4.7 Add 3 mL dH_2O and aspirate at ≤ 3inches of mercury or a low positive pressure (< ~10 psi).

28.6.8.4.8 Wash with 2.0 mL 0.5 M Acetic Acid and aspirate.

28.6.8.4.9 Wash with 3.0 mL of methanol and aspirate under full vacuum/pressure for approximately 30 minutes.

28.6.8.4.10 Wipe the SPE column tips with Kimwipes® (if performing the manual process). Place labeled test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

28.6.8.4.11 Elute drugs by adding 3 mL of freshly prepared 78:20:2 Dichloromethane: Isopropanol: Ammonium Hydroxide to each column. Collect eluate by gravity drain (no vacuum).

28.6.8.5 Add 100 µL of 0.1 N HCl in isopropanol to each tube and vortex briefly.

28.6.8.6 Evaporate to dryness at approximately 50-60°C under nitrogen.

28.6.8.7 Reconstitute in 200 uL 0.01% formic acid in water and 6 mM ammonium formate.

28.6.8.8 Transfer to autosampler vials.

28.6.9 When preparing samples for automated extraction using Hamilton STAR:

28.6.9.1 Add normal volumes of internal standard, water, and phosphate buffer. Vortex, let stand for 5 minutes, and centrifuge as usual.

28.6.9.2 For blood and urine samples, load centrifuged samples into Hamilton tube carriers in numerical order from 1 up to 48 and place onto system.

28.6.9.3 For tissue homogenates, after centrifugation and prior to loading samples into Hamilton tube carriers, separate liquid portion from solid portion(s) of sample and place it into a new tube which will then be loaded onto system.

28.6.9.4 Barcode labeling may be utilized for the identification of samples in the Hamilton system (Appendix E).

28.6.9.5 Once samples are loaded onto system, automated extraction process may begin using the Hamilton system and software.

28.6.9.6 Vortexing of the 48 well plate is to be done at ~550 rpm for 10 minutes.

28.6.9.7 If samples need to be moved from the autosampler vial rack in the Hamilton to the autosampler vial rack in the LCMSMS (typically, for differing rack layouts), a vial check shall be performed and recorded appropriately.

28.7 Quality Control and Reporting

28.7.1 OpiCoc Quality Control and Reporting

28.7.1.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

28.7.1.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

28.7.1.3 6-acetylmorphine may only be reported if morphine is confirmed in the same sample or any other biological specimen from the same individual (e.g., 6-acetylmorphine positive in urine and morphine positive in blood). Depending on case history and circumstances, 6-acetylmorphine may be reported as positive without morphine present after supervisor approval.

28.7.1.4 When a target concentration is above the ULOQ, 1.0 mL of case sample shall be diluted with no more than 19.0 mL of blank matrix for a total dilution volume of 20.0 mL. Alternatively, 0.05 mL of case sample may be used for a dilution of 1/20. If less than 0.05 mL of sample is used for analysis, only qualitative results may be reported.

28.7.1.4.1 Xylazine and dexmedetomidine may only be diluted up to 1/10 (e.g., 1.0 mL sample diluted with up to 9.0 mL blank matrix; 0.1 mL sample diluted with up to 0.9 mL blank matrix).

28.7.1.5 The calibration model for all targets is weighted (1/x) quadratic with the exception of morphine, tramadol, and cocaine which are weighted (1/x) linear. Samples with a concentration greater than the second highest calibrator concentration for a target with a quadratic fit must be repeated for quantitative reporting if the high positive control is outside of acceptable limits.

28.7.1.6 Threshold Control: The TC is spiked at one half of the LOQ however this may be adjusted based upon the validated LODs listed below.

OpiCoc:

Limit of Detection	
Target	LOD (mg/L)
Morphine	0.0025
Oxymorphone	0.001
Hydromorphone	0.0005
Codeine	0.00125
Oxycodone	0.00125
6-Acetylmorphine	0.0005
Hydrocodone	0.000625
Benzoylecgonine	0.0004
Tramadol	0.0025
Cocaine	0.00125
Meperidine	0.00125
Acetyl Fentanyl	0.000125
Cocaethylene	0.00125
Fentanyl	0.0000625
Methadone	0.0025
Xylazine	0.001
Dexmedetomidine	0.001

Fentanyl Derivatives:

Limit of Detection	
Targets	Concentration (mg/L)
Methoxyacetylfentanyl	0.00025
Acetylfentanyl	0.00025
beta-Hydroxythiofentanyl	0.00025
Ocfentanil	0.00025
alpha-Methylacetylfentanyl	0.00025
Despropionylfentanyl	0.00025
Tetrahydrofuranfentanyl	0.00025
Acrylfentanyl	0.00025
para-Fluoroacrylfentanyl	0.00025
ortho-Fluoroacrylfentanyl	0.00025
U-47700	0.0005
Fentanyl	0.00025
para-Fluorofentanyl	0.00025
alpha-Methylfentanyl	0.00025
Furanylfentanyl	0.00025
ortho-Fluorofentanyl	0.00025
Cyclopropylfentanyl	0.00025
U-49900	0.0005
para-Fluoroisobutyrylfentanyl	0.00025
Butyrylfentanyl	0.00025
3-Fluorofentanyl	0.0005
Phenylfentanyl	0.00025
Benzodioxolefentanyl	0.00025
para-Fluorobutyrylfentanyl	0.00025
ortho-Fluoroisobutyrylfentanyl	0.00025
ortho-Fluorobutyrylfentanyl	0.00025
4-Methoxybutyrylfentanyl	0.00025
Valerylfentanyl	0.00025
Carfentanil	0.00025

cis-3-Methylfentanyl	0.000125
trans-3-Methylfentanyl	0.000125

28.7.1.7 Extracted samples are stable for seven days after reconstitution.

28.7.1.8 Calibrator and control solutions may be made in larger than 10 mL volumes, if necessary. The larger volume calibrator solutions need to be made in calibrated glassware.

28.7.1.9 Confirmed results for dexmedetomidine shall be reported as "medetomidine" due to the co-elution of dexmedetomidine and levomedetomidine. Dexmedetomidine was the standard utilized for validation and is the standard used for analysis with this method.

28.7.2 Fentanyl Derivatives Quality Control and Reporting

28.7.2.1 The presence of fentanyl derivatives may only be reported if the instrumental response ratio is equal to or greater than the low threshold control response ratio and all other qualitative identification criteria are met. If the instrumental response of a case sample is greater than the high carryover control, an evaluation of carryover shall be performed.

28.7.2.2 Any targets that are outside of the relative retention time (RRT) acceptance range shall not be reported.

28.7.2.3 Extracted samples are stable for seven days after reconstitution.

28.7.2.4 Control expiration: Expired controls may be used within this method for screening purposes only for the expired reference material. Any presumptive positives determined from expired controls will require confirmation with an unexpired reference material. Mixed controls may have expiration dates set before all components expire.

28.7.3 Hamilton ALH System

28.7.3.1 If barcoding is utilized to identify samples, a vial check is not necessary when removing samples from the Hamilton ALH. See Appendix E for barcode generating instructions.

28.8 References

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29 Buprenorphine, Norbuprenorphine and Naloxone Quantitation and Confirmation by LCMSMS

29.1 Summary

Buprenorphine, norbuprenorphine and naloxone are extracted from biological samples using solid phase extraction (SPE) and confirmed or quantified with LCMSMS. These drugs may be analyzed simultaneously or individually as needed.

Naloxone may not be run with every batch. This can be run by customer request or at the discretion of the toxicology staff assigning testing.

29.2 Specimen Requirements

2 mL of blood or fluid. Note: This method is not suitable for tissue homogenate analysis.

29.3 Reagents and Standards

- Drugs and associated internal standards

Target	Internal Standards
Buprenorphine	Buprenorphine-D_4
Norbuprenorphine	Norbuprenorphine- D_3
Naloxone*	Naloxone- D_5

*May not be run with every batch

- Acetonitrile, Optima (or similar) grade or higher
- Dichloromethane, ACS grade or better
- Methanol, ACS grade or better
- 2-Propanol, ACS grade or better
- Type 1 or LCMS grade water
- Distilled water (dH2O)
- Formic acid, Fisher Optima (or similar) grade or higher
- Glacial Acetic Acid
- Potassium Phosphate
- Potassium hydroxide
- Ammonium Hydroxide

29.4 Solutions, Internal Standard, Calibrators and Controls

29.4.1 1 M Acetic acid: Add 100-200 mL dH_2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH_2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH_2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

29.4.2 0.1 M Phosphate buffer, pH 6.0: Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the solution to 6.0 by the addition of 5.0 M potassium hydroxide while stirring. QS to volume using dH_2O. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Store at room temperature for up to 2 years.

29.4.2.1 5 M Potassium hydroxide: Add 28.05 g potassium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

29.4.3 Elution solvent (dichloromethane, 2-propanol, ammonium hydroxide, 77:20:3): Combine 20 mL of 2-propanol, and 3.0 mL of ammonium hydroxide then add 77 mL of dichloromethane. Mix gently. pH should be between 11-12. Make fresh for each day of use. Note: Additional ammonium hydroxide may be required to achieve the desired pH.

29.4.4 Mobile phase A (Water with 0.1% formic acid): Add 1.0 mL of formic acid to approximately 0.5 L water and qs to 1.0 L with water. Store at room temperature for up to one month.

29.4.5 Mobile phase B (Acetronitrile with 0.1% formic acid): Add 1.0 mL of formic acid to approximately 0.5 L acetonitrile and qs to 1.0 L with acetonitrile. Store at room temperature for up to one month.

29.4.6 Preparation of working internal standard (IS) solution (0.10 mg/L)

29.4.6.1 Dilute 10 µL of each internal standard (buprenorphine- D_4, norbuprenorphine- D_3, naloxone- D_5) stock solution (100 µg/mL) in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7 Preparation of calibrators

29.4.7.1 Working calibrator solution A (CAL A) (2.0 mg/L): Pipette 20 µL of each 1.0 mg/mL standard stock solution (buprenorphine, norbuprenorphine, and naloxone) in a calibrated 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.2 Working calibrator solution B (CAL B) (0.2 mg/L): Pipette 1000 µL of CAL A (2.0 mg/L) in a calibrated 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.3 Working calibrator solution C (CAL C) (0.02 mg/L): Pipette 1000 µL of CAL B (0.2 mg/L) in a calibrated 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.4 To prepare the calibration curve, pipette the following volumes of CAL A, B, or C working calibrator solutions into appropriately labeled 16 x 125 mm screw cap test tubes. DO NOT dry down calibrators with nitrogen or heat. Add 2 mL blank blood to obtain the final concentrations listed below. Mix by vortex.

Calibrator Concentration (mg/L)	Calibrator Working Solution	Volume of Working Solution (µL)
0.020	CAL A – 2.0 mg/L	20
0.015	CAL A – 2.0 mg/L	15
0.010	CAL B – 0.2 mg/L	100
0.0075	CAL B – 0.2 mg/L	75
0.0050	CAL B – 0.2 mg/L	50
0.0025	CAL B – 0.2 mg/L	25
0.0010	CAL C – 0.02 mg/L	100
0.0005	CAL C – 0.02 mg/L	50
0.00025 threshold control	CAL C– 0.02 mg/L	25

29.4.7.5 Preparation of controls

29.4.7.5.1 Working control solution A (CTL A) (2.0 mg/L): Pipette 20 µL of each 1.0 mg/mL standard stock solution (buprenorphine, norbuprenorphine, and naloxone) in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.5.2 Working control solution B (CTL B) (0.2 mg/L): Pipette 1000 µL of CTL A (2.0 mg/L) in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.5.3 Working control solution C (CTL C) (0.040 mg/L): Pipette 2000 µL of CTL B (0.2 mg/L) in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable. Store in freezer.

29.4.7.5.4 To prepare positive controls, the following are provided as examples of concentrations and instructions. Pipette the following volumes of CTL A and B working control solutions into appropriately labeled 16 x 125 mm screw cap test tubes. Do not dry down controls with nitrogen or heat. Add 2 mL blank blood to obtain the final concentrations listed below. Mix by vortex.

Positive Control Concentration (mg/L)	Control Solution	Working Solution Volume (µL)
0.0020	CTL C (40 µg/L)	100
0.0080	CTL B (200 µg/L)	80
0.017	CTL A (2000 µg/L)	17

29.4.7.5.5 Negative control: Blood bank blood or equivalent determined not to contain buprenorphine, norbuprenorphine, or naloxone.

29.5 Apparatus

29.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

29.5.2 Test tubes, conical bottom

29.5.3 Test tube Rotator

29.5.4 Centrifuge capable of 3500 rpm

29.5.5 Solid phase extraction columns (SPE) UCT ZSDAU020

29.5.6 United Chemical Technologies 48 place positive pressure manifold or suitable substitute

29.5.7 Evaporator/concentrator

29.5.8 Vortex mixer

29.5.9 GC autosampler vials with inserts

29.5.10 Typical LCMSMS parameters.

- LC Parameters
- Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size
- Column Thermostat: 50°C
- Solvent A: H_2O with 0.1% formic acid
- Solvent B: Acetonitrile with 0.1% formic acid
- Initial Flow Rate: 0.50 mL/min
- Injection vol.: 10 µL with a minimum 30 second needle wash
- Stop time: 5.50 min
- Post time: Minimum 2.00 min
- Gradient:

Time (minutes)	Solvent A	Solvent B
0.00	98.0	2.0
1.91	70.0	30.0
4.20	60	40

4.21	5	95

29.5.11 Typical MS-MS parameters.

- MSD Parameters:Ionization: ESI
    - Polarity: positive
    - Gas temp: 350°C
    - Drying Gas: 10.0 L/min
    - Nebulizer press: 40 psi
    - Capillary: 4000 V
    - Delta EMV: 400 V
- Time Segments (TS):
    - TS1: 0-2.7 minutes (To Waste)
    - TS2: 2.7-3.1 minutes (Naloxone- D_5, Naloxone)
    - TS3: 3.1-3.5 minutes (Norbuprenorphine- D_3, Norbuprenorphine)
    - TS4: 3.5-4.2 minutes (Buprenorphine- D_4, Buprenorphine)
    - TS5: 4.2-5.5 minutes (To Waste)

Analytes and IS	Transitions (m/z) Q1	Q3	Dwell (ms)	Frag (V)	CE (V)	Cell Accel (V)
Naloxone	328.2	**310.2**	85	160	15	3
		212.1	85	160	39	3
Naloxone- D_5	333.2	**273.2**	85	160	27	3
		258.2	85	160	27	3
Norbuprenorphine	414.3	**396.3**	60	200	29	3
		340.3	60	200	33	3
Norbuprenorphine- D_3	417.3	**101.1**	60	179	39	3
		83.2	60	179	55	3
Buprenorphine	468.3	**414.2**	65	190	35	3
		396.2	65	190	39	3
Buprenorphine- D_4	472.3	**400.2**	65	198	39	3
		101.1	65	198	43	3

Bold transition ions are used for quantification. Frag – Fragment Voltage; CE - collision energy

29.6 Procedure

29.6.1 Label appropriate clean screw cap tubes accordingly, negative, calibrators, control(s), and case sample IDs.

29.6.2 Prepare calibrators and controls. (Note: DO NOT dry down calibrators and controls).

29.6.3 Pipette 2.0 mL of blank blood, calibrators, controls (if not already completed), and case sample bloods, or fluids into appropriately labeled tubes.

29.6.4 Add 25 µL of IS to each tube.

29.6.5 Add 2.0 mL of phosphate buffer.

29.6.6 Cap and vortex briefly to mix, then rotate tubes for 10 min.

29.6.7 Centrifuge at approximately 3500 RPM for 10 min.

29.6.8 Solid phase extraction (unless otherwise instructed column flow rate is approximately 1 mL/min with pressurized air or nitrogen).

- Wash column with 2.0 mL of methanol.
- Wash column with 2.0 mL of dH_2O.
- Wash column with 2.0 mL of phosphate buffer.
- Pour prepared sample on column. Aspirate slowly so sample takes at least two minutes to pass through the column.
- Wash column with 3.0 mL of dH_2O.
- Wash column with 3.0 mL of acetic acid.
- Wash column with 3.0 mL of methanol.
- Dry column for approximately 10 min at maximum pressure.
- Elute column with 3.0 mL of elution solvent by gravity flow into a screw-cap conical centrifuge tube.

29.6.9 Evaporate eluate to dryness at a setting of 40°C under nitrogen, do not dry down at temperatures above 40°C. Ensure that evaporation of samples occurs promptly after elution.

29.6.10 Reconstitute residue with 50 µL of 75% mobile phase A mixed with 25% mobile phase B.

29.6.11 Briefly vortex.

29.6.12 If necessary, centrifuge the tubes for 15 minutes at approximately 2800 rpm.

29.6.13 Transfer to autosampler vials fitted with glass inserts.

29.7 Quality Control and Reporting

29.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

29.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

29.7.3 The calibration models for naloxone and buprenorphine are weighted (1/x) linear. The calibration model for norbuprenorphine is weighted (1/x) quadratic. Due to the quadratic nature of the norbuprenorphine calibration curve, at least three controls, at low medium and high concentrations, must be run across the concentration range in every batch. If the 20 µg/L calibrator is included in the calibration curve, the 17 µg/L control or a control within 15 – 20 µg/L must be included.

29.7.4 Threshold Control: TC is set at the validated LOD for all targets.

29.7.5 When a target concentration is above the ULOQ, samples may be diluted up to 1/20 (e.g., 1/2 - 1.0 mL case diluted with 1.0 mL of matrix; 1/20 - 0.1mL case sample diluted with 1.9 mL matrix).

29.7.6 All compounds are stable up to seven days with the exception of naloxone which is considered stable for three days.

29.7.7 See Toxicology Quality Guidelines

Note: High concentrations of amphetamine (~2.0 mg/L) and some post-mortem sample components may cause reduced internal standard signals for buprenorphine-D_4, norbuprenorphine-D_3, and naloxone-D_5. The examiner should evaluate these on a case-by-case basis and report as present at the examiner's discretion.

29.8 References

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30 Novel Psychoactive Substances Qualitative Screen and Confirmation using LCMSMS

30.1 Summary

Novel psychoactive substances are extracted from biological samples using a solid phase extraction. An aliquot of the extract is qualitatively analyzed by LCMSMS. Targets may be analyzed by panel, different combinations, or individually as needed.

Note: Novel psychoactive substances can be abbreviated as NPS. Formal names and synonyms may be included in Appendix B for the target compounds however the common names listed below will be used for reporting.

30.2 Specimen Requirements

0.5 mL of blood or fluid. Note: This method is not suitable for tissue homogenates.

30.3 Reagents and Standards

- Drug targets and associated internal standards

Panel	Target Compounds	Internal Standards
Research Chemicals	4-APDB	Pentylone-D_3
	5-APDB	Pentylone-D_3
	6-APDB	Pentylone-D_3
	Dibutylone (bk-DMBDB)	Pentylone-D_3
	4/5/6-MAPB	Pentylone-D_3
	Pentylone/N,N-Dimethylpentylone	Pentylone-D_3
	N-Ethylpentylone/N,N-Diethylpentylone	Pentylone-D_3
	5-DBFPV	Pentylone-D_3
	4-Chloro-alpha-PVP	Pentylone-D_3
	PV8	25I-NBOME-D_3
Miscellaneous	Mitragynine	Pentylone-D_3
	Methoxphenidine	Pentylone-D_3
	Tenocyclidine (TCP)	Pentylone-D_3
	3-Methoxy-PCP	Pentylone-D_3
	Clonazolam	25I-NBOMe-D_3

- Potassium phosphate buffer solution concentrate (1 M, pH 6.0, e.g., Fisher)
- Potassium hydroxide
- Potassium phosphate
- Methanol, HPLC grade or higher
- Acetate buffer
- Methylene chloride
- 2-Propanol
- Ammonium hydroxide, Optima (or similar) grade or higher
- Hydrochloric acid, Optima (or similar) grade or higher
- Formic acid, eluent additive ~98%
- Type I or LCMS grade water
- Sodium acetate trihydrate
- Glacial acetic acid

30.4 Solutions, Internal Standard, Calibrators and Controls

30.4.1 0.1 M Potassium Phosphate Buffer, pH 6.0: Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of deionized water. Adjust the pH of the above solution to 6.0 by the addition of 5 M potassium hydroxide while stirring and qs to volume with deionized water. Solution may also be purchased as a 10X concentrate that must be diluted prior to use (e.g., Fisher). Dilute one volume of potassium phosphate buffer solution concentrate with nine volumes of deionized water. Store at room temperature for up to two years.

30.4.1.1 5 M Potassium hydroxide: Add 28.05 g potassium hydroxide to ~80 mL dH2O in a 100 mL volumetric flask, qs to volume with dH2O. Solutions may also be prepared from a concentrate or obtained as a prepared solution. Store at room temperature for up to two years.

30.4.2 0.1 M Acetate Buffer: Weigh 2.93 g sodium acetate trihydrate into a 500 mL volumetric flask. Add approximately 400 mL of deionized water to dissolve sodium acetate. Add 1.62 mL glacial acetic acid. Adjust pH to 4.5 with 1 M acetic acid and qs to volume with deionized water. Store at room temperature for up to two years.

30.4.3 Dichloromethane/isopropanol/ammonium hydroxide (78:20:2): Mix 78 mL dichloromethane with 20 mL isopropanol. Mix well. In hood, add 2 mL ammonium hydroxide. Mix gently. Prepare solution fresh daily.

30.4.4 Mobile Phase A (H_2O with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L of Type I or LCMS grade water. Store at room temperature for up to one month.

30.4.5 Mobile Phase B (Methanol with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L of methanol. Store at room temperature for up to one month.

30.4.6 Reconstitution Solvent (98:2 water:methanol): Add 2.0 mL of methanol to 98.0 mL dH_2O. Different final volumes may be prepared that maintain the 98:2 volume ratio.

30.4.7 0.2% Hydrochloric acid in 2-propanol: Add 1 mL of concentrated HCl (12N) into 500 mL of 2-propanol. Store at room temperature for up to one month.

30.4.8 Working Internal Standard (1.0 mg/L): Pipette 10.0 µL of the 1.0 mg/mL or 100 µL of 0.1 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

30.4.9 Controls

30.4.9.1 Negative control: blank blood or equivalent determined not to contain target compounds.

30.4.9.2 High Threshold Working Standard (10.0 mg/L): Pipette 100 µL of 1.0 mg/mL or 1000 µL of 0.1 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable. This may be made into two different standards due to volume limitations.

30.4.9.3 Low Threshold Working Standard A (1.0/5.0/10.0 mg/L): Pipette the following volumes for each compound given a 1.0 mg/mL stock solution into a 10.0 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

Target Compound	Volume of 1.0 mg/mL Stock Solution (µL)
Dibutylone	10
4/5/6-MAPB	10
5-DBFPV	10

4-APDB	50
5-APDB	50
Pentylone	50
N-Ethylpentylone	50
Tenocyclidine	50
4-Chloro-alpha-PVP	50
3-Methoxy-PCP	50
Mitragynine	50
Methoxphenidine	50
PV8	50
Clonazolam	50
6-APDB	100

30.4.9.4 Low Threshold Working Standard B (0.01/0.05/0.1 mg/L): Pipette 100 µL of Low Threshold Working Standard A into a 10.0 mL volumetric flask and qs to volume with acetonitrile or methanol, whichever is more suitable.

30.4.9.5 Control expiration: Expired controls may be used within this method for screening purposes only. Any presumptive positives determined from expired controls will require confirmation with an unexpired reference material. Mixed controls may have expiration dates set before all components expire which will be documented in an MFR from the Program Manager.

30.4.9.6 Preparation of qualitative threshold controls

Control Level	High Threshold Working Standard 10.0 mg/L (µL)	Low Threshold Working Standard B 0.01/0.05/0.1 mg/L (µL)	Final Concentration Qualitative Control (mg/L)
Low Threshold Control	-	50	0.001/0.005/0.01
High Carryover Control	50	-	1.0

30.5 Apparatus

30.5.1 Agilent Technologies LCMSMS, MassHunter software, compatible computer and printer

30.5.2 Test tubes, round bottom, screw cap tubes, borosilicate glass with Teflon caps

30.5.3 Test tubes, glass tubes, conical bottom

30.5.4 Centrifuge capable of 2,000-3,000 rpm

30.5.5 Solid phase extraction cartridges (UCT CleanScreen ZSDAU020 columns)

30.5.6 Solid phase extraction manifold

30.5.7 Vortex mixer

30.5.8 Heating block

30.5.9 Evaporator/concentrator

30.5.10 GC autosampler vials and inserts

30.5.11 Agilent Technologies LCMSMS Parameters:

30.5.11.1 LC Parameters:

- Column: Agilent Technologies Poroshell 120 EC-C18, 3.0x50 mm 2.7 µm (P.N. 699975-302)

- Column Thermostat: 60ºC
- Mobile Phase A: Water with 0.1% formic acid
- Mobile Phase B: Methanol with 0.1% formic acid
- Flow Rate: 0.7 mL/min
- Injection Volume: 10 µL with a minimum 5 second needle wash
- Stop Time: 16.0 minutes
- Post Time: Minimum 2.0 minutes
- Gradient:

Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	98.0	2.0
2.00	98.0	2.0
7.00	93.0	7.0
13.00	5.0	95.0
14.00	2.0	98.0
15.00	2.0	98.0
16.00	98.0	2.0

30.5.11.2 MSMS Parameters

- Ionization: ESI
- Polarity: Positive
- Gas Temperature: 325ºC
- Nebulizer Pressure: 45 psig
- Capillary: 3500 V
- Drying Gas Flow: 12 L/min
- Delta EMV: 400 V
- Transition Ions: (Note: Target MRMs for all listed compounds may not be removed from the data acquisition method without Program Manager approval due to changes in sensitivity for isomeric compounds. Target transitions are in bold font, qualifier transition is in normal font.) Frag = Fragmentor, CE = Collision Energy, Cell Accel = Cell Accelerator Voltage

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Frag (V)	CE (V)	Cell Accel (V)	Approx. RT (min)	Delta RT (min)
4-APDB	**178.1**	**161.1**	**75**	**4**	**2**	6.280	3
		133.0		16		6.280	
5-APDB	**178.1**	**161.1**	**65**	**4**	**2**	6.870	3
		133.0		20		6.870	
6-APDB	**178.1**	**161.1**	**75**	**4**	**2**	7.600	3
		133.0		20		7.600	
Dibutylone (bk-DMBDB)	236.1	161.1	105	20	2	8.019	3
		86.1		**24**		8.019	
4/5/6-MAPB	190.1	159.1	90	8	2	8.548	3
		131.0		**20**		8.548	
Pentylone-D_3	**239.1**	**221.2**	**100**	**12**	**2**	9.000	3
		191.1		20		9.000	
Pentylone/N,N-Dimethylpentylone	236.1	218.1	85	8	2	9.080	3

		188.1		**16**		9.080	
N-Ethylpentylone/N,N-Diethylpentylone	250.1	202.1	115	16	2	9.100	3
		232.1		**8**		9.100	
5-DBFPV	274.2	133.0	135	28	2	9.596	3
		126.1		**24**		9.596	
Tenocyclidine (TCP)	**250.2**	**165.0**	**70**	**8**	**2**	9.724	3
		86.0		4		9.724	
4-Chloro-alpha-PVP	266.1	126.1	100	28	2	10.022	3
		125.0		**24**		10.022	
3-Methoxy-PCP	**274.2**	**121.1**	**90**	**28**	**2**	10.055	3
		86.1		8		10.055	
Mitragynine	399.2	226.1	130	24	2	10.137	3
		174.1		**32**		10.137	
Methoxphenidine	**296.2**	**211.1**	**100**	**8**	**2**	10.215	3
		117.1		20		10.215	
PV8	260.2	154.1	135	28	2	10.336	3
		91.1		**24**		10.336	
25I-NBOMe-D_3	**431.1**	**124.1**	**120**	**24**	**2**	10.500	3
		92.1		25		10.500	
Clonazolam	**354.1**	**308.1**	**135**	**28**	**2**	10.655	3
		280.1		40		10.655	

30.5.12 Data Analysis Parameters (Note: Relative Retention Time acceptance criteria established for isomeric compounds only.)

Relative Retention Time Acceptance Criterion	
Target	Relative Retention Time Criteria (%)
4-APDB	1.5
5-APDB	1.5
6-APDB	1.5
4/5/6-MAPB	0.5
Pentylone/N,N-Dimethylpentylone	0.3
Dibutylone	1.5
N-Ethylpentylone/N,N-Diethylpentylone	0.3
3-Methoxy-PCP	0.3

30.6 Procedure

30.6.1 Label appropriate clean screw cap tubes accordingly, negative, threshold controls and case sample IDs.

30.6.2 Prepare controls. To eliminate solvent effect, controls shall be dried down under nitrogen/air prior to the addition of blank blood.

30.6.3 Pipette 500 µL of blank blood, controls, and case sample blood/fluids in appropriately labeled tubes.

30.6.4 Add 10 µL of 1.0 mg/L internal standard solution to each tube.

30.6.5 Vortex briefly.

30.6.6 Add 1.0 mL of 0.1 M phosphate buffer (pH 6) to each tube and vortex.

30.6.7 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation.

30.6.8 Solid phase extraction. Place SPE cartridges in the extraction manifold. Throughout the SPE procedure, it is important not to permit the SPE sorbent bed to dry, unless specified. If necessary, add additional solvent/buffer to re-wet.

30.6.8.1 Add 1.0 mL methanol to each column and aspirate.

30.6.8.2 Add 1.0 mL 0.1 M phosphate buffer (pH 6) to each column and aspirate.

30.6.8.3 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with approximately 1-2 mL/minute flow.

30.6.8.4 Add 1.0 mL dH_2O to each column and aspirate.

30.6.8.5 Add 1.0 mL acetate buffer (pH 4.5) to each column and aspirate.

30.6.8.6 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

30.6.8.7 Elute with 1.0 mL methanol. Dry the columns at > 10 inches of Hg for at least 2 minutes.

30.6.8.8 Elute with 3.0 mL of freshly prepared dichloromethane/isopropanol/ammonium hydroxide solution to each column. Collect eluate by gravity drain (no vacuum).

30.6.9 Add 40 µL of 0.2% HCl in isopropanol.

30.6.10 Evaporate samples to dryness at approximately 50ºC under nitrogen.

30.6.11 Reconstitute in 50 µL reconstitution solvent. (Note: Centrifugation may be necessary at this step.)

30.6.12 Transfer to autosampler vials.

30.7 Quality Control and Reporting

30.7.1 The presence of novel psychoactive substances may only be reported if the instrumental response ratio is equal to or greater than the low threshold control response ratio and all other qualitative identification criterion are met. If the instrumental response of a case sample is greater than the high carryover control, an evaluation of carryover shall be performed.

30.7.2 Any targets that are outside of the relative retention time (RRT) acceptance range shall not be reported.

30.7.3 The following table represents extracted sample stability

Target Compound	Stability (Days)
4-APDB	7
5-APDB	7
Dibutylone (bk-DMBDB)	7
6-APDB	7
4/5/6-MAPB	7
Pentylone/N,N-Dimethylpentylone	7
N-Ethylpentylone/N,N-Diethylpentylone	7
5-DBFPV	7
Tenocyclidine (TCP)	7
4-Chloro-alpha-PVP	7
3-Methoxy-PCP	7

Methoxphenidine	7
PV8	7
Clonazolam	7
Mitragynine	2

30.7.4 As this method can be used for screening, it shall be reported with the screened list on the Certificate of Analysis. The language shall be in the following format:

Novel psychoactive substances – (insert here)

The COA should reflect the panel and/or targets screened for that are listed within this procedure.

30.8 Notes

The following were validated with this method but are no longer analyzed. The MSMS parameters for these compounds are listed below and shall remain in the instrumental method.

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Frag (V)	CE (V)	Cell Accel (V)	Aprox. RT (min)	Delta RT (min)
25B-NBOMe	380.1	91.1	105	58	2	10.687	3
		121.1		**16**		10.687	
25I-NBF	**416.1**	**291.0**	**135**	**20**	**2**	10.729	3
		275.9		32		10.729	
25I-NBMD	442.1	286.1	130	16	2	10.753	3
		135.0		**28**		10.753	
25I-NBOMe	**428.1**	**121.1**	**120**	**20**	**2**	10.855	3
		91.1		58		10.855	
25I-NBOMe-D_3	**431.1**	**124.1**	**120**	**24**	**2**	10.500	3
		92.1		25		10.500	
25C-NBOMe	**336.1**	**121.0**	**95**	**20**	**2**	10.588	3
		91.1		54		10.588	
25I-NBOH	**414.1**	**291.0**	**130**	**20**	**2**	10.627	3
		107.0		32		10.627	
25H-NBOMe	302.2	121.1	120	16	2	10.232	3
		91.1		**40**		10.232	
Methiopropamine	**156.1**	**125.0**	**75**	**8**	**2**	3.062	3
		97.0		20		3.062	
3-fluorophenmetrazine	**196.1**	**115.0**	**105**	**32**	**2**	5.348	3
		109.0		32		5.348	
PB-22	**359.2**	**214.1**	**90**	**8**	**2**	12.599	3
		144.0		40		12.599	
5F-AB-PINACA	**349.2**	**304.2**	**70**	**12**	**2**	11.464	3
		233.1		20		11.464	
AB-FUBINACA	369.2	253.1	95	24	2	11.666	3
		109.1		**56**		11.666	
ADB-FUBICA	**382.2**	**252.1**	**85**	**16**	**2**	11.869	3
		109.0		48		11.869	
AB-PINACA	331.2	286.2	70	12	2	12.067	3

		215.0		**24**		12.067	
4F/5F-AMB	**364.2**	**233.1**	**115**	**20**	**2**	12.090	3
		145.0		50		12.090	
3F-AMB	**364.2**	**233.1**	**115**	**24**	**2**	12.090	3
		145.0		44		12.090	
4F-ADB	378.2	318.2	130	12	2	12.096	3
		233.1		**24**		12.096	
5F-PB-22	**377.2**	**232.1**	**80**	**12**	**2**	12.097	3
		144.0		40		12.097	
AMB-FUBINACA	384.2	253.0	125	20	2	12.222	3
		109.0		**50**		12.222	
SDB-006	321.2	214.1	145	20	2	12.339	3
		91.1		**48**		12.339	
FUB-MDMB (MDMB-FUBINACA)	398.2	253.1	105	24	2	12.458	3
		109.0		**48**		12.458	
MAB-CHMINACA (ADB-CHMINACA)	**371.2**	**241.1**	**110**	**24**	**2**	12.588	3
		145.0		44		12.588	
MMB-CHMICA	**371.2**	**240.1**	**115**	**12**	**2**	12.647	3
		144.0		40		12.647	

30.9 References

30.9.1 L. Moses and R. Wagner, Qualitative Analysis of Novel Psychoactive Substances using LCMSMS In-house Development, 2017.

30.9.2 L. Moses and R. Wagner, Qualitative Analysis of Novel Psychoactive Substances using LCMSMS Validation, 2018.

30.9.3 Ambach, Lars *et al.,* Detection and quantification of 56 new psychoactive substances in whole blood and urine by LC-MS/MS. Bioanalysis 7(9), 1119-1136, 2015.

30.9.4 Bertol, Elisabetta *et al*., A novel screening method for 64 new psychoactive substances and 5 amphetamines in blood by LC-MS/MS and application to real cases. Journal of Pharmaceutical and Biomedical Analysis 129, 441-449, 2016.

30.9.5 Scientific Working Group for Forensic Toxicology (SWGTOX) Standard practices for method validation in forensic toxicology. *JAT* 2013, 37, 452-474.

31 Miscellaneous Basic Drug Quantitation and Confirmation by LCMSMS

31.1 Summary

Miscellaneous basic drugs including fluoxetine, paroxetine, sertraline, quetiapine, hydroxyzine, and cyclobenzaprine are extracted from biological samples using solid phase extraction (SPE) and confirmed or quantified with liquid chromatography tandem mass spectrometry using multiple reaction monitoring mode. Drug targets may be analyzed in different combinations or separately as needed.

31.2 Specimen Requirements

1 mL of blood, fluid, or tissue homogenate. Note: Tissue homogenate shall be analyzed qualitatively only.

31.3 Reagents and Standards

- Drugs and internal standards

Drug	Internal Standards
Fluoxetine	Fluoxetine-D_6
Paroxetine	Paroxetine- D_6
Sertraline	Sertraline-D_3
Quetiapine	Quetiapine-D_8
Hydroxyzine	Quetiapine-D_8
Cyclobenzaprine	Cyclobenzaprine-D_3

- Acetonitrile, Optima (or similar) grade or higher
- Glacial Acetic Acid
- Ammonium hydroxide
- Ethyl Acetate
- Dichloromethane
- Methanol, ACS grade or better
- 2-Propanol, ACS grade or better
- Type I or LCMS grade water
- Distilled water (dH_2O)
- Formic acid, Fisher Optima (or similar) grade or higher
- n-Hexane
- Potassium Phosphate

31.4 Solutions, Internal Standards, Calibrators, and Controls

31.4.1 1 M Acetic acid: Add 100-200 mL dH_2O to a 1 L volumetric flask. Add 57.5 mL glacial acetic acid and qs to volume with dH_2O. Alternatively, add 28.8 mL glacial acetic acid and qs to 500 mL in a volumetric flask (partially filled with dH_2O). Alternatively add 5.75 mL of glacial Acetic Acid to a 100 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

31.4.2 0.1 M Phosphate buffer, pH 6.0: Weigh 13.61 g of KH_2PO_4 and transfer into a 1 L volumetric flask containing approximately 800 mL of dH_2O. Adjust the pH of the solution to 6.0 by the addition of 5.0 M potassium hydroxide while stirring. QS to volume using dH_2O. Store at room temperature for up to 2 years. This may also be purchased as a concentrate and diluted following the instructions.

31.4.3 Elution solvent (dichloromethane, 2-propanol, ammonium hydroxide, 78:20:2): Combine 20 mL of 2-propanol with 2.0 mL of ammonium hydroxide then add 78 mL of dichloromethane. Mix gently. pH should be between 11-12. Make fresh for each day of use. Note: additional ammonium hydroxide may be required to obtain the desired pH.

31.4.4 Mobile phase A (water with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L Type I or LCMS grade water. Store at room temperature for up to one month.

31.4.5 Mobile phase B (acetonitrile with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L Optima (or similar) grade or higher acetonitrile. Store at room temperature for up to one month.

31.4.6 Preparation of working internal standard solution (10 µg/mL)

31.4.6.1 Dilute 1 mL of 100 µg/mL or 100 µL of 1 mg/mL of each internal standard (paroxetine-d6, quetiapine-d8, fluoxetine-d6, cyclobenzaprine-d3, sertraline-d3) stock solution in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable.

31.4.7 Preparation of calibrators

31.4.7.1 Working calibrator solution A (10 µg/mL): Dilute 100 µL of each 1.0 mg/mL standard stock solution (paroxetine, fluoxetine, sertraline, quetiapine, hydroxyzine, and cyclobenzaprine) in a calibrated 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable.

31.4.7.2 Working calibration solution B (1 µg/mL): Dilute 1000 µL of 10 µg/mL standard solution A in a calibrated 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable.

31.4.8 To prepare the calibration curve, pipette the following volumes of working calibrator solutions into appropriately labeled screw cap test tubes. To eliminate a solvent effect, calibrators may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to obtain the final concentrations listed below.

Calibrator Concentration (mg/L)	Volume of 1 µg/mL Working Solution (µL)	Volume of 10 µg/mL Working Solution (µL)
1.0		100
0.75		75
0.50		50
0.20		20
0.10		10
0.050	50	
0.020	20	
0.010	10	
TC (0.005)	5	

31.4.9 Preparation of controls

31.4.9.1 Working control solution A (10 µg/mL): Dilute 1000 µL of each 100 µg/mL standard stock solution, or 100 µL of each 1 mg/mL standard stock solution (paroxetine, fluoxetine, sertraline, quetiapine, hydroxyzine, and cyclobenzaprine) in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable.

31.4.9.2 Working control solution B (1 µg/mL): Dilute 1000 µL of 10 µg/mL standard solution A in a 10 mL volumetric flask with acetonitrile or methanol, whichever is more suitable.

31.4.9.3 To prepare positive controls, pipette the following volumes of the working control solution into appropriately labeled screw cap test tubes. To eliminate a solvent effect, controls may be dried under nitrogen/air prior to the addition of blank blood. Add 1 mL blank blood to obtain the final concentrations listed below.

Positive Control Concentration (mg/L)	Volume of 1 µg/mL Working Solution (µL)	Volume of 10 µg/mL Working Solution (µL)
0.030	30	
0.40		40
0.75		75
0.80		80

31.4.9.4 Negative control: Blood bank blood or equivalent determined not to contain paroxetine, fluoxetine, sertraline, quetiapine, hydroxyzine, or cyclobenzaprine.

31.4.9.5 Due to the quadratic nature of the quetiapine and hydroxyzine calibration curve, at least three controls at low, medium and high concentrations must be analyzed across the concentration range in every batch. If the 1.0 mg/L calibrator is included in the calibration curve, the 0.75 mg/L control or a control between the 0.50 mg/L and 1.0 mg/L calibrators must be included.

31.5 Apparatus

31.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

31.5.2 Test tubes, round bottom, borosilicate glass

31.5.3 Test tubes, conical bottom

31.5.4 Centrifuge capable of 3500 rpm

31.5.5 Solid phase extraction columns (SPE) UCT ZSDAU020

31.5.6 United Chemical Technologies 48 place positive pressure manifold or suitable substitute

31.5.7 Evaporator/concentrator

31.5.8 Vortex mixer

31.5.9 GC autosampler vials with inserts

31.5.10 Typical LC-MS-MS parameters.

LC Parameters
Column: Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size
Column Thermostat: 40°C
Solvent A: H_2O with 0.1% formic acid
Solvent B: Acetonitrile with 0.1% formic acid
Initial Flow Rate: 0.50 mL/min
Injection vol.: 1.0 µL with a minimum 10 second needle wash
Stop time: 6.0 min
Gradient: Initial 10% B
5.00 minutes 50% B
5.01 minutes 90% B
6.00 minutes 90% B
Post time minimum 2 minutes

31.5.11 Typical MS-MS parameters.

- MSD Parameters:
  Scan: Dynamic MRM
  Ionization: ESI
  Polarity: positive
  Gas temp: 350°C
  Drying Gas: 10.0 L/min
  Nebulizer press: 40 psi
  Capillary: 4000 V
  Delta EMV: 400 V

- Dynamic MRM (Delta RT for all analytes and IS = 2 min) (Bold underlined transition ions are used for quantification. Frag – Fragment Voltage; CE - collision energy)

Analytes and IS	Transitions (m/z)		Frag (V)	Cell Accel (V)	CE (V)	Approx. RT (min)
	Q1	Q3				
Quetiapine	384.2	**279.1**	140	4	25	3.1
		253.1	140	4	21	
Quetiapine-D_8	392.2	**258.1**	140	4	25	3.1
		286.1	140	4	29	
Paroxetine	330.2	**192.1**	110	4	21	3.9
		70.1	110	4	33	
Paroxetine-D_6	336.2	**198.2**	140	4	21	3.9
		76.1	140	4	37	
Hydroxyzine	375.2	**201.1**	110	4	17	4.1
		165.1	110	4	77	
Cyclobenzaprine	276.18	**215**	126	4	47	4.2
		202.1	126	4	55	
Cyclobenzaprine-D_3	279.2	**215.1**	132	4	52	4.2
		205.1	132	4	24	
Fluoxetine	310.1	**148.1**	80	4	4	4.5
		44.1	80	4	9	
Fluoxetine-D_6	316.18	**154.1**	94	4	8	4.5
		44	94	4	12	
Sertraline	306.08	**159**	94	4	28	4.7
		275	94	4	8	
Sertraline-D_3	309.1	**159**	85	4	32	4.7
		275	85	4	8	

31.6 Procedure

31.6.1 Prepare calibrators and controls.

31.6.2 Add 1.0 mL of case specimens/blank blood to appropriately labeled tubes.

31.6.3 Add 50 µL of internal standard solution and vortex briefly.

31.6.4 Add 2.0 mL deionized water to each tube. Vortex briefly and let stand for 5 minutes.

31.6.5 Centrifuge at approximately 3500 RPM for 15 min to achieve separation. Transfer supernatant to clean tubes and discard the tube with the remaining pellet.

31.6.6 Add 2.0 mL of phosphate buffer and vortex.

31.6.7 Solid phase extraction (unless otherwise instructed, column flow rate is approximately 1 mL/min).

31.6.7.1 Add 3.0 mL of hexane to each column and aspirate.

31.6.7.2 Add 3.0 mL of methanol to each column and aspirate.

31.6.7.3 Add 3.0 mL of dH_2O to each column and aspirate.

31.6.7.4 Add 1.0 mL of phosphate buffer to each column and aspirate.

31.6.7.5 Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with approximately 1-2 mL/minute flow.

31.6.7.6 Add 3.0 mL of dH_2O to each column and aspirate.

31.6.7.7 Add 3.0 mL of dH_2O to each column and aspirate.

31.6.7.8 Add 2.0 mL of 1.0 M acetic acid to each column and aspirate.

31.6.7.9 Add 3.0 mL of methanol to each column and aspirate.

31.6.7.10 Dry column for approximately 2 minutes at maximum pressure or flow.

31.6.7.11 Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube.

31.6.7.12 Elute column with 3.0 mL of freshly prepared methylene chloride/isopropanol/ammonium hydroxide elution solvent by gravity flow into a screw-cap conical centrifuge tube.

31.6.8 Evaporate eluate to dryness at approximately 50°C under nitrogen.

31.6.9 Reconstitute with 100 µL of mobile phase A.

31.6.10 Vortex briefly.

31.6.11 If necessary, centrifuge the tubes for 15 minutes at approximately 2800 rpm.

31.6.12 Transfer to an autosampler vial fitted with a glass insert.

31.7 Quality Control and Reporting

31.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

31.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

31.7.3 The validated LOD for all targets is 0.0025 mg/L. The TC for this method is set at 0.0050 mg/L.

31.7.4 The calibration models for quetiapine and hydroxyzine are quadratic and weighted (1/x). The calibration models for paroxetine, cyclobenzaprine, fluoxetine, and sertraline are linear and weighted (1/x).

31.7.5 Dilution

31.7.5.1 Small volume dilutions: use less than 1 mL of sample and dilute up to 1 mL. All targets can be diluted up to 1/20 (50 µL sample, 950 µL blank matrix).

31.7.5.2 Large volume dilutions: use 1 mL of sample and dilute to larger volumes than 1 mL. Sertraline can be diluted up to 1/4 (1 mL of sample, 3 mL of blank matrix) and other targets can be diluted up to 1/20 (1 mL of sample, 19 mL blank matrix).

31.7.6 Extracted samples are stable for 7 days for all targets.

31.7.7 If analyzing fluoxetine or sertraline, solvent/matrix blanks shall be run between all calibrators, controls, and samples to assist in the evaluation of carryover. If these targets are not analyzed, blanks are not necessary.

31.7.8 See Toxicology Quality Guidelines.

31.8 References

31.8.1 A. de Castro, M. Concheiro, O. Quintela, A. Cruz, M. López-Rivadulla; “LC-MS/MS method for the determination of nine antidepressants and some of their main metabolites in oral fluid and plasma Study of correlation between venlafaxine concentrations in both matrices”; Journal of Pharmaceutical and Biomedical Analysis 48 (2008): 183-193.

31.8.2 M. Fisichella, L. Morini, C. Sempio, A. Groppi; “Validation of a multi-analyte LC-MS/MS method for screening and quantification of 87 psychoactive drugs and their metabolites in hair”; Analytical and Bioanalytical Chemistry (2014); 406 (16): 3497-506.

31.8.3 C. Harris, T. Wright, J. Kuhlman; Virginia Department of Forensic Science; In-house method development miscellaneous basic drugs by LCMSMS, 2020.

31.8.4 R. Wagner, A. Siddiqi; Virginia Department of Forensic Science; Expansion of the Validation of Miscellaneous Basic Drug Quantitation and Confirmation by Solid Phase Extraction Using LCMSMS, 2022.

31.8.5 R. Wagner, A. Siddiqi; Virginia Department of Forensic Science; Validation of Sertraline in the Miscellaneous Basic Drug Quantitation and Confirmation by Solid Phase Extraction Using LCMSMS, 2022.

32 Barbiturate Quantitation and Confirmation By LCMSMS

32.1 Summary

Barbiturates are extracted from biological samples using a liquid-liquid extraction. An aliquot of the extract is quantitated and confirmed by LCMSMS. Drug targets may be analyzed in different combinations or separately as needed.

32.2 Specimen Requirements

0.1 mL blood, fluid or tissue homogenate

32.3 Reagents and Standards

- Drug targets and associated internal standards

Target	Internal Standard
Butabarbital	Butalbital-D_5
Butalbital	Butalbital-D_5
Pentobarbital	Pentobarbital-D_5
Phenobarbital	Phenobarbital-D_5
Secobarbital	Secobarbital-D_5

- Methanol, Optima grade or higher
- Acetonitrile, Optima grade or higher
- Type I or LCMS grade water
- Ammonium acetate, ≥99.99% trace metals basis
- Hydrochloric acid, ACS or higher grade
- n-Hexane, Optima grade or higher
- Ethyl acetate, Optima grade or higher

32.4 Solutions, Internal Standard, Calibrators and Controls

32.4.1 Mobile Phase A (H_2O with 5 mM Ammonium Acetate): Add approximately 0.385 grams of ammonium acetate to 1.0 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

32.4.2 Mobile Phase B (Methanol): 1.0 L of methanol.

32.4.3 0.1 M HCl: Add 830 µL of concentrated hydrochloric acid into 100 mL volumetric flask that is partially filled with dH_2O and qs to volume with dH_2O. Store at room temperature for up to two years.

32.4.4 1:9 hexane:ethyl acetate: Add 90 mL of ethyl acetate to 100 mL graduated cylinder. Add 10 mL of n- hexane. Store at room temperature for up to two years.

32.4.5 90/10 water/methanol reconstitution solvent: Add 1 mL methanol to 9 mL water, mix well. Store at room temperature for up to one month.

32.4.6 Preparation of calibrators.

32.4.6.1 Working internal standard solution (20 µg/mL): Pipette 200 µL of 1.0 mg/mL stock solution of deuterated standards into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

32.4.6.2 Working standard solution (100 µg/mL): Pipette 1.0 mL of the 1.0 mg/mL stock solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

32.4.6.3 Working standard solution (10 µg/mL): Pipette 1.0 mL of the 100 µg/mL working standard solution into a 10 mL volumetric flask and qs to volume with methanol or acetonitrile, whichever is more suitable.

32.4.6.4 To prepare the calibration curve, pipette the following volumes of the 100 µg/mL and 10 µg/mLworking standard solutions into appropriately labeled 16 x 100 mm or 16 x 125 mm screw cap test tubes. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood. Add 0.1 mL blank blood to obtain the final concentrations listed below.

Amount of 100 µg/mL stock solution (µL)	Amount of 10 µg/mL stock solution (µL)	Final concentration of Barbiturates (mg/L)
40		40
30		30
20		20
10		10
	50	5
	25	2.5
	10	1
	5	TC (0.5)

32.4.7 Controls

32.4.7.1 Barbiturate Controls. Controls may be from an external source or prepared in-house using standards from different manufacturers or lot numbers than those used to prepare the calibrators. The 15 mg/L control level will be used for control chart tracking.

Amount of 100 µg/mL stock solution (µL)	Amount of 10 µg/mL stock solution (µL)	Final concentration of Barbiturates (mg/L)
32		32
15		15
	30	3

32.4.7.2 Negative control. Blood bank blood or equivalent determined not to contain barbiturates or other targets.

32.5 Apparatus

32.5.1 Test tubes, round bottom, borosilicate glass with Teflon caps

32.5.2 Centrifuge capable of 2000-3000 rpm

32.5.3 Evaporator/concentrator

32.5.4 Vortex mixer

32.5.5 GC autosampler vials with inserts

32.5.6 LC Parameters

- Column: Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm particle size
- Column Thermostat: 60 °C
- Mobile Phase A: H_2O with 5 mM Ammonium Acetate
- Mobile Phase B: Methanol
- Initial Flow Rate: 0.7 mL/min

- Injection vol.: 10.0 µL with a minimum 3 second needle wash
- Stop time: 12 min
- Gradient:

Initial	10% B
9.5 minutes	45% B
10.5 minutes	90% B
11.5 minutes	90% B
12 minutes	10% B
Post time	Minimum 1 minute

32.5.7 MS-MS parameters:

- MSD Parameters:

- Ionization: ESI
- Polarity: Negative
- Gas temp: 350 °C
- Drying Gas: 10.0 L/min
- Nebulizer press: 40 psi
- Capillary: 4000 V
- Delta EMV: 400 V

32.5.8 Transition Ions (bold product ions are the quantitation transitions) (Delta RT = 2 min)

Compound	Precursor Ion	Product Ions	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	RT (approx.)
Phenobarbital	231.1	188.2	95	4	5	4.386
		42.1		16		
Phenobarbital-D_5	236.1	193.1	95	0	5	4.445
		42.1		16		
Butabarbital	211.1	168	95	12	5	5.837
		42		15		
Butalbital	223.1	180.1	95	12	5	6.440
		42.1		16		
Butalbital-D_5	228.1	185.1	80	10	5	6.396
		42.1		12		
Pentobarbital/Amobarbital	225.1	182.2	95	12	5	8.065
		42.1		16		
Pentobarbital-D_5	230.2	187.1	85	18	5	8.022
		42.1		24		
Secobarbital	237.1	194.1	95	4	5	8.961
		42.1		12		
Secobarbital-D_5	242.2	199.2	100	0	5	8.927
		42.1		16		

32.6 Procedure

32.6.1 Label clean screw cap centrifuge tubes appropriately with calibrators, controls and case sample IDs.

32.6.2 Prepare calibrators and controls. To eliminate a solvent effect, calibrators and controls shall be dried under nitrogen/air prior to the addition of blank blood.

32.6.3 Add 0.1 mL case specimens/blank blood to the appropriately labeled tubes.

32.6.4 Add 50 µL of the 20 µg/mL internal standard working solution to each tube.

32.6.5 Add 20 µL of 0.1 M HCl to each tube and vortex briefly.

32.6.6 Add 0.4 mL of 1:9 hexane:ethyl acetate to each tube and vortex for approximately 15 seconds. Ensure that the blood in the bottom of the tube mixes thoroughly with the extraction solvent.

32.6.7 Centrifuge at approximately 2800 rpm for 15 minutes to achieve separation.

32.6.8 Transfer topmost layer into test tubes. Avoid transferring any of the precipitate into the test tube.

32.6.9 Evaporate to dryness at approximately 50-60°C under nitrogen.

32.6.10 Reconstitute in 100 µL of 90/10 water/methanol. (Note: Centrifugation may be necessary at this step)

32.6.11 Transfer to autosampler vials.

32.7 Quality Control and Reporting

32.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte.

32.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

32.7.3 The validated LOD for pentobarbital and secobarbital is 0.25 mg/L and for butabarbital, butalbital, and phenobarbital the LOD is 0.5 mg/L. The TC for this method is set at 0.5 mg/L.

32.7.4 The calibration model for butalbital is quadratic and weighted (1/x) and all other targets is linear and weighted (1/x).

32.7.5 Dilutions: All targets may be diluted up to 1/20 (e.g., 0.05 mL sample with 0.95 mL matrix) with this method.

32.7.6 Extracted samples are stable for 7 days except for butabarbital which is stable for 6 days.

32.7.7 If a confirmed response is detected for pentobarbital/amobarbital, the sample should be analyzed using the GCMS-based barbiturate method for differentiation and confirmation.

32.7.8 See Toxicology Quality Guidelines

32.8 References

32.8.1 Crapps, D. and Wagner, R. Barbiturates quantitation and confirmation by LCMSMS method development. Virginia Department of Forensic Science. **2020**.

32.8.2 Zhang, X., Lin, Z., Li, J., et al. Rapid Determination of nine barbiturates in human whole blood by liquid chromatography-tandem mass spectrometry. *Drug Testing and Analysis*. 9, 588-595, **2017**.

32.8.3 ANSI/ASB Standard 036 Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. **2019**.

33 Cannabinoid Quantitation and Confirmation by Supported Liquid Extraction Using LCMSMS

33.1 Summary

Δ^9-tetrahydrocannabinol (Δ^9-THC), 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH, Δ^9-Carboxy-THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-OH-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), 11-hydroxy- Δ^8-tetrahydrocannabinol (Δ^8-OH-THC), 11-nor-9-carboxy- Δ^8-tetrahydrocannabinol (Δ^8-THC-COOH, Δ^8-Carboxy-THC), and 9R- $\Delta^{6a,10a}$-tetrahydrocannabinol (9R-$\Delta^{6a,10a}$-THC) are extracted from biological samples by a supported liquid extraction (SLE). An aliquot of the extract is quantitated and/or confirmed by LCMSMS. CBD, Δ^8-THC, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC are analyzed qualitatively only. Drug targets may be analyzed in different combinations or separately as needed.

33.2 Specimen Requirements

0.5 mL blood, fluid, or urine. Note: Urine will only be qualitatively assessed. This method shall not be used for tissue homogenates.

33.3 Reagents and Standards

- Drug targets and internal standards

Targets	Internal Standards
(-)Δ^9-Tetrahydrocannabinol (Δ^9-THC)	(-)Δ^9-Tetrahydrocannabinol-D_3 (Δ^9-THC-D_3)
(-)Δ^8-Tetrahydrocannabinol (Δ^8-THC)	(-)Δ^9-Tetrahydrocannabinol-D_3 (Δ^9-THC-D_3)
(-)11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol (Δ^9-Carboxy-THC, Δ^9-THC-COOH)	(±)11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol-D_3 (Δ^9-Carboxy-THC-D_3)
(±)11-Hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-OH-THC)	(±)11-Hydroxy-Δ^9-tetrahydrocannabinol-D_3 (Δ^9-OH-THC-D_3)
Cannabidiol (CBD)	Cannabidiol-D_3 (CBD-D_3)
Additional qualitative targets that may not be analyzed with every batch.	
(-)11-hydroxy- Δ^8-tetrahydrocannabinol (Δ^8-OH-THC)	(±)11-Hydroxy-Δ^9-tetrahydrocannabinol-D_3 (Δ^9-OH-THC-D_3)
11-nor-9-carboxy- Δ^8-tetrahydrocannabinol (Δ^8-THC-COOH, Δ^8-Carboxy-THC)	(±)11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol-D_3 (Δ^9-Carboxy-THC-D_3)
9R- $\Delta^{6a,10a}$-tetrahydrocannabinol (9R-$\Delta^{6a,10a}$-THC)	(-)Δ^9-Tetrahydrocannabinol-D_3 (Δ^9-THC-D_3)

- dH_2O
- Type 1 or LCMS grade water
- Ammonium formate, LCMS grade or higher
- Formic acid, eluent additive for LCMS
- Ethyl acetate, Fisher Optima (or similar) grade or higher
- n-Hexane, Fisher Optima (or similar) grade or higher
- Methanol, Fisher Optima (or similar) grade or higher
- Acetonitrile, Fisher Optima (or similar) grade or higher
- Dichlorodimethylsilane, Sigma Aldrich 98.5+% (or similar) grade or higher
- Toluene, Fisher ACS (or similar) grade or higher

33.4 Solutions, Internal Standard, Calibrators and Controls

33.4.1 5% Dichlorodimethylsilane in Toluene: Add 50 mL of dichlorodimethylsilane into a 1000 mL volumetric flask and qs with toluene. Store at room temperature for up to 2 years.

33.4.2 0.1% Formic Acid in Water: Pipette 1 mL of formic acid into a 1000 mL volumetric flask half filled with dH_2O and qs to volume with dH_2O. Different final volumes may be prepared with the concentration set at 0.1% (e.g., 0.1 mL of formic acid into 100 mL dH_2O). Store at room temperature for up to 1 month.

33.4.3 Mobile Phases for Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm

33.4.3.1 Mobile Phase A (H_2O with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

33.4.3.2 Mobile Phase B (80:20 Methanol:Acetonitrile): Combine 800 mL of methanol and 200 mL of acetonitrile for a total volume of 1.0 L. Store at room temperature for up to one month.

33.4.4 Mobile Phases for Poroshell 120 PFP 3.0 x 100 mm, 2.7 µm

33.4.4.1 Mobile Phase A (H_2O with 0.1% formic acid and 5mM ammonium formate): Add 1.0 mL of formic acid and approximately 0.315 grams of ammonium formate to 1.0 L of Type I or LCMS grade H_2O. Store at room temperature for up to one month.

33.4.4.2 Mobile Phase B (Methanol with 0.1% formic acid): Add 1.0 mL of formic acid to 1.0 L of methanol. Store at room temperature for up to one month.

33.4.5 Calibrators and Internal Standard

33.4.5.1 Stock internal standard solution (1/2/5 µg/mL): Pipette 10 µL of the 1.0 mg/mL (or 100 µL of 0.10 mg/mL) certified reference materials of Δ^9-THC-D_3, 200 µL of 0.1 mg/mL cannabidiol-D_3 and Δ^9-OH-THC-D_3, and 50 µL of 1.0 mg/mL Δ^9-Carboxy-THC-D_3 into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.2 Working internal standard solution (0.2/0.4/1.0 µg/mL): Pipette 2.0 mL of the 1/2/5 µg/mL stock internal standard solution of deuterated standards into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol)..

33.4.5.3 Δ^9-THC stock standard calibrator solution (0.1 mg/mL Δ^9-THC): Pipette 1.0 mL of 1.0 mg/mL certified reference material (CRM) standard into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.4 Working calibrator standard solution (1/2/5 ug/mL): Pipette 100 µL of the Δ^9-THC stock standard calibrator solution and 20 µL/50 µL of 1.0 mg/mL CRM standard (Δ^9-OH-THC/Δ^9-Carboxy-THC) into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.5 Working calibrator standard solution (0.5/1/2.5 µg/mL): Pipette 50 µL of the Δ^9-THC stock standard calibrator solution and 10 µL/25 µL of 1.0 mg/mL CRM standard (Δ^9-OH-THC/Δ^9-Carboxy-THC) into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.6 Working calibrator standard solution (0.05/0.1/0.25 µg/mL): Pipette 1 mL of the 0.5/1/2.5 µg/mL working calibrator standard solution into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.7 CBD, Δ^8-THC, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC working qualitative standard solution (1 µg/mL): Pipette 10 µL of the 1.0 mg/mL CRM standard into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.8 CBD, Δ^8-THC, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC working qualitative standard solution (0.1 µg/mL): Pipette 1 mL of the 1 µg/mL standard solution into a 10 mL volumetric flask and qs to volume with an appropriate solvent (e.g., acetonitrile or methanol).

33.4.5.9 To prepare the calibration curve, pipette the following volumes of the 1/2/5 µg/mL, 0.5/1/2.5 µg/mL or the 0.05/0.1/0.25 µg/mL working calibrator standard solution into appropriately labeled silanized glass test tubes. The two highest calibrators may be prepared with either the 1/2/5 or 0.5/1/2.5 µg/mL calibration solutions. Calibrators and controls shall not be dried down under any circumstances (i.e., nitrogen, heat). Add 0.5 mL blank blood to obtain the final concentrations listed below.

Amount of 1/2/5 µg/mL calibrator solution (µL)	Amount of 0.5/1/2.5 µg/mL calibrator solution (µL)	Amount of 0.05/0.1/0.25 µg/mL calibrator solution (µL)	Final concentration of cannabinoids (mg/L)*
50	(or 100)		0.100/0.200/0.500
25	(or 50)		0.050/0.100/0.250
	25		0.025/0.050/0.125
	10		0.010/0.020/0.050
		50	0.005/0.010/0.025
		25	0.0025/0.0050/0.0125
		10	0.001/0.002/0.005

*Note: The final concentration of cannabinoids (mg/L) is delineated as Δ^9-THC/Δ^9-OH-THC/Δ^9-Carboxy-THC.

33.4.6 Controls

33.4.6.1 Negative control: Blood bank blood or equivalent determined not to contain cannabinoids.

33.4.6.2 Cannabinoid Controls: If Δ^8-OH-THC is included, do NOT combine working qualitative standard solutions with working control standard solutions in preparation of batch controls. The 0.030/0.060/0.150 mg/L control shall be used for control chart tracking if the statewide control is not available. Spike the following for the final control concentration levels:

Amount of 1/2/5 µg/mL solution (µL)	Amount of 0.5/1/2.5 µg/mL solution (µL)	Amount of 0.05/0.1/0.25 µg/mL solution (µL)	Amount of 1.0 µg/mL qualitative solution (µL)	Amount of 0.1 µg/mL qualitative solution (µL)	Final concentration of cannabinoids (mg/L)*
35			25		0.070/0.140/0.350/0.050
	30				0.030/0.060/0.150
		30		25	0.003/0.006/0.015/0.0050
			25		0.050
				25	0.0050
				10	0.0020**

*NOTE: The final concentration of cannabinoids (mg/L) is delineated as Δ^9-THC/Δ^9-OH-THC/Δ^9-Carboxy-THC. The Δ^8-THC, CBD, Δ^8-OH-THC, Δ^8-THC-COOH, 9R-$\Delta^{6a,10a}$-THC final concentration is listed as a separately created control.
**NOTE: This optional control is prepared solely for the purpose of a threshold control for Δ^8-THC and CBD at the validated LOD. This shall not be used as a control for the other qualitative targets.

33.5 Apparatus

33.5.1 Agilent Technologies LCMSMS, MassHunter software, compatible computer and printer

33.5.2 Test tubes, silanized borosilicate glass, round bottom

33.5.3 Test tubes, conical bottom

33.5.4 SLE cartridges (Biotage Isolute SLE 1.0 mL sample PN 820-0140-C)

33.5.5 Evaporator/concentrator

33.5.6 Vortex mixer

33.5.7 GC autosampler vials with inserts

33.5.8 Agilent Technologies LCMSMS parameters: (Note: If the LCMSMS is equipped with the autosampler thermostat, the temperature may be set to 4°C to help minimize evaporative losses.)

33.5.8.1 PFP Column LC Parameters

- Column: Poroshell 120 Pentafluorophenyl (PFP) 3.0 x 100 mm, 2.7 µm (PN 695975-308)
- Column Thermostat: 50°C
- Solvent A: H_2O with 0.1% formic acid and 5mM ammonium formate
- Solvent B: Methanol with 0.1% formic acid
- Flow Rate: 0.50 mL/min
- Injection Volume: 5 µL with minimum 5 second needle wash
- Stop Time: 11.5 minutes
- Post Time: 1.5 minutes minimum
- Gradient:

Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	32	68
10.00	32	68
10.50*	5	95
11.50	5	95

*May be adjusted to 11 minutes for elution of 9R-$\Delta^{6a,10a}$-THC.

33.5.8.2 MS-MS parameters

- MSD Parameters:

  Ionization: ESI
  Polarity: Positive
  Gas Temperature: 350°C
  Drying Gas: 10.0 L/min
  Nebulizer Pressure: 40 psi
  Capillary: 4000 V
  Delta EMV: 400 V

- Transition Ions (Dynamic MRM, bold formatting indicates quantitation transition)

  - Time Segments (TS)
  - TS1 – 0-2.9 minutes (To Waste)
  - TS2 – 2.9-10.5 minutes (To MS)
  - TS3 – 10.5-11.5 minutes (To Waste) (Note: this time segment and the gradient end time may be extended to allow for late elution of targets)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Approx. Retention Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Δ^8-OH-THC	331.2	**201**	3.4	105	20	7
		193.1			20	
Δ^9-OH-THC	331.2	**313.2**	3.9	105	8	7
		193.1			20	
Δ^9-OH-THC-D_3	334.2	**316.2**	3.9	120	8	7
		196.3			20	
Δ^8/Δ^9-Carboxy-THC	345.2	**299.1**	4.5	125	16	7
		193.1			24	
Δ^9-Carboxy-THC-D_3	348.2	**330.1**	4.5	125	12	7
		302.1			16	
Cannabidiol	315.2	**193.1**	4.6	110	20	7
		123			32	
Cannabidiol-D_3	318.2	**196.1**	4.6	110	20	7
		123			32	
Δ^8/Δ^9-THC	315.2	**193**	9.5	120	20	7
		122.9			32	
Δ^9-THC-D_3	318.2	**196**	9.5	120	20	7
		123			32	
9R-$\Delta^{6a,10a}$-THC	315.2	**193**	10.5	120	20	7
		122.9			32	

Note: The Δ^8/Δ^9 transitions and instrumental settings are analyzed separately in data analysis. On the PFP column, Δ^8 isomers elute before Δ^9 isomers. 9R-$\Delta^{6a,10a}$-THC elutes after Δ^9-THC.

33.5.8.3 C18 Column LC Parameters

- Column: Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm (PN 699975-302)
- Column Thermostat: 50°C
- Solvent A: H_2O with 0.1% formic acid
- Solvent B: 80:20 Methanol:acetonitrile
- Flow Rate: 1.0 mL/min
- Injection Volume: 10 µL with minimum 5 second needle wash
- Stop Time: 11.0 minutes
- Post Time: 1.5 minutes minimum
- Gradient:

Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	40	60
1.00	40	60
7.00	23	77
11.00	5	95

33.5.8.4 MS-MS parameters

- MSD Parameters:

  Ionization: ESI
  Polarity: Positive
  Gas Temperature: 350°C
  Drying Gas: 10.0 L/min
  Nebulizer Pressure: 40 psi
  Capillary: 4000 V
  Delta EMV: 400 V

- Transition Ions (bold formatting indicates quantitation transition)

  - Time Segments (TS)
  - TS1 – 0-0.1 minutes (To Waste)
  - TS2 – 0.1-9.0 minutes (To MS)
  - TS3 – 9.0-11.0 minutes (To Waste)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Approx. Retention Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Δ^8-OH-THC	331.2	**201**	3.4	105	20	7
		193.1			20	
Δ^9-OH-THC	331.2	**313.2**	3.8	105	8	7
		193.1			20	
Δ^9-OH-THC-D_3	334.2	**316.2**	3.8	120	8	7
		196.3			20	
Δ^8/Δ^9-Carboxy-THC	345.2	**299.1**	4.3	125	16	7
		193.1			24	
Δ^9-Carboxy-THC-D_3	348.2	**330.1**	4.3	125	12	7
		302.1			16	
Cannabidiol	315.2	**193.1**	4.7	110	20	7
		123			32	
Cannabidiol-D_3	318.2	**196.1**	4.7	110	20	7
		123			32	
Δ^8/Δ^9-THC	315.2	**193**	6.8	120	20	7
		122.9			32	
Δ^9-THC-D_3	318.2	**196**	6.8	120	20	7
		123			32	
9R-$\Delta^{6a,10a}$-THC	315.2	**193**	7.3	120	20	7
		122.9			32	

Note: The Δ^8/Δ^9 transitions and instrumental settings are analyzed separately in data analysis. On the C18 column, Δ^9–THC elutes before Δ^8-THC, Δ^9– OH–THC elutes before Δ^8-OH-THC, Δ^8–Carboxy–THC elutes before Δ^9-Carboxy-THC. 9R-$\Delta^{6a,10a}$-THC elutes after Δ^8-THC.

33.6 Procedure

33.6.1 Silanizing Glassware: Round bottom borosilicate test tubes used in initial sample preparation shall be silanized prior to use. Commercially available silanized test tubes may be used.

33.6.1.1 Fill appropriate glassware with a 5% dichlorodimethylsilane in toluene solution.

33.6.1.2 Allow glassware to silanize under standard laboratory conditions for at minimum 20 minutes.

33.6.1.3 Remove silanizing solution and perform a series of rinses:

33.6.1.3.1 Toluene
33.6.1.3.2 Methanol
33.6.1.3.3 Toluene
33.6.1.3.4 Methanol

33.6.1.4 Dry silanized glassware in the oven at approximately 80°C for at least 20 minutes, exercising caution to vent vapors appropriately. Alternatively, dry glassware under standard laboratory conditions.

NOTE: Silanizing agents can be corrosive to other materials, other equipment in hoods should be removed to prevent corrosion. Silanizing solution may be used more than once.

33.6.2 Prepare calibrators and controls. Calibrators and controls shall not be dried down under any circumstances (i.e., nitrogen, heat).

33.6.3 Add 0.5 mL case specimens/blank blood to the appropriately labeled silanized test tubes.

33.6.4 Add 200 µL of 0.1% formic acid in water and vortex briefly.

33.6.5 Add 25 µL of the 0.2/0.4/1.0 µg/mL internal standard working solution to each tube.

33.6.6 Optionally, the samples may be centrifuged at approximately 2500 rpm for 15 minutes.

33.6.7 Decant sample onto SLE cartridge and allow to incubate for 5 minutes after slightly drawing the matrix onto the cartridge frit.

NOTE: Prior to the start of the SLE, place conical bottom collection tubes under the cartridges in the manifold for collection. All eluates are collected.

33.6.8 Add 3.0 mL ethyl acetate and allow to incubate for 10 minutes prior to elution. After 10 minutes, pressure or vacuum may be applied, if necessary, to complete elution.

33.6.9 Add 3.0 mL n-hexane and allow to incubate for 15 minutes prior to elution. After 15 minutes, pressure or vacuum may be applied, if necessary, to complete elution.

33.6.10 Evaporate samples to dryness at approximately 50°C under nitrogen.

33.6.11 Reconstitute samples in 50 µL of methanol. (Note: Centrifugation may be necessary at this step.)

33.6.12 Transfer to GC autosampler vials with inserts for LCMSMS analysis.

33.7 Quality Control and Reporting

33.7.1 The LOQ for this procedure is defined as the lowest acceptable calibrator concentration used in the calibration curve for each analyte, limited to the following parameters:

33.7.1.1 Δ^9-THC: 0.0010 mg/L, except for postmortem blood (reporting limit = 0.0025 mg/L)

33.7.1.2 Δ^9-OH-THC: 0.0020 mg/L, except for postmortem blood (reporting limit = 0.0050 mg/L)

33.7.1.3 Δ^9-Carboxy-THC: 0.0050 mg/L

33.7.1.4 When evaluating postmortem specimens, the entire acceptable calibration range shall be used and no samples shall be reported quantitatively below the above stated reporting limits.

33.7.2 The ULOQ for this procedure is defined as the highest acceptable calibrator concentration used in the calibration curve for each analyte.

33.7.3 The LOD for this method is equal to the LOQ for all targets (listed above) except for Δ^9-Carboxy-THC. The validated LOD for Δ^9-Carboxy-THC is 0.0025 mg/L. The validated LOD for Δ^8-THC, and CBD is 0.0020 mg/L.

33.7.3.1 The LOD for Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC is 0.0050 mg/L.

33.7.4 Data Analysis

33.7.4.1 Quantitative and Qualitative (C18):

33.7.4.1.1 Quantitative: Δ^9-THC, Δ^9-OH-THC, and Δ^9-Carboxy-THC should be processed quantitatively using the C18 analytical column data.

33.7.4.1.2 Qualitative: Δ^8-THC, CBD, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC shall be processed qualitatively using the C18 analytical column data.

33.7.4.2 Qualitative (PFP):

33.7.4.2.1 Δ^9-THC, Δ^9-OH-THC, Δ^9-Carboxy-THC, Δ^8-THC, CBD, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC shall be processed qualitatively using the PFP analytical column data.

33.7.4.2.2 At minimum, the 0.070/0.140/0.350/0.050mg/L high control, 0.0030/0.0060/0.015/0.0050 mg/L low control, and negative control shall be used in the evaluation of data for Δ^9-THC, Δ^9-OH-THC, Δ^9-Carboxy-THC, Δ^8-THC, CBD, Δ^8-OH-THC, Δ^8-THC-COOH, and 9R-$\Delta^{6a,10a}$-THC.

33.7.5 Targets shall meet, at minimum, qualitative identification criteria on both columns to be reported.

33.7.6 The best fit calibration model is quadratic weighted 1/x for all analytes using area.

33.7.7 Internal standard response for the Cannabinoid Quantitation and Confirmation by Supported Liquid Extraction using LCMSMS may vary greater than 200% (+100%). Samples with an internal standard response greater than 200% (+100%) in this assay may be excluded from the requirement at 2.4.4.4.

33.7.8 Dilutions: A minimum of 0.5 mL of case sample shall be diluted with no more than 4.5 mL of blank matrix (1/10 dilution ratio maximum).

33.7.9 Extracted sample stability:

33.7.9.1 Extracted samples are stable for six days after reconstitution when evaluating with the C18 analytical column.

33.7.9.2 Extracted samples are stable for five days after reconstitution when evaluating with the PFP analytical column (except Δ^8-THC in antemortem blood, which was stable for four days).

33.7.9.3 Δ^8-OH-THC and 9R-$\Delta^{6a,10a}$-THC are stable for five days in both columns. Δ^8-THC-COOH is stable for five days on the PFP column and three days on the C18 column.

33.7.10 Interferences were noted on both columns during validation, as follows:

33.7.10.1 Interferences based on qualifier ratio and retention time:

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^9-OH-THC		Δ^8-OH-THC
Δ^8-OH-THC		Δ^9-OH-THC
Δ^9-Carboxy-THC		Δ^8-Carboxy-THC
Δ^8-Carboxy-THC		Δ^9-Carboxy-THC
Cannabidiol		
Δ^9-THC	9S-Δ^7-THC	exo-THC
Δ^8-THC	9R-Δ^7-THC	Δ^8-Iso-THC, 9R-Δ^7-THC, 9S-Δ^7-THC

33.7.10.2 Interferences based on instrumental response:

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^9-OH-THC	CBDVA	Δ^8-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^8-OH-THC		Δ^9-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^9-Carboxy-THC		Δ^8-Carboxy-THC

Δ^8-Carboxy-THC		Δ^9-Carboxy-THC
Cannabidiol	CBG	(±)-9-nor-9α-hydroxyhexahydrocannabinol
Δ^9-THC	9S-Δ^7-THC, CBL	exo-THC
Δ^8-THC	9R-Δ^7-THC	Δ^8-Iso-THC, 9R-Δ^7-THC, 9S-Δ^7-THC, CBL

33.7.10.3 Interferences with Δ^9-OH-THC-D_3: 8(S)-hydroxy-9(S)hexahydrocannabinol produces signal in quantifier window outside of ±3% RT acceptance range. (±)-9-nor-9β-hydroxyhexahydrocannabinol produces a signal in quantifier window only at the correct RT on the C18 column and not on the PFP column.

33.7.11 Qualifier acceptance range for Δ^8/Δ^9-OH-THC, Δ^9-OH-THC-D_3 is ±30% due to the low qualifier ratio (Refer to 2.5.2.4 for ANSI/ASB 098 ion/transition ratio acceptance ranges).

33.7.12 Δ^8-THC and cannabidiol shall be assessed qualitatively. Cannabidiol may be reported from a single aliquot if Δ^9-THC, Δ^8-THC, or their metabolites are present. Two aliquots are necessary to report 9R-$\Delta^{6a,10a}$-THC, and also cannabidiol in the absence of Δ^9-THC, Δ^8-THC, or their metabolites.

33.7.13 Results for Δ^8-THC shall be reported as “Delta-8-tetrahydrocannabinol/9R-delta-7-tetrahydrocannabinol”.

33.7.14 Results for 9R-$\Delta^{6a,10a}$-THC shall be reported as “9R-delta-6a,10a-tetrahydrocannbinol/Delta-10-tetrahydrocannabinol”.

33.7.15 See Toxicology Quality Guidelines.

33.8 References

33.8.1 D. Herr, A. Siddiqi, R. Wagner, Cannabinoids Method Development Summary, **2022**.

33.8.2 A. Siddiqi, R. Wagner, Validation Summary, Cannabinoids Quantitation and Confirmation by Supported Liquid Extraction using LCMSMS. **2023**.

33.8.3 A. Siddiqi, R. Wagner, Validation Summary Addendum, Validation of Δ^8-Tetrahydrocannabinol Quantitation and Confirmation by Supported Liquid Extraction using LCMSMS. **2024**.

33.8.4 A. Siddiqi, R. Wagner, Validation Summary, Validation of Qualitative Analysis of Cannabinoids by Supported Liquid Extraction Using LCMSMS. **2023**.

33.8.5 ANSI/ASB 036, Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. **2019**.

33.8.6 ANSI/ASB 098, Standard for Mass Spectral Analysis in Forensic Toxicology. 1st Edition. **2023**.

Appendix A - Abbreviations

The following is a list of abbreviations commonly used in the Section. This list has been generated to assist in the interpretation of case files. The abbreviations are appropriate written in either lower or upper case, with and without punctuation such as periods. Empirical formulas, chemical, mathematical and shorthand abbreviations are equally acceptable and are not listed here. In all instances, abbreviations used in note taking should be readily interpretable within the context of the subject material and in conjunction with the associated Certificate of Analysis.

A.1 General Abbreviations

Abbreviations	Definitions
/nor	desmethyl metabolite
?	unidentified/unknown
A/N, AN, ABN, BAN, A/N/B, ANB	acidic/neutral/base drugs
ABC	Alcoholic Beverage Commission
Abs	absorbance
Accd	accepted
Acids	acidic drugs
ACN, acn	acetonitrile
ADM	administrative
Alc	alcohols/alcohol screen
ALH	Automated liquid handling
Anal	analysis
Ant	anterior
AP	Abused panel (ELISA)
Ave, Avg	average
Bases	basic drugs
BB	Blank Blood
Blk	Blank
BAC	blood alcohol content
Bld	blood
Bldy	bloody
Brn	brain
BD	Breakdown
BD prod	breakdown product
BPB	Brown paper bag
BSC	Base screen
BSQ	Base quant
BT	Batch threshold
b/w, B/W	between
c, o, f, m, t, x	cocaine, opioid, fentanyl, methadone, tramadol, xylazine (note: specific to opicoc analyses and may be used in any combination/order)

C, cont	container
Cal, Calib	calibrator
c/o	Carryover
cav fl	cavity fluid
CE	Collision energy
Cell Accel	Cell accelerator
Cer	Cerilliant
Chol	Cholesterol
CHROMAT, chrom	chromatograph
cntl, ctl, cont, con, ctrl	control
Conc	concentrated/concentration
conf, conf'd	confirmed
Cpds	compounds
CRM	Certified reference material
CSF	cerebral spinal fluid
CT	Clear top
Derive, deriv	Derivative
DAD	Diode array detector
DCM, $MeCl_2$	Dichloromethane, methylene chloride
dec fl, decomp fl	decomposition fluid
dH2O, DiH2O	deionized water
dil, diln	dilution/diluted
DNE	did not extract
DP	ELISA duid panel
DOA	Drugs of Abuse
EIC	Extracted ion chromatogram
ELISA	Enzyme Linked Immunosorbant Assay
EtOAc	ethyl acetate
Ext	extraction/extracted
Exp	Expires, expiration
EIP	extracted ion profile
F	femoral
FA	Formic acid
FD	Found
FID	flame ionization detector
Form	formaldehyde
FRAGS, frag	Fragments, fragmentor voltage
Fs	full scan
Full	full toxicology panel
gst, gast	gastric
Gastric, gst, gc	gastric contents
GC	gas chromatograph/gas chromatography

GC-MS	gas chromatograph/mass spectrometer
GT	greater than
H, Ht	heart
Hb	hemoglobin
HB, Hbl	heart blood
Hex	Hexane
HbCO	carboxyhemoglobin
HBLD, Hbl, hosp	hospital blood
HDR	High dynamic range
Homo	homogenate
HS	Hospital sample
HSGC	Headspace gas chromatography
HSPB	heat sealed plastic bag
ID	identification
I	Iliac
IH, I.H.	In-house
Immunal	Immunalysis
Imp	Impurities
int, integ	Integration
Int Std, ISTD, IS	internal standard
IT, ITM	item
IVC	inferior vena cava
L	left
Lav	Lavender
LCMS	liquid chromatography/mass spectrometry
LCMSMS	liquid chromatography/ tandem mass spectrometry
LDR	Low dynamic range
Lipo	Lipomed
Liq	liquid
Liv, lvr	liver
LL	liquid/liquid
LLE	liquid/liquid extraction
LLOQ	lower limit of quantitation
LME	Local medical examiner
LOD	limit of detection
LOQ	limit of quantitation
LT	less than, light (typically written as Lt)
Man	Manual
MC	Multicomponent
MI	Manual integration
MPA	Mobile Phase A
MPB	Mobile Phase B

MS	mass spectrometer
MSD	mass spectrometer detector
Metab	Metabolite
Mod	moderate or moderately
N, neg	negative
NA	not analyzed
N/A	not available, not applicable
NCI	negative chemical ionization
ND	not detected or none detected
NDD	no drugs detected
Neutrals	neutral drugs
NOA	no other acidic or neutral drugs
NOB	no other alkali extractable drugs
NPD	nitrogen phosphorous detector
OpiCoc, opicoc, Opi-Coc, opi-coc	Opioid, Cocaine, Benzoylecgonine, Cocaethylene Quantitation and Confirmation by LCMSMS
O:, op, OP'D, OPN'D	opened
ORG, orig	original
PdCl2	palladium chloride
Pf	purge fluid
PGT, PRGT	present greater than
Pk	peak
Pl fl	pleural fluid
PLB	plastic bag
PLC	plastic container
prep'd	prepared
PLT, PRLT	present less than
PM	Preventive maintenance, Program Manager, Post Mortem, Procedures Manual
PMP	ELISA Postmortem Panel
Pos	Positive, position (on Alcohol QC Worksheet)
Poss	Possibly
Pr	present
Prob	probably
Pur	purge
Q	quantity
QA	quality assurance
QC	quality control
QNS, QNSFA	quantity not sufficient for analysis
QNS, QNSFFA	quantity not sufficient (insufficient) for further analysis
QQQ	liquid chromatography/mass spectrometry triple quadrupole
Qs	add quantity sufficient to bring up to volume
Qual	Qualitative

Quant	Quantitation, Quantitative
R	Right, review
R:, rec'd, rcvd	received
RRT	relative retention time
Rel	Related compounds
Relinq	relinquished
RI, reinj, reinject	reinjection
RO	Reverse osmosis
Rpt, rpt, RPT	repeat
RR	Response ratio, relative response
RT	retention time
Samp	Sample
SBPB	Sealed brown paper bag
SBX	sealed box
SC	Subclavian
scr, scrn	screen
SD	standard deviation
SENV	sealed envelope
Ser	Serum
SIM	selected ion monitoring
Sl	slightly
SLE	Supported liquid extraction
SPB	sealed plastic bag
Solv	Solvent
s/p	Serum/plasma
SPE	solid phase extraction
SPLB	sealed plastic bag
SPLC	sealed plastic container
Std	standard
Subclav	Subclavian
Supraclav	supraclavian or supraclavicular
SW	statewide
Syr	syringe
Sys	systemic/system
sys imps	systemic impurities
SZLPB	Sealed ziplock plastic bag
T, tgt	Target
T1	Tray 1
T2	Tray 2
TC	Threshold control
TIC	total ion chromatogram
THIA	toluene/hexane/isoamyl alcohol

TO	Toxicology-Other
Tox, TX	Toxicology
Tr	trace
UFA, unsuit, Uns	Unsuitable for analysis
ULOL	upper limit of linearity
ULOQ	upper limit of quantitation
Unid	unidentified
UoM	Uncertainty of Measurement
UR	urine
UV	ultraviolet spectrophotometer
UV-VIS	Ultraviolet/visible spectrophotometer
V	vial
VC	vena cava
VH, vit	vitreous humor
vol, vols	volatile(s)
WB	Whole blood
WQA	Wine Quality Assurance
Wkstd	working standard
WS	Working stock
XP	ELISA extra panel
ZLPB	Ziplock plastic bag
ZLPB/T	Ziplock plastic bag sealed with tape

A.2 Drug Names

Abbreviations	**Definitions**
9S-Δ^7-THC	9S-(-)Δ^7-Tetrahydrocannabinol
9R-Δ^7-THC	9R-(-)Δ^7-Tetrahydrocannabinol
Δ^8-THC	(-)Δ^8-Tetrahydrocannabinol
Δ^8-iso-THC	Δ^8-iso-Tetrahydrocannabinol
Δ^9-THC	(-)Δ^9-Tetrahydrocannabinol
Δ^9-THC-D_3	(-)Δ^9-Tetrahydrocannabinol-D_3
Δ^8-Carboxy-THC, Δ^8-THC-COOH	(-)11-nor-9-Carboxy-Δ^8-tetrahydrocannabinol
Δ^9-Carboxy-THC, Δ^9-THC-COOH	(-)11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol
Δ^9-Carboxy-THC-D_3	(±)11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol-D_3
Δ^9-OH-THC-D_3	(±)11-Hydroxy-Δ^9-tetrahydrocannabinol-D_3
Δ^8-OH-THC	(±)11-Hydroxy-Δ^8-tetrahydrocannabinol
Δ^9-OH-THC	(±)11-Hydroxy-Δ^9-tetrahydrocannabinol
Δ^{10}-THC	(-)Δ^{10}-Tetrahydrocannabinol
(6aR,9R)-Δ^{10}-THC	(6aR,9R)-Δ^{10}-Tetrahydrocannabinol
(6aR,9S)-Δ^{10}-THC	(6aR,9S)-Δ^{10}-Tetrahydrocannabinol
3FF	3-fluorofentanyl

4-ANPP, desprop	Despropionyl fentanyl
4MBF	4-methoxybutyrylfentanyl
6AM, 6MAM, MAM	6-acetylmorphine
7-AC	7-aminoclonazepam
Ace	acetone
afent	acetylfentanyl
alp, alpraz	alprazolam
ami, amit, amitrip	amitriptyline
amobarb, amob	amobarbital
Amox	amoxapine
Amp	amphetamine
Amps	amphetamine type drugs
APAP	acetaminophen
ASA	salicylate
Atomox	atomoxetine
barb, barbs, BR	barbiturates
BE, BZG	benzoylecgonine
BE-d3	benzoylecgonine d3
Benz, benzos, BZ, benzo	benzodiazepines
benztr, benztrp	benztropine
Bromphen	brompheniramine
BUP, bupren	buprenorphine
Buprop	Bupropion
Butal	butalbital
Busp	buspirone
C/M	carisoprodol/meprobamate
Caff	caffeine
Cannabs	cannabinoids
Carbam	carbamazepine
Carboxy-THC, THC-COOH	THC carboxylic acid
cariso, caris, CAR	carisoprodol
Cbd, CBD	cannabidiol
CBDVA	cannabidivarinic Acid
CBG	cannabigerol
CBL	cannabicyclol
Cbn	cannabinol
cis-3-MF	cis-3-methylfentanyl
CO	carbon monoxide
CMA, Ceterz MeOH Ad	Ceterizine Methanol Adduct
CE	cocaethylene
CHLORDIAZ	chlordiazepoxide
Chlorphen	chlorpheniramine

Chlorprom	chlorpromazine
CIT, citalo, cital	citalopram
Clomip	clomipramine
Clonaz, clo	clonazepam
Cloz	Clozapine
COC	cocaine
coc metab	cocaine metabolite (benzoylecgonine)
Cod	codeine
Cot	cotinine
CYCLOBENZ, cyclobenz, cycbnz	cyclobenzaprine
Desip	Desipramine
DEX, DXM, dextro	dextromethorphan
DEXMED	dexmedetomidine
dia, DIAZ	diazepam
DFE	difluoroethane
diltz, diltaz, diltiaz	diltiazem
DIP, DPH, DIPH, DIPHEN	diphenhydramine
DOX, doxep	doxepin
Doxyl	doxylamine
EG, ETH GLY	ethylene glycol
ETOH	ethanol
exo-THC	exo-tetrahydrocannabinol
FBF	fluorobutyrylfentanyl
Fent, FEN	fentanyl
Fent deriv, fentalogs, FA, fentanyl der, fent der	Fentanyl derivatives or fentanyl analogs (both versions are synonymous)
FIBF	fluoroisobutyrylfentanyl
FLUNITRAZ, flunit	flunitrazepam
FLUOX	fluoxetine
Fluraz	flurazepam
Fluvox	fluvoxamine
Gabap, Gaba	Gabapentin or gamma-aminobutyric acid
GBL	gamma butyrolactone
GHB	gamma hydroxybutyrate
Gluteth	glutethimide
Halop	haloperidol
HCD, HYC, hydrocod	hydrocodone
HYM, hydromorph	hydromorphone
HXZ	hydroxyzine
Imip	imipramine
IPA, iso, isoprop, ISOP	isopropyl alcohol or isopropanol
ketam, ket	ketamine
lamot, lamo	lamotrigine

Levam	levamisole/tetramisole
levet	levetiracetam
lid, lido	lidocaine
Loper	loperamide
lor, loraz	lorazepam
LOX	loxapine
MC	multi-component
mCPP	m-chlorophenylpiperazine
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MDO, Mdon, MTD, MDN	methadone
MDPV	3,4-methylenedioxypyrovalerone
Mecliz	meclizine
MED, MEDET	Medetomidine
MeOH	methanol
MeO	Methoxy
Meper	meperidine
Mepro	meprobamate
Metax	metaxalone
meth, methamp	methamphetamine
methapyr, mp	methapyrilene
Methocarb	methocarbamol
Metoclo	metoclopramide
mFBF	meta-fluorobutyrylfentanyl
mFF	meta-fluorofentanyl
mFIBF	meta-fluoroisobutyrylfentanyl
Midaz	midazolam
MIRTAZ	mirtazapine
Mitrag	mitragynine
MJ	marijuana
mor, morph	morphine
MPZ	methadone/pcp/zolpidem
Mthphen, MPD	methylphenidate
Nalox, nalox	naloxone
Nic	nicotine
Norbup	Norbuprenorphine
Norchlor	norchlorcyclizine
nor, nordiaz	nordiazepam
Norflox	norfluoxetine
norprop, norpropx	norpropoxyphene
Nortrip	nortriptyline
n-prop	n-propanol

oFAF	ortho-fluoroacrylfentanyl
oFBF	ortho-fluorobutyrylfentanyl
oFF	ortho-fluorofentanyl
oFIBF	ortho-fluoroisobutyrylfentanyl
OH-THC	11-hydroxy- Δ9-tetrahydrocannabinol
OLANZ	olanzapine
OP, opi	opiate(s)
Orphan	orphenadrine
OXAZ	oxazepam
oxcarbaz, oxcarb	oxcarbazepine
OXC, oxy, oxycod	oxycodone
OXM, oxymor	Oxymorphine, oxymorphone
PAROX	paroxetine
p-clamp, pclamp	p-chloroamphetamine
PCP	phencyclidine
pentobarb, pentob	pentobarbital
pFAF	para-fluoroacrylfentanyl
pFBF	para-fluorobutyrylfentanyl
pFF	para-fluorofentanyl
p/mFF	Para/meta-fluorofentanyl
p/m	Para/meta-
pFIBF	para- fluoroisobutyrylfentanyl
Phenmet	phenmetrazine
PHENOBARB, pheno, phenob	phenobarbital
Phenaz	phenazepam
Phent	phentermine
PROMETH	promethazine
PROP GLY, PG	propylene glycol
Propox	propoxyphene
Pseudo	pseudoephedrine
Ritacid	ritalinic acid
QUET	quetiapine
secobarb, secob	secobarbital
SERT	Sertraline
Sertis	sertraline internal standard
Tapen	tapentadol
TCA, TRI	Tricyclic antidepressants
TEMAZ	temazepam
THC	tetrahydrocannabinol
THCCOOH, carboxy-THC	11-nor-9-carboxy- Δ9-tetrahydrocannabinol
THFF	tetrahydrofuranfentanyl
topir	topiramate

Triaz	triazolam
Thiorid	thioridazine
TRAM, tram	tramadol
trans-3-MF	trans-3-methylfentanyl
TRAZ	trazodone
MCPP, mCPP, CPP	trazodone metabolite - meta chlorophenylpiperazine
VA, valp, VPA	valproic acid
VENLA	venlafaxine
Verap	verapamil
XYL, xyla	xylazine
Zolp	zolpidem
zonis	zonisamide
ZZZ	Zolpidem, zaleplon, zopiclone

A.3 Case History

Abbreviations	**Definitions**
Aa	automobile accident
ACI	acute coronary insufficiency
ASCVD	Arteriosclerotic cardiovascular disease
Ass	assault
BBR	burned beyond recognition
BFT	blunt force trauma
BP	blood pressure
c/o	complained of
CAD	coronary artery disease
CKD	chronic kidney disease
COD	cause of death
COPD	chronic obstructive pulmonary disease
CS	Controlled substance
D	driver
Decomp	Decomposed
DFSA	drug-facilitated sexual assault
DFC	drug-facilitated crime
DIB	dead in bed
Dec	Decedent
Dep	depression
DM	diabetes mellitus (diabetic)
Dx	diagnosis
DZ	disease
EtOHism	alcoholism

Fx	fracture
GI	gastrointestinal
GSW	gunshot wound
H	Homicide, hospital
HASCVD, HACVD	hypertensive arteriosclerotic cardiovascular disease
h/o	history of
Hdz	heart disease
Hrnd	heart related natural death
Ht	heart
Hem	hemorrahage
HTN	hypertension
Hx	history
IH	in-house
L#	Lot number
Lac	laceration
LEA	law enforcement agency
LEO	law enforcement officer
LKA	last known alive
LSA	last seen alive
MEO	medical examiner office
Mc	motorcycle
MCC	motorcycle collision
ME	medical examiner
MOD	manner of death
MRN	medical record number
MV	motor vehicle
MVA	motor vehicle accident
MVC	motor vehicle collision
Nat	natural
NPS	Novel psychoactive substance
OD	overdose
OTJ	on the job
P, pass	passenger
PD	police department
PE	pulmonary embolism
Ped	pedestrian
Perf	perforated
PMP	prescription monitoring program
Rx	prescription
PTB	presumed to be
Psych	Psychiatric
r/o	rule out

s/i	suicidal ideations
s/a	suicidal attempts
S, suic	suicide
SCHIZ	Schizophrenia
SGW	shotgun wound
SD	sudden death
SDH	subdural hematoma
Sgsw	single gunshot wound
SUD	sudden unexpected death
SUID	sudden unexpected infant death
Surg	surgery/surgical
SW	search warrant
Sx	sexual
SA	sexual assault
SZ	seizure
UDS	urine drug screen
Unresp	unresponsive
V	vehicle/vehicular
Vic	victim

A.4 DUI/DUID Evidence Codes – DUID Worksheet

Abbreviations	Definitions
CBW	Certificate of Blood Withdrawal
OA	Name order different on RFLE.
QN	Quantity insufficient for further analysis
01	Received broken vial. No analysis possible. Blood and tube discarded.
02	Container not sealed. Vial sealed.
03	Last name on Certificate of Blood Withdrawal not present.
04	Blood vial was not provided by the Department.
05	Blood was coagulated when received. No analysis was possible.
06	Spelling of last name on CBW is questionable.
07	Spelling of first name on CBW is questionable.
08	Name order on CBW is questionable.
09	First name on CBW is not legible.
10	Last name on CBW is not legible.
11	The quantity of blood received was insufficient for analysis.
12	First and last names on CBW are not legible.
13	There is no information on the CBW.
14	Spelling of first and last names on the CBW is questionable.
24	Container sealed. Vial not sealed.
27	Neither vial nor container was sealed.
30	There was no CBW submitted.
31	CBW was detached from the vial at the perforation.
33	The vial number on the vial does not match the vial number on the CBW.
41	Blood vial cracked and leaking when received.
44	Vial number is not complete.
53	CBW not fully recoverable from vial.

55	Vial cap loose. No blood available for analysis.
58	CBW not recoverable from vial.
65	The attached is a photocopy of the vial label.
66	Vial leaking.
67	No analysis performed.
68	Sample may be analyzed if resubmitted with identifying information.
69	No name on CBW.
70	CBW was not attached to the vial.

Appendix B – Testing Panel Summary

Methods:
Immunoassay (EIA)
Gas Chromatography/Mass Spectrometry (GCMS)
Liquid Chromatography (LC)
Spectrophotometry (S)
Headspace Gas Chromatography (HSGC)
Gas Chromatography (GC)
Liquid Chromatography/Tandem Mass Spectrometry (LCMSMS)

List of drugs is not all-inclusive. Additional qualitative analyses may be performed at the discretion of a toxicologist. The ability to meet the below quantitative analyses is dependent upon the availability of certified reference materials (CRM). The availability of CRM changes routinely therefore if there is a question regarding the ability to perform an analysis, please contact your local toxicology section or the Toxicology Program Manager.

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Δ^8-Tetrahydrocannabinol (Δ^8-THC)	0.5 mL		X	X	LCMSMS	33
Δ^9-Tetrahydrocannabinol (Δ^9-THC)	0.5 mL		X	X	LCMSMS	33
11–Hydroxy–Δ^9-tetrahydrocannabinol (Δ^9-OH-THC)	0.5 mL		X	X	LCMSMS	33
11–Hydroxy–Δ^8-tetrahydrocannabinol (Δ^8-OH-THC)	0.5 mL		X		LCMSMS	33
(-)11-nor-9-carboxy-Δ^9-Tetrahydrocannabinol (Δ^9-THC-COOH, Δ^9-Carboxy-THC)	0.5 mL		X	X	LCMSMS	33
(-)11-nor-9-carboxy-Δ^8-Tetrahydrocannabinol (Δ^8-THC-COOH, Δ^8-Carboxy-THC)	0.5 mL		X		LCMSMS	33
Benocyclidine (BCP) (formal name: 1-[1-(1-benzothiophen-2-yl)cyclohexyl]-piperidine	2 mL		X		GCMS (LLE)	9
1,4-Butanediol	0.2 mL		X	X	LCMSMS	15
25B-NBOMe (formal name: 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine; synonyms: 2C-B-NBOMe)	0.5 mL		X		LCMSMS	30
25C-NBOMe (formal name: 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine; Synonym: 2C-C-NBOMe)	0.5 mL		X		LCMSMS	30
25H-NBOMe (formal name: 2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine; synonym: 2C-H-NBOMe)	0.5 mL		X		LCMSMS	30
25I-NBF (formal name: N-(2-fluorobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine; synonym: 2C-I-NBF)	0.5 mL		X		LCMSMS	30
25I-NBMD (formal name: N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(4-iodo-2,5-	0.5 mL		X		LCMSMS	30

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
dimethoxyphenyl)ethanamine; synonym: Cimbi-29)						
25I-NBOH (formal name: 2-(((4-iodo-2,5-dimethoxyphenethyl)amino)methyl)phenol; synonym: 2C-I-NBOH)	0.5 mL		X		LCMSMS	30
25I-NBOMe (formal name: 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine; synonym: 2C-I-NBOMe)	0.5 mL		X		LCMSMS	30
(2-methylaminopropyl)benzofuran (MAPB)	2 mL		X		GCMS	9
3F-AMB (formal name: methyl (1-(3-fluoropentyl)-1H-indazole-3-carbonyl)-L-valinate; synonym: 3-fluoro-AMP)	0.5 mL		X		LCMSMS	30
3-fluorofentanyl	1mL		X	X	LCMSMS	11, 28
3-fluorophenmetrazine	0.5 mL		X		LCMSMS	30
3-methoxy-PCP	2 mL, 0.5mL		X		GCMS, LCMSMS	9, 30
3-methylfentanyl	2 mL, 1 mL		X		GCMS, LCMSMS	28
4-APDB (formal name: 2,3-dihydro-α-methyl-4-benzofuranethanamine; synonym: 4-(2-Aminopropyl)-2,3-dihydrobenzofuran)	0.5 mL		X		LCMSMS	30
4-chloro-α-PVP (formal name: 1-(4-chlorophenyl)-2-(1-pyrrolidinyl)-1-pentanone)	0.5 mL		X		LCMSMS	30
4F-ADB (formal name: methyl (2S)-2-(1-(4-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate; synonym: 4-fluoro-MDMB-PINACA)	0.5 mL		X		LCMSMS	30
4F/5F-AMB (formal name: methyl (1-(4/5-fluoropentyl)-1H-indazole-3-carbonyl)-L-valinate; synonym: 4/5-fluoro-AMB)	0.5 mL		X		LCMSMS	30
4/5/6-MAPB (formal name: 1-(benzofuran-4/5/6-yl)-N-methylpropan-2-amine; synonym: 4/5/6-(2-Methylaminopropyl)Benzofuran)	0.5 mL, 2mL		X		LCMSMS, GCMS (LLE)	30, 9
4-methoxybutyrylfentanyl	1mL		X	X	LCMSMS	11, 28
4-methyl-N-ethylcathinone (4-MEC)	2 mL		X		GCMS (LLE)	9
5-APDB (formal name: 2,3-dihydro-α-methyl-5-benzofuranethanamine)	0.5 mL		X		LCMSMS	30
5-DBFPV	0.5 mL		X		LCMSMS	30

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
(formal name: 1-(2,3-dihydrobenzofuran-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one; synonym: 3-desoxy-3,4-MDPV)						
5F-AB-PINACA (formal name: N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(5-fluoropentyl)-1H-indazole-3-carboxamide)	0.5 mL		X		LCMSMS	30
5F-PB-22 (formal name: 1-(5-fluoropentyl)-8-quinolinyl ester-1H-indole-3-carboxylic acid; synonym: 5-fluoro-QUPIC)	0.5 mL		X		LCMSMS	30
6-APDB (formal name: 2,3-dihydro-α-methyl-6-benzofuranethanamine)	0.5 mL		X		LCMSMS	30
6-acetylmorphine	2 mL, 1 mL		X	X	GCMS, LCMSMS	11, 28
7-Aminoclonazepam	1 mL		X	X	LCMSMS	25
7-Aminoflunitrazepam	1 mL		X	X	LCMSMS	25
8-aminoclonazolam	1 mL		X		LCMSMS	25
9R- $\Delta^{6a,10a}$-tetrahydrocannabinol (9R-$\Delta^{6a,10a}$-THC)	0.5 mL		X		LCMSMS	33
AB-FUBINACA (formal name: N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3-carboxamide)	0.5 mL		X		LCMSMS	30
AB-PINACA (formal name: (S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide)	0.5 mL		X		LCMSMS	30
Acetaminophen	0.1 mL, 2 mL		X	X	EIA, LCMSMS, GC, GCMS	8, 9, 22
Acetone	0.1 mL, 1-2 gm tissue		X	X	HSGC	7
Acetylcodeine	2 mL		X		GCMS	9
Acetyl fentanyl	2 mL, 1 mL		X	X	GCMS, LCMSMS	11, 28
Acrylfentanyl	1mL		X		LCMSMS	11, 28
ADB-FUBICA (formal name: N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indole-3-carboxamide)	0.5 mL		X		LCMSMS	30
Alcohol Panel	0.1 mL, 1-2 gm tissue	X	X	X	HSGC	7
alpha-hydroxyalprazolam	1 mL		X	X	LCMSMS	25
alpha-hydroxymidazolam	1 mL		X	X	LCMSMS	25

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
alpha-hydroxytriazolam	1 mL		X	X	LCMSMS	25
alpha-methylacetylfentanyl	1mL		X		LCMSMS	11, 28
alpha-methylfentanyl	1mL		X		LCMSMS	11, 28
alpha-PVP (alpha pyrrolidinovalerophenone)	1 mL		X	X	GCMS, LCMSMS	9, 26
Alprazolam	1 mL		X	X	LCMSMS	25
Amantadine	2 mL		X		GCMS	9
AMB-FUBINACA (formal name: N-[[1-[(4-fluorophenyl)methyl]-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester; synonym: MMB-FUBINACA, FUB-AMB)	0.5 mL		X		LCMSMS	30
Amitriptyline	2 mL		X	X	GCMS, GC	9
Amobarbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Amoxapine	2 mL		X	X	GCMS, GC	9
Amphetamine	1 mL		X	X	LCMSMS	26
Antimony	See method	X			Reinsch test	21
Arsenic	See method	X			Reinsch test	21
Atomoxetine	2 mL		X		GCMS	9
Benocyclidine	2 mL		X		GCMS	9
Benzocaine	2 mL		X		GCMS, GC	9
Benzodioxolefentanyl	1mL		X	X	LCMSMS	11, 28
Benzonatate	2 mL		X		GCMS, GC	9
Benzoylecgonine	1 mL		X	X	LCMSMS	28
Benztropine	2 mL		X		GCMS, GC	9
beta-Hydroxythiofentanyl	1mL		X		LCMSMS	11, 28
Bismuth	See method	X			Reinsch test	21
Blood Alcohol Concentration (BAC)	0.1 mL		X	X	HSGC	7
Bromazepam	1 mL		X		LCMSMS	25
Bromazolam	1 mL		X		LCMSMS	25
Brompheniramine	2 mL		X	X	GCMS, GC	9
Bupivacaine	2 mL		X		GCMS	9

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Buprenorphine	0.1 mL, 2 mL	X	X	X	EIA, LCMSMS	8, 29
Bupropion	2 mL, 0.2 mL		X	X	GCMS, LCMSMS	9, 26
Buspirone	2 mL		X		GCMS	9
Butabarbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Butalbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Butylone	2 mL		X		GCMS	9
Butyryl fentanyl	2 mL, 1mL		X	X	GCMS, LCMSMS	11, 28
Caffeine	2 mL		X	X	GCMS, GC	9
Cannabidiol	0.5 mL		X	X	LCMSMS	33
Carbamazepine	0.2 mL, 2mL		X	X	LCMSMS, GC, GCMS	27, 9
Carbinoxamine	2 mL		X		GCMS, GC	9
Carbon Monoxide	5 mL	X	X	X	Chemical, S	10
Carfentanil	1mL		X	X	LCMSMS	11, 28
Carisoprodol	1-2 mL, 0.2 mL		X	X	GCMS, GC	9, 14
Cetirizine	2 mL		X		GCMS	9
Chlordiazepoxide	1 mL		X	X	LCMSMS	13, 25
Chlorodifluoroethane	2 mL		X		HSGC, GCMS	20
Chlorodifluoromethane	2 mL		X		HSGC, GCMS	20
Chloroethane	2 mL		X		HSGC, GCMS	20
Chloroform	2 mL		X		HSGC, GCMS	20
Chloroquine	2 mL		X		GCMS	9
Chlorpheniramine	2 mL		X	X	GCMS, GC	9
Chlorphentermine	2 mL		X		GCMS, GC	9
Chlorpromazine	2 mL		X	X	GCMS, GC	9
cis-3-methylfentanyl	1mL		X	X	LCMSMS	11, 28
Citalopram	2 mL		X	X	GCMS, GC	9
Clomipramine	2 mL		X	X	GCMS, GC	9
Clonazepam	1 mL		X	X	LCMSMS	25
Clonazolam	1 mL, 0.5 mL		X		LCMSMS	25, 30

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Clonidine	2 mL		X		GCMS	9
Clozapine	2 mL		X	X	GCMS, GC	9
Cocaethylene	1 mL		X	X	LCMSMS	28
Cocaine	1 mL		X	X	LCMSMS	28
Codeine	1 mL		X	X	LCMSMS	28
Crotonylfentanyl	2 mL, 1mL		X	X	GCMS (LLE), LCMSMS	9, 11
Cyclopropylfentanyl	2 mL, 1mL		X	X	GCMS (LLE), LCMSMS	9, 11, 28
Cyclobenzaprine	2 mL, 1mL		X	X	GCMS, GC, LCMSMS	9, 31
Deschloroketamine	2 mL		X		GCMS (LLE)	9
Desipramine	2 mL		X	X	GCMS, GC	9
Despropionyl fentanyl	2 mL, 1mL		X		GCMS, LCMSMS	11, 28
Dexmedetomidine	1 mL		X	X	LCMSMS	28 (LLE)
Dextromethorphan	2 mL, 1 mL		X	X	GCMS, GC, EIA	8, 9
Diazepam	1 mL		X	X	LCMSMS	25
Dibutylone	0.5 mL		X		LCMSMS	30
Dicyclohexylamine	2 mL		X		GCMS (LLE)	9
Dicyclomine	2 mL		X		GCMS, GC	9
N,N-Diethylpentylone	0.5 mL		X		LCMSMS	30
Difluoroethane	2 mL		X		HSGC, GCMS	20
Diltiazem	2 mL		X	X	GCMS, GC	9
N,N-Dimethylpentylone	0.5 mL		X		LCMSMS	30
Diphenhydramine	2 mL, 1 mL		X	X	GCMS, GC, EIA	8, 9
Dothiepin	2 mL		X		GCMS, GC	9
Doxepin	2 mL		X	X	GCMS, GC	9
Doxylamine	2 mL		X	X	GCMS, GC	9
Ephedrine	1 mL		X		LCMSMS	26
Ethanol	0.1 mL, 1-2 gm tissue		X	X	HSGC	7
Ethyl acetate	2 mL		X		HSGC, GCMS	20
n-Ethylhexedrone	2 mL		X		GCMS	9

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Ethylone	1 mL		X		GCMS, LCMSMS	9, 26
Ethylpentylone (synonym: N-Ethylpentylone)	2 mL, 0.5 mL		X		GCMS (LLE), LCMSMS	9, 30
Ethylphenidate	2 mL		X		GCMS	9
Etizolam	1 mL		X		LCMSMS	25
Etomidate	2 mL		X		GCMS	9
Eutylone	2 mL		X		GCMS (LLE)	9
Fentanyl	2 mL, 1 mL		X	X	GCMS, LCMSMS	11, 28
Flecainide	2 mL		X	X	GCMS, GC	9
Flualprazolam	1 mL		X		LCMSMS	25
Flubromazolam	1 mL		X		LCMSMS	25
Flubromazepam	1 mL		X		LCMSMS	25
Flunitrazepam	1 mL		X	X	LCMSMS	25
Fluoxetine	2 mL, 1 mL		X	X	GCMS, GC, LCMSMS	9, 31
Flurazepam	1 mL		X	X	LCMSMS	25
Flurazepam (N-desalkyl)	1 mL		X	X	LCMSMS	25
Fluvoxamine	2 mL		X	X	GCMS	9
FUB-MDMB (formal name: N-[[1-[(4-fluorophenyl)methyl]-1H-indazol-3-yl]carbonyl]-3-methyl-L-valine; synonym: MDMB-FUBINACA)	0.5 mL		X		LCMSMS	30
Furanylfentanyl	2 mL, 1mL		X	X	GCMS, LCMSMS	9, 11, 28
Gabapentin	0.2 mL		X	X	LCMSMS	27
GBL (gamma-butyrolactone)	0.2 mL		X		LCMSMS	15
GHB (gamma hydroxybutyric acid)	0.2 mL		X	X	LCMSMS	15
Glutethimide	2 mL, 1 mL		X		GCMS	9
Haloperidol	2 mL		X	X	GCMS, GC	9
Hydrocodone	1 mL		X	X	LCMSMS	28
Hydromorphone	1 mL		X	X	LCMSMS	28
Hydroxyzine	2 mL, 1 mL		X	X	GCMS, LCMSMS	9, 19, 31
Ibuprofen	2 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 22

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Imipramine	2 mL		X	X	GCMS, GC	9
Isopropanol	0.1 mL, 1-2 gm tissue		X	X	HSGC	7
Isopropylphenidate	2 mL		X		GCMS	9
Ketamine	2 mL		X	X	GCMS, GC	9
Lacosamide	0.2 mL		X	X	LCMSMS	27
Lamotrigine	2 mL, 0.2 mL		X	X	GCMS, LCMSMS	9, 27
Levamisole/tetramisole	2 mL		X		GCMS, GC	9
Levetiracetam	0.2 mL, 2 mL		X	X	LCMSMS, GC, GCMS	27, 9
Licarbazepine	0.2 mL, 2 mL		X	X	LCMSMS, GC, GCMS	27, 9
Lidocaine	2 mL		X	X (elevated)	GCMS, GC	9
Loratidine	2 mL		X		GCMS, GC	9
Lorazepam	1 mL		X	X	LCMSMS	25
Loxapine	2 mL		X	X	GCMS, GC	9
MAB-CHMINACA (formal name: N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide)	0.5 mL		X		LCMSMS	30
Maprotiline	2 mL		X	X	GCMS, GC	9
MDA	1 mL		X	X	LCMSMS	26
MDMA	1 mL		X	X	LCMSMS	26
MDPV	1 mL		X	X	LCMSMS, GCMS	9, 26
Meclizine	2 mL		X		GCMS, GC	9
Meperidine	2 mL, 1 mL		X	X	GCMS, GC, LCMSMS	9, 28
Mephedrone	1 mL		X	X	LCMSMS	26
Mepivicaine	2 mL		X		GCMS, GC	9
Meprobamate	1-2 mL, 0.2 mL		X	X	GCMS, GC	9, 14
Mercury	See method	X			Reinsch test	21
meta-fluorobutyrylfentanyl	1mL		X		LCMSMS	28
meta-fluorofentanyl	1mL		X		LCMSMS	28
meta-fluoroisobutyrylfentanyl	1mL		X		LCMSMS	28

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Metaxalone	2 mL		X	X	GCMS, GC	9
Methadone	2 mL, 1 mL		X	X	GCMS, GC, LCMSMS	9, 28
Methamphetamine	1 mL		X	X	LCMSMS	26
Methanol	0.1 mL		X	X	HSGC	7
Methcathinone	1 mL		X	X	LCMSMS	26
Methedrone	1 mL		X	X	LCMSMS	26
Methocarbamol	2 mL		X	X	GCMS, GC	9
Methoxetamine	2 mL		X		GCMS	9
Methoxyacetylfentanyl	2 mL, 1mL		X	X	GCMS (LLE), LCMSMS	9, 11, 28
Methoxphenidine	0.5 mL		X		LCMSMS	30
Methylene chloride	2 mL		X		HSGC, GCMS	20
Methylone	2 mL		X	X	LCMSMS, GCMS	9, 26
Methylphenidate	2 mL		X		GCMS	9
Metoclopramide	2 mL		X		GCMS	9
Mexiletine	2 mL		X		GCMS	9
Midazolam	1 mL		X	X	LCMSMS	25
Mirtazapine	2 mL		X	X	GCMS, GC	9
Mitragynine	2 mL, 0.5 mL		X		GCMS (LLE), LCMSMS	9, 30
MMB-CHMICA (formal name: methyl (1-(cyclohexylmethyl)-1H-indole-3-carbonyl)-L-valinate; synonym: AMB-CHMICA)	0.5 mL		X		LCMSMS	30
Molindone	2 mL		X		GCMS	9
Morphine	1 mL		X	X	LCMSMS	28
Naloxone	2 mL		X	X	LCMSMS	29
Naproxen	0.5 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 22
Nefazodone	2 mL		X		GCMS	9
Nicotine	2 mL		X		GCMS	9
Norbuprenorphine	2 mL		X	X	LCMSMS	29
Nordiazepam	1 mL		X	X	LCMSMS	25

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Nordoxepin	2 mL		X	X	GCMS, GC	9
Norfluoxetine	2 mL		X	X	GCMS, GC	9
Norketamine	2 mL		X	X	GCMS, GC	9
Normeperidine	2 mL		X	X	GCMS, GC	9
Norpropoxyphene	2 mL		X	X	GCMS, GC	9
Nortriptyline	2 mL		X	X	GCMS, GC	9
Ocfentanil	1mL		X	X	LCMSMS	11, 28
Olanzapine	2 mL		X	X	GCMS, GC	9
Ondansetron	2 mL		X		GCMS	9
Orphenadrine	2 mL		X	X	GCMS, GC	9
ortho-fluoroacrylfentanyl	1mL		X		LCMSMS	11, 28
ortho-fluorobutyrylfentanyl	1mL		X	X	LCMSMS	11, 28
ortho-fluorofentanyl	1mL		X	X	LCMSMS	11, 28
ortho-fluoroisobutyrylfentanyl	1mL		X	X	LCMSMS	11, 28
Oxazepam	1 mL		X	X	LCMSMS	25
Oxcarbazepine	0.2 mL, 2 mL		X	X	LCMSMS, GC, GCMS	27, 9
Oxycodone	1 mL		X	X	LCMSMS	28
Oxymorphone	1 mL		X	X	LCMSMS	28
para-fluoroacrylfentanyl	1mL		X		LCMSMS	11, 28
para-Fluorobutyrylfentanyl	2 mL, 1mL		X	X	GCMS, LCMSMS	11, 28
para-fluorofentanyl	1mL		X		LCMSMS	11, 28
para-Fluoroisobutyrylfentanyl	2 mL, 1mL		X	X	GCMS, LCMSMS	11, 28
Paroxetine	2 mL, 1mL		X	X	GCMS, LCMSMS	9, 31
PB-22 (formal name: 1-pentyl-8-quinolinyl ester-1H-indole-3-carboxylic acid; synonym: QUPIC)	0.5 mL		X		LCMSMS	30
Pentazocine	2 mL		X	X	GCMS, GC	9
Pentobarbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Pentylone	0.5 mL		X		LCMSMS	30
Phenazepam	1 mL		X	X	LCMSMS	25

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Phencyclidine	2 mL		X	X	GCMS, GC	9
Phendimetrazine	2 mL		X		GCMS	9
Pheniramine	2 mL		X		GCMS, GC	9
Phenmetrazine	2 mL		X		GCMS	9
Phenobarbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Phensuximide	2 mL		X		GCMS, GC	9
Phentermine	1 mL		X	X	LCMSMS	26
Phenylfentanyl	1mL		X	X	LCMSMS	11, 28
Phenyltoloxamine	2 mL		X		GCMS, GC	9
Phenytoin	0.2 mL, 2 mL		X	X	LCMSMS, GC, GCMS	27, 9
Pramoxine	2 mL		X		GCMS	9
Pregabalin	0.2 mL		X	X	LCMSMS	27
Primidone	2 mL		X		GCMS	9
Procaine	2 mL		X		GCMS, GC	9
Promethazine	2 mL		X	X	GCMS, GC	9
Propanolol	2 mL		X		GCMS	9
Propofol	2 mL		X		GCMS	9
Propoxyphene	2 mL		X	X	GCMS, GC	9
Protriptyline	2 mL		X		GCMS, GC	9
Pseudoephedrine	1 mL		X	X	LCMSMS	26
PV8 (formal name: 1-phenyl-2-(1-pyrrolidinyl)-1-heptanone; synonym: α-PHPP)	0.5 mL		X		LCMSMS	30
Pyrazolam	1 mL		X		LCMSMS	25
Quetiapine	2 mL, 1 mL		X	X	GCMS, LCMSMS	18, 31
Quinine/quinidine	2 mL		X		GCMS	9
Salicylate, salicylic acid	0.1 mL		X	X	EIA, LCMSMS	8, 22
SDB-006 (formal name: 1-pentyl-N-(phenylmethyl)-1H-indole-3-carboxamide)	0.5 mL		X		LCMSMS	30
Secobarbital	2 mL, 1 mL, 0.1 mL		X	X	GCMS, LCMSMS	9, 12, 32
Sertraline	2 mL, 1 mL		X	X	GCMS, GC, LCMSMS	9, 31

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Strychnine	2 mL		X		GCMS	9
Tapentadol	2 mL		X	X	GCMS, GC	9
Temazepam	1 mL		X	X	LCMSMS	25
Tenocyclidine (synonym: TCP)	0.5 mL		X		LCMSMS	30
Terfenadine	2 mL		X		GCMS	9
Tetrafluoroethane	2 mL		X		HSGC, GCMS	20
Tetrahydrocannabinol (THC)	1 mL		X	X	LCMSMS	24
Tetrahydrocannabinol, 11-hydroxy (OH-THC)	1 mL		X		LCMSMS	24
THC Carboxylic Acid (THC-COOH)	1 mL		X	X	LCMSMS	24
Tetrahydrofuranfentanyl	1mL		X		LCMSMS	11, 28
Thiopental	2 mL		X		GCMS	9
Thioridazine	2 mL		X		GCMS, GC	9
Thiothixine	2 mL		X		GCMS, GC	9
Tizanidine	2mL		X		GCMS (SPE)	9
Toluene	2 mL		X		HSGC, GCMS	20
Topiramate	0.2 mL		X	X	LCMSMS	27
Tramadol	2 mL, 1 mL, 0. 1 mL		X	X	EIA, GCMS, GC, LCMSMS	8, 9, 28
trans-3-methylfentanyl	1mL		X	X	LCMSMS	11, 28
Trazodone	2 mL		X	X	GCMS, GC	9
Triazolam	1 mL		X	X	LCMSMS	25
Trifluroperazine	2 mL		X		GCMS, GC	9
Trihexyphenidyl	2 mL		X		GCMS, GC	9
Trimethylbenzamide	2 mL		X		GCMS, GC	9
Trimipramine	2 mL		X	X	GCMS, GC	9
U-47700	2 mL, 1mL		X		GCMS (LLE), LCMSMS	9, 11, 28
U-49900	2 mL, 1mL		X	X	GCMS (LLE), LCMSMS	9, 11, 28
Valerylfentanyl (pentanoyl fentanyl)	1mL		X		LCMSMS	11, 28
Valproic Acid	1 mL		X	X	GCMS	16
Venlafaxine	2 mL		X	X	GCMS, GC	9

Toxicology Laboratory Procedure	Specimen Requirement	Screen	Qual. Testing	Quant. Testing	Method(s)	Chapter(s)
Verapamil	2 mL		X	X	GCMS, GC	9
Xylazine	2 mL, 1 mL		X	X	GCMS, LCMSMS	9 , 28 (LLE)
Xylene	2 mL		X		HSGC, GCMS	20
Zaleplon	1 mL		X	X	LCMSMS	25
Zolpidem	2 mL		X	X	LCMSMS, GCMS, GC	9, 25
Zonisamide	0.2 mL		X	X	LCMSMS	27
Zopiclone	1 mL		X	X	LCMSMS	25

Appendix C – Return Codes

The following list contains codes that may be used on the Toxicology Summary Worksheet to indicate the disposition of evidence.

481	The evidence is being returned under separate cover.
482	The evidence is returned herewith.
483	The evidence is being retained for personal pickup.
484	The evidence will be returned via registered mail.
486	The results of other requested examinations will be reported separately.
487	The requested examination was terminated at the request of (name and title) on (date).
488	The disposition of the evidence and the results of other requested examinations are the subject of another report.
489	The evidence will be returned via United Parcel Service.
490	The evidence will be returned via Fedex.
48A	The evidence is being returned to the Central Laboratory where it will be available for personal pickup.
48B	The evidence is being returned to the Northern Laboratory where it will be available for personal pickup.
48C	The evidence is being returned to the Eastern Laboratory where it will be available for personal pickup.
48D	The evidence is being returned to the Western Laboratory where it will be available for personal pickup.
48E	The evidence will be available at the Central Laboratory after you have received the results of all requested examinations.
48F	The evidence will be available at the Northern Laboratory after you have received the results of all requested examinations.
48G	The evidence will be available at the Eastern Laboratory after you have received the results of all requested examinations.
48H	The evidence will be available at the Western Laboratory after you have received the results of all requested examinations.
48I	The evidence is being returned to the Western Laboratory.
48J	The submitted evidence is available for pickup at the Northern Laboratory.
48K	The evidence is being returned to the Eastern Laboratory.
48L	The evidence is being returned to the Central Laboratory.
48M	The evidence is being returned to the Northern Laboratory.
48T	The evidence is being returned to the Office of the Chief Medical Examiner.

Appendix D – Method Abbreviations

The following list contains abbreviations that may be used on the Toxicology Summary and Toxicology DUI-DUID Summary Worksheets to indicate the method used for an analysis.

Abbreviation	Method Title
ABN or Base or A/N	Acid/Base/Neutral Drug Screen and Quantitation by GC and GCMS
BAC	Alcohols by Headspace Gas Chromatography
CO	Carboxyhemoglobin Saturation Determination by Spectrophotometry with Confirmation by Microdiffusion
CannabSLE or Cannabs-SLE or Cannabs-33	Cannabinoid Quantitation and Confirmation by Supported Liquid Extraction using LCMSMS
FentDeriv	Fentanyl Derivatives Quantitation and Confirmation by LCMSMS
Barbs	Barbiturate Quantitation and Confirmation by LCMSMS
BarbsGC	Barbiturate and Acid Drug Quantitation and Confirmation by GC and GC-MS
Chlordiaz	Chlordiazepoxide Quantitation and Confirmation by LCMSMS
Cariso	Carisoprodol and Meprobamate Quantitation and Confirmation by GC and GC-MS
GHB	GHB, GBL, and 1,4-Butanediol Quantitation and Confirmation by LCMSMS
Valproic	Valproic Acid Quantitation and Confirmation by GC-MS
Fent	Fentanyl Quantitation and Confirmation by GC-MS
Quet	Quetiapine Quantitation and Confirmation by GC-MS
Hydroxy	Hydroxyzine Quantitation and Confirmation by GC-MS
Vol	Volatile Screen and Confirmation by Headspace GC and GC-MS
Metals	Heavy Metals by Reinsch Test
NSAID	NSAID Quantitation and Confirmation by LCMSMS
Cannabs or Cannabs-24	Cannabinoid Quantitation and Confirmation by LCMSMS
Benz	Benzodiazepines, Zolpidem, Zopiclone, and Zaleplon (Benz-ZZZ) Quantitation and Confirmation by LCMSMS
Amp or APD	Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS
AED	Anti-Epileptic Drugs Quantitation and Confirmation by LCMSMS
OpiCoc	Opioid, Cocaine, Benzoylecgonine, Cocaethylene Quantitation and Confirmation by LCMSMS
BUP	Buprenorphine, Norbuprenorphine, and Naloxone Quantitation and Confirmation by LCMSMS
NPS	Novel Psychoactive Substances Qualtitative Screen and Confirmation using LCMSMS
MiscBase	Miscellaneous Basic Drug Quantitation and Confirmation by LCMSMS

Appendix E - Barcode Generation Instructions

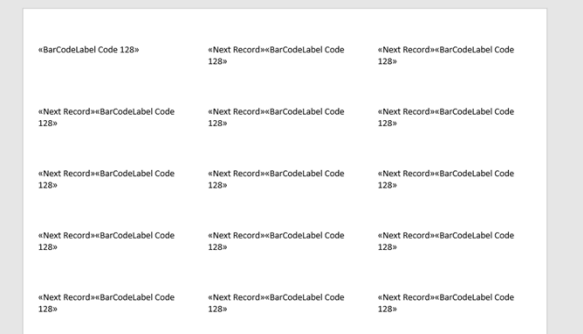
Note: Use only Avery 5160 Easy Peel Address Labels

Ensure that the correct 220-F156 Toxicology Barcode Batch Worksheet is saved on the computer/network drive and that the file location is known. Ensure that the BarCodes tab is correctly populated with the information from the "Sample/FS#" and "Item #" columns.

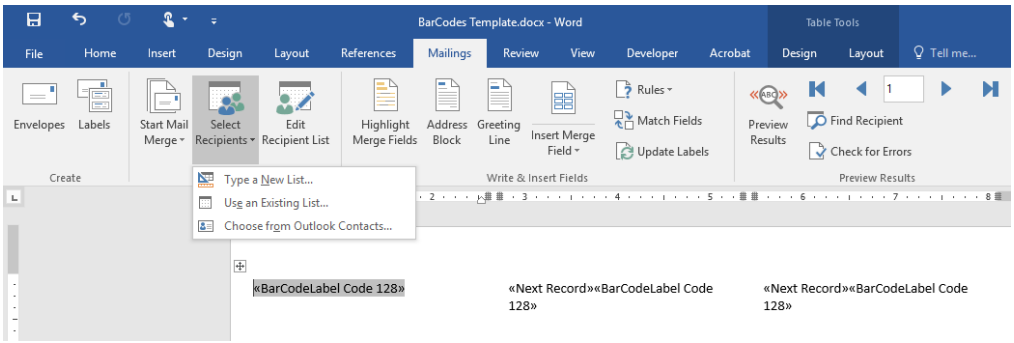
	Analyst(s):	
	Sample / FS #	**Item #**
1	0.01 mg/L	
2	0.02 mg/L	
3	0.05 mg/L	
4	0.10 mg/L	
5	0.25 mg/L	
6	0.50 mg/L	
7	1.0 mg/L	
8	X19-123	TX1
9	X19-124	TX2
10	X19-125	TX3
11	X19-126	TX4
12	X19-127	TX5
13	X19-128	TX6
14	X19-129	TX7
15	X19-130	TX8
16	X19-131	TX9
17	X19-132	TX10

C
BarCodeLabel
0.01 mg/L
0.02 mg/L
0.05 mg/L
0.10 mg/L
0.25 mg/L
0.50 mg/L
1.0 mg/L
X19-123 TX1
X19-124 TX2
X19-125 TX3
X19-126 TX4
X19-127 TX5
X19-128 TX6
X19-129 TX7
X19-130 TX8
X19-131 TX9
X19-132 TX10
X19-133 TX11
X19-134 TX12
X19-135 TX13
X19-136 TX14

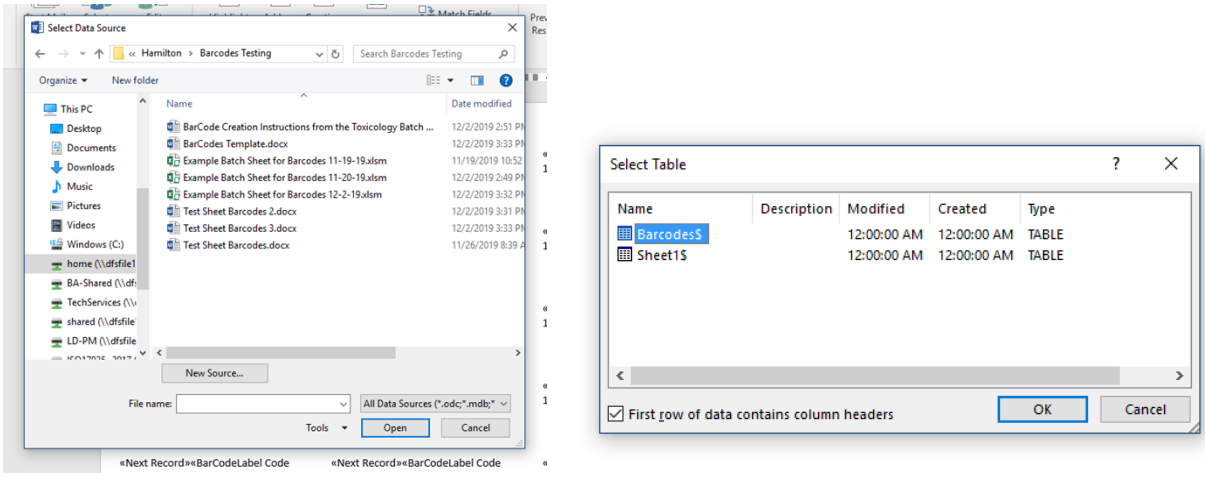
Open the "BarCodes Template" word document which is located in the Tools folder of the Toxicology section of Qualtrax. Upon opening the file, there may be a pop-up window, please select "No" as the file will not work correctly if you select "Yes." It should appear similar to the image below.



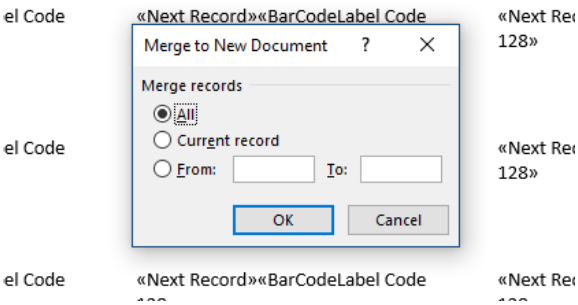
In the "Mailings" menu, click on "Select Recipients" and click on "Use an Existing List…".



The pop up selection window will be where you choose the Batch Worksheet file. Click on the file to select it and click on "Open". The next pop up window will be where the table of information for the barcode entries is chosen. Select the table called "Barcodes$" and click "OK".



In the "Mailings" menu, select "Finish & Merge" and select "Edit Individual Documents…". In the pop up menu, ensure that "All" is selected and click "OK".



The sheet should now appear as a table of barcodes with the data below. The sheet can now be printed at any laserjet printer with Avery 5160 labels loaded.


0.01 mg/L
0.02 mg/L
0.05 mg/L
0.10 mg/L
0.25 mg/L
0.50 mg/L
1.0 mg/L
X19-123 TX1
X19-124 TX2
X19-125 TX3
X19-126 TX4
X19-127 TX5
X19-128 TX6
X19-129 TX7
X19-130 TX8