TOXICOLOGY ANALYTICAL METHODS

Table of Contents

Revision History

Revision #	Description of Changes
1	Original Version: Combination of methods. Changes made during combination: removal of quantitative references in AM #11, Adjustments made to the control and calibration requirements in AM #13 and AM #14, specified required steps for use of calibrated pipette in LC methods.
2	Addition of LCQQQ ToxBox Methods, minor formatting corrections, removal of running blanks from LCQQQ Benzo/Z-drugs and Cannabinoid methods based on evaluation for carry over. Clarification made for LCMS confirmations in AM 19, Added requirements for ToxBox to AM 21. Added tolerances of balance checks to AM #17.
3	Corrected footer in AM #3, addition of AM 28 Multi-Drug Confirmation by LCQQQ, corrected volumes of low control to add in AM #14, addition of required instrument parameters to all extraction AM's, removed requirement for calibrated pipettes in AM 13 and AM 14, removed cannabinol from scope in AM 14 and made cannabidiol optional, removed methadone from scope of AM 1.
4	Removed Lipomed exclusion in AM #14, corrected oxazepam glucuronide spiking amounts in AM #13, clarified wording for cals and controls in AM #7, added additional requirement for after pipettes are calibrated, added requirement to checking of authenticated reference materials, changed LOD of carboxy-THC to 10ug/mL in AM #27.

Toxicology AM #1: Enzyme Immunoassay Screening for Drugs-of-Abuse in Urine

1.0 Background/References

1.1 Background

Enzyme Multiplied Immunoassay Technique (EMIT) is a competitive binding assay based on the enzyme activity of drug-labeled Glucose-6-Phosphate Dehydrogenase (G-6-P-DH). Glucose-6-Phosphate Dehydrogenase (G-6-P-DH) catalyzes the conversion of the substrate Glucose-6-Phosphate (G-6-P), this activity simultaneously results in the conversion of the co-factor Nicotinamide-Adenine Dinucleotide (NAD) to the reduced form NADH. This conversion results in a subsequent increase in absorbance at 340 nm. Both the degree of absorbance and reaction rate is monitored spectrophotometrically.[2]

Note that in this analytical method, the terms calibrator and calibration are not used in the ISO/IEC 17025:2005 sense. The manufacturer term calibrator refers to a urine sample with a known drug reference material concentration. This spiked urine is used for a one point calibration to establish a direct relationship between an amount of drug in the spiked urine and the degree/rate of absorbance as described below. The terms calibrator and calibration are used to coincide with the terminology in manufacturer manuals and package inserts.

For drugs-of-abuse applications, the G-6-P-DH is labeled with the particular drug, which the assay is designed to detect.[9,10] In the EMIT reaction, a drug in a urine specimen competes with the drug-enzyme for the binding site on a drug or drug-class specific antibody. The amount of NADH produced during the EMIT reaction is directly proportional to the amount of drug present. The EMIT reaction takes place over a finite time interval.[9,10] The rate of NADH production is what is used to provide a preliminary indication of the presence of a drug or drug class in the urine specimen. The initial set-up of the automated chemistry analyzer used for the EMIT reaction monitors the rates of production of NADH for various calibrators and controls containing known concentrations of drug. This information is then used to detect drugs in an unknown sample. The following examples outline how the EMIT reaction detects drugs-of-abuse.

1.1.1 Example 1: Specimen Contains Drug(s) of Interest

- An aliquot of the urine, Reagent A and Reagent B are added together.
- The drug in the urine and the drug-labeled enzyme compete with each other for available binding sites on the antibody.
- As the concentration of the drug in the urine is higher than that of the drug-labeled enzyme, a larger proportion of the free drug will bind to the antibody.
- The antibody binding sites become filled predominantly with drug from the sample.

- This results in a higher proportion of drug-labeled enzyme unbound in solution.
- This enzyme is available to breakdown G-6-P.
- When G-6-P is broken down, along with the product, a single Hydrogen ion is released.
- The NAD reacts with the H+ forming NADH.
- The amount of NADH formed is directly proportional to the amount of free drug in the urine. Thus more NADH indicates more drug(s) is present in the urine specimen.
- NADH has an absorbance wavelength of 340nm (UV).

1.1.2 Example 2: Specimen Contains No Drug(s) of Interest

- An aliquot of the urine, Reagent A and Reagent B are added together.
- The drug in the urine and the drug-labeled enzyme compete with each other for available binding sites on the antibody.
- Due to the absence of competing drug, much of the drug-labeled enzyme will bind to the antibody.
- Due to the effect of steric hindrance - the active site on the enzyme is blocked by the sheer size of the antibody.
- Bound enzyme therefore cannot breakdown G-6-P.
- *Note - In the absence of drug, some enzyme-labeled drug does remain free and some NADH is formed. Thus a negative does have some measurable absorbance. This absorbance is clearly differentiated from the absorbance of the cut-off calibrator by a defined level of separation. In addition to the absorbance change, the rate of conversion from NAD to NADH is monitored and must occur within established time limits.*
- With less hydrogen ions liberated, significantly less NADH is formed.
- A low reading at 340nm indicates the absence of drug.

1.2 References

1.2.1 Thompson, S.G., Principles for Competitive-Binding Assays. in: Clinical Chemistry: Theory, Analysis, Correlation, edited by Kaplan, L.A., Pesce, A.J. and Kazmierczak, S.C., pp. 246-260, Mosby, 2003.

1.2.2 Hand, C. and Baldwin, D., Immunoassays in: Clarke's Analytical Forensic Toxicology, edited by Jickells, S. and Negrusz, A., pp. 375-391, Pharmaceutical Press, 2008.

1.2.3 E.M.I.T. Urine Screening Procedure, Montana Department of Justice Forensic Sciences Division, Courtesy of Jim Hutchison, May 2008.

1.2.4 Enzyme Multiplied Immunoassay (EMIT) Enzymatic Assays for Drug Screening in Urine, Whole Blood Extracts and Other Biological Fluids, Washington State Toxicology Laboratory, Courtesy of Melissa Pemberton, August 2008.

1.2.5 Viva-Jr™ Operator's Manual, Article No.: 6002-940-410, Version number: 01/04-06.

1.2.6 Viva-Jr System Operations Guide, T268, 6/25/07, D01373.

1.2.7 Viva-E Operator's Manual, Article No.: 6002-380-410-01, Version number: 1.0/08-04

1.2.8 Viva-E System Operations Guide, T216, 6/4/07, D01320.

1.2.9 Leedam, D.C., EMIT Basic Power Point Presentation, February 1997 (Provided by Siemens during training October 16, 2008.)

1.2.10 Syva Package Inserts for Emit II Plus Assays

- Amphetamines: 9C122UL.4DS_A
- Benzodiazepine: 9F022UL.10DS_B
- Cannabinoid: 9N022UL.9DS_A
- Cocaine: 9H522UL.4DS_A
- Methadone: 9E022UL.9DS¬_A
- Opiate: 9B322UL.10DS_A

2.0 Scope

2.1 This analytical method employs EMIT for the qualitative screening for drugs-of-abuse in urine specimens. EMIT is commonly used for the detection of drugs-of-abuse in urine. The EMIT assays are run on a microprocessor-controlled automatic chemistry analyzer. The assay results are intended as only a preliminary analytical test result. Confirmatory analysis is performed with an instrument such as a gas chromatograph or liquid chromatograph equipped with a mass selective detector. If EMIT results are reported out, the report must clearly state that the results are from initial screening and confirmatory testing may be requested.

As indicated in the table below, each assay in use has an established administrative threshold or cut-off. For this reason, a negative result does not indicate that no drug is present; the concentration of the drug may be less than the administrative cut-off, or a drug may have poor cross-reactivity to the assay. For this reason there may be situations where confirmation of an analyte may be pursued even if a negative result is indicated for the compound or a class of compounds in question.

Assay	***Calibrator***	***Urine Cut-off***
Amphetamines	d-Methamphetamine	500ng/mL
Benzodiazepines	Lormetazepam	300ng/mL
Cannabinoids	11-Nor-9-Carboxy-THC	50ng/mL
Cocaine Metabolite/-M	Benzoylecgonine	300ng/mL
Opiates	Morphine	300ng/mL

3.0 Equipment/Reagents

3.1 Equipment

3.1.1 Viva-Junior Analyzer

3.1.2 Disposable polyethylene pipettes

3.1.3 Disposable 1 mL plastic specimen cups

3.1.4 Disposable 13X75 polypropylene tubes

3.1.5 *15mL* HDPE Bottle

3.1.6 *30mL* HDPE Bottle

3.2 Reagents

3.2.1 DI water

3.2.2 Syva EMIT Assay Kits

3.2.2.1 Antibody/Substrate Reagent 1:

Antibodies to drug(s) of interest, bovine serum albumin, Glucose-6-Phosphate (G-6-P), Nicotinamide Adenine Dinucleotide (NAD+), preservatives, and stabilizers.

3.2.2.2 Enzyme Reagent 2:

Drug(s) of interest labeled with bacterial Glucose-6-Phosphate Dehydrogenase (G-6-P-DH), Tris buffer, bovine serum albumin, preservatives, and stabilizers.

3.2.3 Manufacturer Provided Assay Reagents

3.2.3.1 0.1N Hydrochloric Acid (Cleaning Solution A)

3.2.3.2 0.1N Sodium Hydroxide (Cleaning Solution B)

3.2.3.3 System Solution (Added to DI water for rinsing)

3.2.3.4 Sodium Hypochlorite (Needle Rinse)

3.3 Reference Material

3.3.1 EMIT Cut-off Calibrators

3.3.1.1 The following table indicates which level of EMIT urine calibrator contains the selected cut-off concentration.

Assay/Selected Cutoff (ng/mL)	Level 1	Level 2	Level 3	Level 4
Amphetamine/500		☠		
Benzodiazepine/300				☠
Carboxy-THC/50			☠	
Cocaine-M/300			☠	
Opiate/300	☠			

3.3.2 EMIT Urine Controls

3.3.2.1 EMIT® Urine Controls

- EMIT® Level 0/Negative Control
- EMIT® Level 5/High Positive Control

Assay/Cutoff (ng/mL)	Level 0	Level 5
Amphetamine/500	0	2000
Benzodiazepine/300	0	1000
Carboxy-THC/50	0	200
Cocaine-M/300	0	1000
Opiate/300	0	4000

3.3.3 Commercially Obtained Enzyme Immunoassay Positive Urine Controls

3.3.3.1 Obtain positive urine controls with concentrations which challenge each EMIT® assay at below, just above or well above the cut-off for each assay. Ideally the control should contain the analytes that are present in calibrators. Positive control can be obtained through BIORAD, UTAK or other suitable vendor.

3.3.4 Negative Control

3.3.4.1 Drug-free urine. Negative control can be provided in-house or obtained through BIORAD, UTAK or other suitable vendor.

4.0 Procedure

4.1 Analysis Procedure

4.1.1 Analyzer Calibration and Pre-run Controls

EMIT calibrators are used to set-up the analyzer for each assay at the selected assay cut-off. These cut-offs will be programmed during analyzer installation.

4.1.1.1 To confirm that the analyzer is properly calibrated for each assay, controls are analyzed and evaluated. Prior to each casework run, a minimum of an EMIT Level 0/negative and an EMIT Level 5/High positive control must be run. Two additional urine controls, commercially obtained (see section 3.3.3) should be included.

4.1.1.2 For the Viva-Junior Analyzer, the calibration for urine assays is valid as long as analyzer provides appropriate responses for controls.

4.1.1.3 Validity of calibration is verified by:

4.1.1.3.1 Comparing calibrator reaction rates against those of last calibration.

4.1.1.3.2 Controls responding appropriately as outlined in 4.1.1.4 & 4.1.1.5.

4.1.1.4 Appropriate control responses are:

4.1.1.4.1 Level 0/Negative Control indicating negative response.

4.1.1.4.2 Level 5/High Control indicating positive response.

4.1.1.4.3 Commercially obtained controls responding appropriately.

4.1.1.4.4 Comparison of control reaction rates against those of previous controls, analyzed with current calibration, indicates no significant change.

4.1.1.4.5 Evaluation of assay/level specific manufacturer provided rate separations indicates suitable separations between the following:

- Negative control/Level 0 and cut-off.
- Cut-off and High control/Level 5.

4.1.1.5 There are no absolute evaluation criteria due to variation between analyzers and assays. However, at the discretion of the analyst, any significant departure from previous values should warrant recalibration of the analyzer.

4.1.1.6 If controls fail, the instrument calibrators must be run followed by analysis of additional urine controls.

4.1.2 In-Run Controls

4.1.2.1 In each casework analysis run, a minimum of one negative and one positive urine control must be included in rotor sample positions. Refer to section 3.3.2 for urine control options.

4.1.2.2 Appropriate control responses are:

- Negative urine control indicating negative response consistent with that observed for EMIT Level 0/Negative control.
- Positive urine controls indicating appropriate positive response relative to cut-off.
- No significant change is noted when control reaction rates are compared to those of previous controls analyzed with current calibration.

4.1.3 Sample Run Preparation

4.1.3.1 Program instrument with laboratory numbers and urine control information.

4.1.3.2 *1mL* Plastic Cups (Pediatric)

- Dispense urine unknowns, negative and positive urine controls into EMIT immunoassay cup. Do not overfill cup.
- Based on sample volumes and dead volume, minimum sample to run our selected assays is .171uL.
- Place cup into pediatric adapter and load in designated position on sample rotor.

4.1.3.3 *13mm x 75mm* Tubes

- Dispense urine unknowns, negative and positive urine controls into tube. Do not overfill tube.
- Based on sample volumes and dead volume, minimum sample to run our selected assays is .421uL.
- Place tube into designated position of sample rotor.

4.2 Viva Junior Operation and Maintenance[5,6]

4.2.1 Daily required Maintenance:

4.2.1.1 Fill Water reservoir and add 15mL Siemens System Solution

4.2.1.2 Check for air bubbles in lines

4.2.1.3 Run Needle Rinse

4.2.1.4 Run Blank Rotor

4.2.2 Monthly/Quarterly Required Maintenance:

Note: Documentation is not required for Monthly, Quarterly Maintenance or as needed maintenance. This maintenance does not affect the results of analysis.

4.2.2.1 Rinse and dry Water Reservoir with .1N Sodium Hydroxide

4.3 Detection Criteria

4.3.1 Positive Case Sample Result

4.3.1.1 Provided that calibration and control evaluation indicate that analyzer has quality assurance conditions suitable for use, a positive result for a sample is indicated by a change in absorbance at a rate value (dABS/m) of equal to or greater than the Cut-off Calibrator.

4.3.2 Elevated Absorbance

4.3.2.1 At the discretion of an analyst, confirmatory techniques may be applied to samples that exhibit an elevated absorbance rate. An elevated absorbance rate is that greater than that of the negative control/Level 0 but less than the cut-off calibrator. If data for confirmatory techniques supports the presence of an analyte, the analyte may be reported as present. In addition, samples with compounds that have low cross reactivity may be confirmed and reported with a negative screen result.

4.3.3 Negative Result

4.3.3.1 A negative result for a sample is indicated by a change in absorbance at a rate that is less than the Cut-off Calibrator. Special considerations may apply as outlined above (4.2).

4.4 Distribution of Assay Information

4.4.1 Electronic copy of EIA analysis report must be attached to the case in LIMS. Case results are also to be recorded in the LIMS system.

4.4.2 A copy of data for calibrators and controls may be stored electronically in a central location.

4.4.3 Original data for calibration and controls for each analysis will be stored centrally in the laboratory, until archiving or destruction. If an electronic copy is created the hard copies need not be retained, if electronic copies are maintained on a network drive.

4.5 Quality Assurance Requirements

4.5.1 Refer to Toxicology AM# 19 for storage requirements.

4.5.2 Refer to Toxicology AM #21 for authentication of reference material requirements.

Toxicology AM #2: General Extraction of Urine for Basic/Neutral or Acidic/Neutral Compounds

1.0 Background/References

1.1 Background

These extractions are extensions of the TOXI-LAB TOXI-A and TOXI-B thin layer chromatography (TLC) drug detection systems. The samples are extracted as with the TLC system, however, instead of concentrating the extract onto a disc, the solvent extract is concentrated and placed into an automated liquid sampler (ALS) vial for analysis by a gas chromatograph equipped with a mass selective detector (GC/MSD). Discussions of TLC and GC/MS theory can be found in most college-level chemistry and/or instrumental texts. In 2013 the TOXI-LAB line was discontinued. An equivalent product, De-Tox Tubes by Dyna-Tek, were evaluated and found to be a suitable replacement.

2.0 Scope

2.1 This procedure describes the extraction of drug compounds from urine. Depending upon the pKa of a drug compound, either De-Tox Tubes A or B (or verified equivalents) are used. Basic and neutral compounds are extracted with an A tube. Addition of urine to the De-Tox A tube results in the sample becoming alkaline and basic and neutral drugs thus extract into a solvent mixture. The B tube is used for acidic and neutral compounds. Urine placed into the De-Tox B tube becomes acidic resulting in acidic and neutral compounds being extracted into a solvent mixture. Either resulting extract is analyzed by full scan GC/MS in EI mode.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Tube Rocker

3.1.2 Evaporative concentrator and appropriate concentration cups or tubes

3.1.3 Laboratory Centrifuge capable of 3000 rpm

3.1.4 Laboratory oven or waterbath

3.1.5 Fixed and/or adjustable volume pipettes, and appropriate tips.

3.1.6 Automated Liquid Sampler (ALS) vials

3.1.7 GC/MS Vial Microinserts

3.1.8 Gas Chromatograph equipped with a mass selective detector and a low bleed (5%-Diphenyl-95%-Dimethylsiloxane copolymer) capillary column.

3.2 Reagents

3.2.1 De-Tox Tubes A and B (or equivalent Toxi Tubes)

3.2.2 b-Glucuronidase Solution

3.2.3 2M Acetate buffer, pH 4.8

3.3 Qualitative Controls

3.3.1 Positive control

3.3.1.1 Tube A positive control may be commercially obtained or prepared in-house. At a minimum, the control must contain at least one phenethylamine at an approximate concentration between 500 and 3000 ng/mL, and one opiate at an approximate concentration between 300 and 3000 ng/mL.

3.1.1.2 Tube B positive control may also be commercially obtained or prepared in-house. At a minimum, the control must contain two barbiturates at an approximate concentration between 300 and 1000 ng/mL.

3.3.2 Negative Urine

3.3.2.1 Negative urine can be commercially obtained or in-house urine verified to be negative for drugs of interest. Refer to Toxicology AM#19 for additional details.

3.3.3 Morphine-Glucuronide Positive and Negative Controls for Optional Enzymatic Hydrolysis Step

3.3.3.1 Commercially-obtained control or in-house spiked urine containing morphine-glucuronide should be used. The same negative urine must be used to prepare both the positive and negative glucuronide controls for in-house spiking. Morphine-glucuronide should be used for these controls and must be at a minimum concentration of 375ng/mL. The positive and negative glucuronide controls are used to demonstrate the glucuronidase cleavage was effective.

3.4 Qualitative Non-Extracted Reference Material

3.4.1 Run necessary reference material as indicated by examination of GC/MSD data. Reference material mixes may be used.

3.4.2 Dilute reference material as necessary. A suggested dilution for a 1mg/mL solution is 1 in 3 parts of appropriate solvent.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 De-Tox Tubes-A Extraction (Basic and Neutral Compounds)

4.1.1.1 Label DE-TOX TUBES A and ALS vials with micro-inserts for negative control, positive control and appropriate laboratory case numbers.

4.1.1.2 Transfer approx. 5 mL of casework, negative and positive control urine to appropriate DE-TOX TUBE A (pH=9).

4.1.1.3 Agitate the DE-TOX tube to break up salts. This assists in reducing the occurrence of emulsions.

4.1.1.4 Rock DE-TOX TUBE A for at least 10 minutes.

4.1.1.5 Centrifuge tube at ~2500-3000 rpm for ~10 minutes.

4.1.1.6 Transfer solvent and evaporate to ~100-300uL.

4.1.1.7 Transfer solvent to labeled GC/MS ALS vial with micro-insert.

4.1.1.8 OPTIONAL: Analyst may, at their discretion, perform an enzymatic hydrolysis on a sample aliquot prior to the above De-Tox Tube A extraction. If done, this must be done in addition to the regular (non-hydrolyzed) extraction of the sample. Positive and negative morphine glucuronide controls (see section 3.3.3 of this method) should be run in addition to the regular controls required by the method.

4.1.1.8.1 Optional Enzyme Hydrolysis: To 4.5mL of urine, add 150µL of 2M acetate buffer and vortex. To all but the glucuronidase negative control, add 100µL of Kura BG-100 β-glucuronidase solution. Cap and vortex gently to mix. Place in a 60°C laboratory oven or waterbath for 2 hours. Allow sample to cool before proceeding with steps 4.1.1.2 through 4.1.1.7.

4.1.2 De-Tox Tubes-B Extraction (Acidic and Neutral Compounds)

4.1.2.1 Label DE-TOX TUBES B and ALS vials with microinserts for negative control, positive control and appropriate laboratory numbers.

4.1.2.2 Transfer approx. 4.5 mL of casework, negative and positive control urine to appropriate DE-TOX TUBE B (pH=4.5).

4.1.2.3 Rock DE-TOX TUBE B for at least 10 minutes.

4.1.2.4 Centrifuge tube at ~2500-3000 rpm for ~10 minutes.

4.1.2.5 Transfer solvent and evaporate to ~100-300uL.

4.1.2.6 Transfer solvent to labeled GC/MS ALS vial with microinsert.

4.2 Preparation for Analysis Run

4.2.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.2.2 Load samples, reference materials, blanks and controls into the quadrant rack as noted in the sequence table.

4.2.3 GC-MSD Analysis Parameters

4.2.3.1 Refer to instrument method for current analysis parameters.

4.2.3.1.1 Instrument Run Parameters

- Inlet at 280 splitless injection, injection volume: 1ul
- Oven at 80 degrees Celsius, hold 2.5 minutes
- Ramp 25 degrees Celsius
- Final temperature: 300 degrees Celsius, hold at least 7.5 minutes.
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.2.3.2 Current analysis method must be stored centrally as a hard or electronic copy. Setting the instrument parameters to store with the data file is also approved.

4.2.4 Detection and Identification Criteria

4.2.4.1 The presence of a drug compound is indicated if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

NOTE: Early eluting drugs, as well as drugs known to have similar retention times and mass spectral fragmentation patterns (e.g. Phentermine and Methamphetamine), may not differ from the retention time of the applicable reference material by more than ±0.1 minutes.

4.3 Quality Assurance Requirements

4.3.1 Refer to applicable sections of Toxicology AM #19 and Toxicology AM #21 for additional quality assurance and reference material authentication requirements.

4.4 Analysis Documentation

4.4.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the iLIMS system.

4.4.2 Original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed until archiving, or electronically on a shared drive.

4.4.3 A copy of controls may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Qualitative Confirmation of Basic and Neutral Drug Compounds in Urine - De-Tox Tube A Extraction

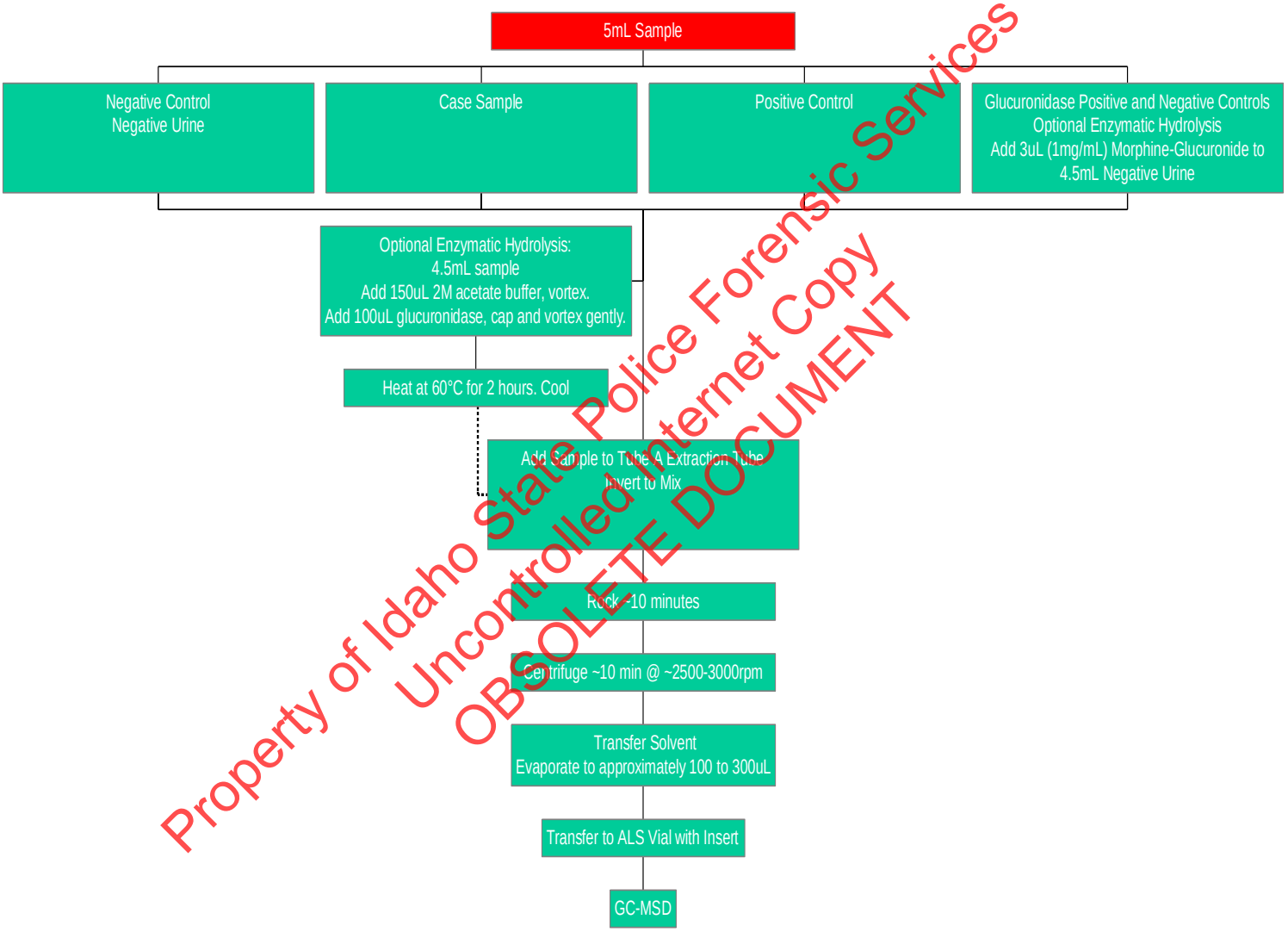
5.1.1 Reagents

5.1.1.1 De-Tox Tube A

5.1.2 Qualitative Controls

5.1.2.1 Positive control (at least one phenethlyamine 500-3000 ng/mL and at least one opiate 300-3000 ng/mL)

5.1.2.2 Negative Urine



5.2 Qualitative Confirmation of Acidic Drug Compounds in Urine - De-Tox Tube B Extraction

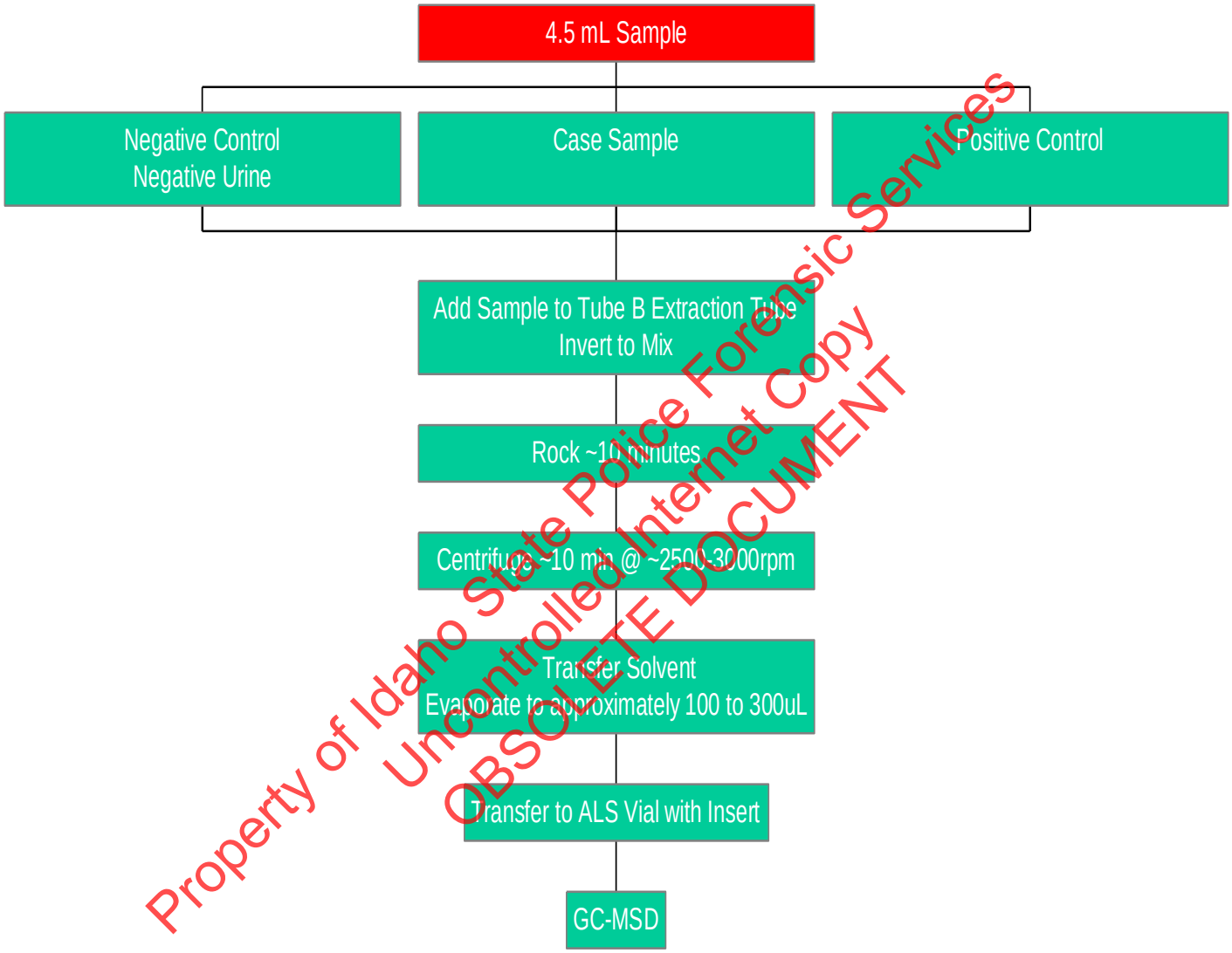
5.2.1 Reagents

5.2.1.1 DE-Tox Tube B

5.2.2 Qualitative Controls

5.2.2.1 Positive Control (at least 2 barbiturates 300 and 1000 ng/mL)

5.2.2.2 Negative Urine



Toxicology AM #3: Qualitative 11-nor-9-THC-D9-COOH (Carboxy-THC) in Urine

1.0 Background/References

1.1 Background

Cannabis sativa use dates back to 2700 B.C.[2,5] Marijuana (MJ) refers to a mixture of the leaves and flowering tops.[3] The smoke from burning cannabis includes 61 different cannabinoids.[2,6] The major active ingredient in marijuana is delta–9-tetrahydrocannabinol (D9-THC). The D9-THC content varies from 2 to 10% with an average of four to five percent. The quality of marijuana is reported to have improved over the last 20 years due to superior cultivation practices. The medicinal effects of MJ include anti-nausea, muscle relaxing, anticonvulsant and reduction of intraocular pressure.[6] Cannabis therefore has found use as an antiemetic to deal with the nausea associated with anticancer chemotherapy and for relief for those suffering from glaucoma. The debate continues on medical use and the complete legalization of the drug.

Several factors come into play when considering the behavioral effects of (D9)-THC. These include the route of administration (smoked or ingested), THC concentration of the plant (dose), the experience of the user, the user's vulnerability to psychoactive effects, and the setting of the use.[5,6] The desirable effects of MJ include an increased sense of well-being, mild euphoria, relaxation and a mild sedative-hypnotic effect.[5,6] Its clinical effects are similar to those of alcohol and the anti-anxiety agents.[5] The side effects of MJ use include impairment of cognitive functions, alteration of the user's perception of time and distance, reaction time, learning and short-term memory.[2,5,6] MJ has been shown to interfere with a person's ability or willingness to concentrate. Cannabis causes temporal disintegration such that the individual loses the ability to store information in the short term and is easily distracted.[2] Impairment from use is thought to last from 4 to 8-hours with more recent studies reporting 3 to 6 hours. Dr. Huestis reported that most behavioral and physiological effects return to baseline within three to six hours after use with residual effects in specific behaviors for up to 24 hours.

Impairment of coordination and tracking behavior has been reported to persist several hours beyond the perception of the high.[6] Due to the variable period of impairment, the relating of urine Carboxy-THC to the time of use, and thus impairment, requires the development of the scenario surrounding the stop for DUI. The presence of Carboxy-THC in urine only indicates exposure to MJ at some previous, indeterminate time.

The physiological effects may include an increase in heart rate and blood pressure, conjunctival suffusion, vasodilation, dry mouth and throat and a decrease in respiratory rate. The individual may also experience increased hunger (munchies).

D9-THC is rapidly metabolized to the inactive metabolite, Carboxy-THC.[1,4,5,6] In urine, this major metabolite, Carboxy-THC is pursued due to D9-THC only being present in minute quantities.[6] Carboxy-THC in urine has been conjugated with glucuronic acid to improve excretion. The detection time of Carboxy-THC in urine following marijuana use varies dependent upon various pharmacological factors such as the dose obtained, the route of administration, and the rates of metabolism and excretion.[1] D9-THC is deposited in body fat due to its high lipid solubility. It is slowly released from this storage depot over time.[1] The amount of D9-THC stored in fat is a function of the amount, frequency and potency of drug exposure. The detection time can therefore vary from days to months.

1.2 References

1.2.1 Huestis, M.A., Mitchell, J.M. and Cone, E.J. Detection Times of Marijuana Metabolites in Urine by Immunoassay and GC-MS J. Anal. Tox. 19:443-449, 1995.

1.2.2 Huestis, M. Marijuana. pp. 269-304. in: Principles of Forensic Toxicology, Third Edition. Levine, B. ed., AACC, 2010.

1.2.3 Cannabis. in: Clark's Isolation and Identification of Drugs. pp. 423-425, Moffat, A.C. ed., Pharmaceutical Press:London, 1986.

1.2.4 Drug Evaluation and Classification Training Manual, U.S. Dept. of Transportation, 1993.

1.2.5 Julien, R.M. Marijuana: A Unique Sedative-Euphoriant-Psychedelic Drug. in: A Primer of Drug Action. pp. 319-349, W.H. Freeman and Company: NewYork, 1998.

1.2.6 O'Brien, C.P. Drug Addiction and Drug Abuse. pp. 572-573. in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth edition, Hardman, J.G. ed., McGraw-Hill, 1996.

2.0 Scope

2.1 This method is to qualitatively confirm the presence of a major metabolite of marijuana, Carboxy-THC, in urine specimens.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Tube Rocker

3.1.2 Laboratory Centrifuge capable of 3500 rpm

3.1.3 Waterbath

3.1.4 Drybath

3.1.5 Evaporative Concentrator equipped with nitrogen tank.

3.1.6 pH Indicator Strips

3.1.7 Glassware

3.1.7.1 16X100mm tubes

3.1.7.2 16X144mm tapered tip centrifuge tubes

3.1.7.3 Caps for 16mm OD tubes

3.1.7.4 GC/MS ALS vials

3.1.7.5 GC/MS vial microinserts

3.1.8 Gas Chromatograph equipped with a mass selective detector and a nonpolar capillary column (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl).

3.2 Reagents

Refer to Toxicology AM#23 for solution preparation instructions. Purity of chemicals must be ACS Grade or equivalent.

3.2.1 1N KOH

3.2.2 Saturated Potassium Phosphate Monobasic pH approx. 1.8

3.2.3 87:13 Hexane with Ethyl Acetate (v/v)

3.2.4 Ethyl acetate

3.2.5 Silylating Agent (select from): BSTFA/1% TMCS or MSTFA

3.3 Standards/Reference Material

3.3.1 Stock Standard Solution

- 100ug/mL (+) 11-nor-9-carboxy-D9-THC

3.3.2 Working Standard Solution (1800ng/mL)

3.3.2.1 Add 180uL Stock Solution to 9.82mL Methanol. Other volumes may be prepared. Document preparation on appropriate log sheet. Solution is stable for 1-year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.

3.4 Qualitative Controls

3.4.1 Positive Controls

3.4.1.1 A minimum of one spiked 60ng/mL and one commercial Carboxy-THC containing control must be analyzed in each batch of samples.

3.4.1.2 60ng/mL Carboxy-THC Spiked Control

3.4.1.2.1 Add 3mL of the same lot of negative urine used to prepare the negative control to extraction tube. Add 100uL of working standard solution, and vortex.

3.4.1.3 Suitable nominal concentration range for commercial control is 15ng/mL to 150ng/mL.

3.4.2 Negative Control

3.4.2.1 Negative urine commercially obtained or in-house urine verified to be negative for drugs of interest.

4.0 Procedure

4.1 Extraction Method

4.1.1 Initial set-up

4.1.1.1 Label extraction tubes, tapered bottom derivatization tubes and GC/MS vials with microinserts for the negative control, spiked positive control, commercial positive control(s), and casework samples.

4.1.2 Sample Preparation

4.1.2.1 Transfer 3 mL urine specimen, negative urine, spiked positive control and commercial positive control(s) to extraction tubes.

4.1.3 Sample Hydrolysis

4.1.3.1 Add 0.5mL 1.0N KOH to each extraction tube.

4.1.3.2 Vortex gently to mix.

4.1.3.3 Check resulting pH.

4.1.3.4 pH must be > 12. If pH <12, add additional 0.5mL of KOH.

4.1.3.5 Place in 40°C water bath for 15 minutes.

4.1.3.6 Allow samples to cool before proceeding with solvent extraction.

4.1.4 Extraction

4.1.4.1 If original pH was > 12:

4.1.4.1.1 Add 1.5mL Saturated Phosphate Buffer (pH 1.8).

4.1.4.1.2 Add 3mL Hexane/Ethyl Acetate (87:13).

4.1.4.1.3 Rock for 10 minutes.

4.1.4.2 If original pH was < 12:

4.1.4.2.1 Add 3.0mL Saturated Phosphate Buffer (pH 1.8).

4.1.4.2.2 Add 4mL Hexane/Ethyl Acetate (87:13).

4.1.4.2.3 Rock for 10 minutes.

4.1.4.3 Centrifuge tubes at ~3500 rpm for 10 minutes.

4.1.4.4 Transfer upper organic phase from tube into labeled tapered bottom tube.

4.1.4.5 Evaporate solvent to dryness, under a gentle stream of nitrogen, at ~37°C.

4.1.5 Derivatization

4.1.5.1 To dried extract in tapered bottom tubes, add 50uL ethyl acetate and 50uL silylating reagent.

4.1.5.2 Cap tubes.

4.1.5.3 Vortex.

4.1.5.4 Heat tube for 15 minutes in 95°C drybath.

4.1.5.5 Remove from heat and allow samples to cool.

4.1.5.6 Transfer derivative to labeled GC/MS ALS vial with microinsert.

4.2 Gas Chromatograph/Mass Spectrometry (GC/MS)

4.2.1 Preparation for Analysis Run

4.2.1.1 Into Sequence log table, enter information for case samples, controls and pre-sample solvent blanks. A 60ng/mL spiked positive control should run both early and late in the sequence.

4.2.1.2 Load case samples, controls and solvent blanks into the quadrant rack(s) as noted in the sequence table.

4.2.2 GC-MSD Acquisition Parameters

4.2.2.1 Refer to instrument method for current acquisition parameters.

4.2.2.1.1 Instrument Run Parameters

- Inlet at 280 Celsius, splitless injection, injection volume: 1uL
- Oven at 185 degrees Celsius
- Ramp 30 degrees Celsius
- Final temperature: 285 degrees Celsius, hold 4 minutes

4.2.2.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.2.2.3 Acquire sample data in SIM (selected ion monitoring) utilizing the ions 371, 473 and 488.

4.2.3 Detection and Identification Criteria

4.2.3.1 Retention Time

4.2.3.1.1 Identification requires a peak within ±0.1 minutes of the retention time established for Carboxy-THC with the in-run control(s).

4.2.3.2 Ion ratios - Selective Ion Monitoring (SIM)

4.2.3.2.1 Carboxy-THC ion ratio for the early and late 60 ng/mL control must be calculated and averaged. This mean ratio must be compared to ratio obtained from casework and the mean of the 60ng/mL control samples. Ratio between monitored ions, 371:473 and 371:488, must agree within ±20%.

4.2.3.2.1.1 Incorrect Ratios

4.2.3.2.1.1.1 If the casework or control sample ion ratios do not agree within ~20% due to high concentration of c-THC in the sample, the extract may be diluted and run within 72 hours of the last positive control. If the time frame is greater, the controls must also be run with the diluted samples.. Alternatively, carboxy-THC in the sample may be confirmed using full scan data, provided a derivatized reference material is also run in full scan mode. The analyte may be confirmed from full scan data if there are no significant differences in the mass spectral data as compared to the appropriate reference material and the retention time is within ±0.1 minutes of the appropriate reference material.

4.2.3.2.1.1.2 Assessment of relative strength of case sample to 60 ng/mL control. The response of case samples will be compared to a 60 ng/mL control sample. The analyst will pick either of the responses from the positive control and divide the response of the 371 ion by 5; this will be defined as the approximate minimum response. The approximate minimum response will be documented in the analyst's notes. -The analyst will compare this response to the response for each case sample.

4.2.3.2.1.1.3 If the response for the case sample is less than the approximate minimum response established by the control. Carboxy-THC will generally not be confirmed. If it is below the minimum response, it is at the analyst's discretion whether or not to call the drug. Other factors such as enzyme screen results and the sample response in relation to the baseline must be considered and noted in the analyst's notes.

4.3 Quality Assurance Requirements

4.3.1 Refer to relevant sections of Toxicology AM #19 and Toxicology AM #21 for additional quality assurance and reference material authentication requirements.

4.4 Analysis Documentation

4.4.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the iLIMS system.

4.4.2 Original data for controls will be compiled for each analysis run and must be stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.4.3 A copy of data for controls may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

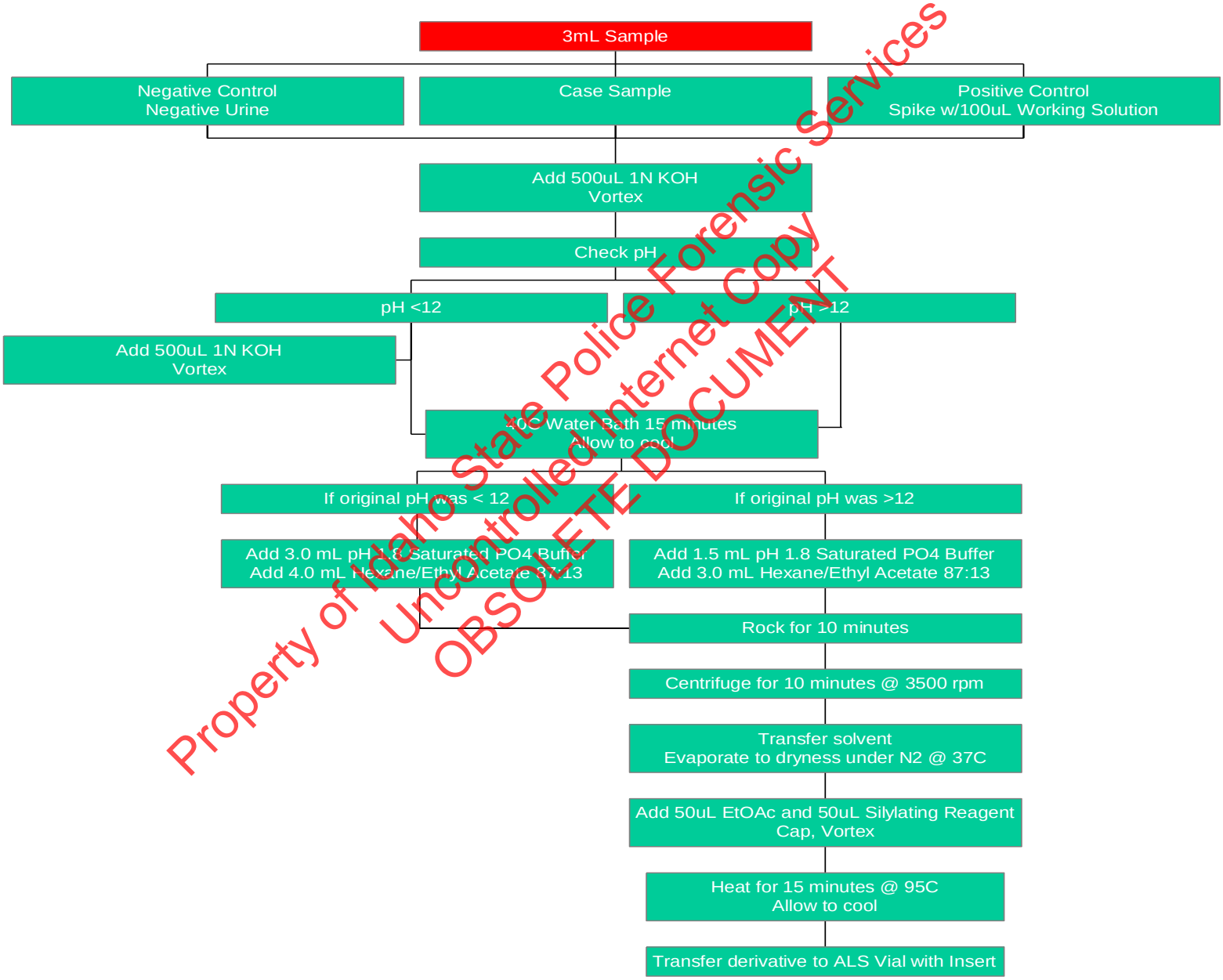
5.1 Reference Material

5.1.1 Stock Standard Solution

- 100µg/mL 11-nor-9-carboxy-Δ^9-THC

5.1.1.1 Working Standard Solution (1800ng/mL)

- Add 180µL Stock Solution to 9.82mL Methanol. Other volumes may be prepared. Solution is stable for one-year, or the expiration date of the stock reference material used (whichever is earlier) when stored under refrigeration or in a freezer.



Toxicology AM #4: Cocaine and Cocaine Metabolites in Urine

1.0 Background/References

1.1 Background

Cocaine is a naturally occurring alkaloid derived from leaves of the South American shrub, Erythroxylon coca. Cocaine also can be produced synthetically. Cocaine is one of the most potent stimulants of the central nervous system due to its mechanism of action, which involves blocking reuptake of stimulatory neurotransmitters. Cocaine is used licitly as a local anesthetic in ophthalmology and health care settings (e.g. biopsy, wound care). The positive effects of cocaine include an increased mental awareness and alertness, a sense of clarity and feelings of elation. The fictional detective Sherlock Holmes used cocaine for its transcendently stimulating and mind clarifying properties, to the displeasure of Doctor Watson. As with all drugs, the effects of cocaine depend on the dosage, the form in which it is taken, and the route of administration. Other significant factors include the setting or circumstances in which the drug is used and the expectations of the user. Side effects can include pupillary dilation, restlessness, dizziness, dyskinesia, tremor, dysphoria, and paranoia. Additional major side effects of cocaine use are a consequence of discontinued use. If the user does not re-administer the drug, they may experience increased anxiety, agitation, restlessness and the disturbance of normal sleep patterns, which leads to fatigue. Due to these effects following cocaine use, an individual's ability to operate a motor vehicle may be impaired both during and following cocaine use.

Routes of administration include snorting, injection and smoking. The metabolism of cocaine and its metabolites involves hydrolysis, transesterification and N-demethylation. Cocaine metabolites detectable in urine include benzoylecgonine, ecgonine methyl ester, norcocaine and various arylhydroxy- and arylhydroxymethoxy- metabolites. The duration of action of cocaine is limited by its rate of metabolism since its major metabolites are inactive.

1.2 References

1.2.1 UCT CLEAN SCREEN® Extraction Columns Application Manual.

1.2.2 Telepchak, M.J., August, T.F. and Chaney, G., Drug Methods for the Toxicology Lab, pp. 204 - 209. in: Forensic and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

1.2.3 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

2.0 Scope

2.1 This procedure outlines the use of the 200mg CLEAN SCREEN® DAU SPE column for the extraction of the cocaine metabolite Benzoylecgonine along with Cocaine and additional metabolite Ecgonine Methyl Ester, from urine. The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances.

The cation exchanger will allow the anionic sorbent (-) to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption. The nonpolar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[2] The cation exchanger component of the phase is effective for compounds which are present in the urine sample in a cationic form bonding ionically to the sorbent.

To maximize the ionic character of analytes, the urine is adjusted with a pH 6 100mM phosphate buffer, and loaded onto a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. Analytes are retained by ionic interaction of the amine functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water and a weak aqueous buffer, to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. When the column is dry the analytes of interest are recovered from the column with a basic organic solvent mixture. Following elution from the SPE column, the extract is derivatized for qualitative confirmation on a gas chromatograph equipped with a mass selective detector (GC/MSD).

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 200 mg CLEAN SCREEN® Extraction Column

3.1.2 Disposable inserts for SPE manifold ports (optional)

3.1.3 Tube Rocker

3.1.4 Vortex Mixer

3.1.5 Dry-bath or Laboratory Oven

3.1.6 Evaporative concentrator equipped with nitrogen tank

3.1.7 Vacuum Manifold/pump

3.1.8 Fixed and adjustable volume single channel air displacement pipettes, and appropriate tips, capable of accurate and precise dispensing of volumes indicated

3.1.9 pH indicator strips

3.1.10 16 x 100mm Screw-top Glass Tube

3.1.11 Screw Cap for 16mm O.D. tube

3.1.12 {Optional} 16X144mm tapered tip centrifuge tubes

3.1.13 Automated Liquid Sample (ALS) vials

3.1.14 GC/MS Vial Micro-insert

3.1.15 Gas Chromatograph equipped with a mass selective detector and a nonpolar capillary column with a phase composition comparable to 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%-diphenyl

3.2 Reagents

Refer to Toxicology AM# 23 for Solution Preparation Instructions

3.2.1 Methylene Chloride (Certified ACS Grade)

3.2.2 Isopropanol (Certified ACS Grade)

3.2.3 Ammonium Hydroxide (Certified ACS Grade)

3.2.4 Methanol (Certified ACS Grade)

3.2.5 Ethyl Acetate (Certified ACS Grade)

3.2.6 Deionized/Distilled (DI) water

3.2.7 100mM Phosphate buffer, pH 6.0

3.2.8 100mM Monobasic Sodium Phosphate

3.2.9 100mM Dibasic Sodium Phosphate

3.2.10 100mM HCl

3.2.11 Elution Solvent

3.2.11.1 Mix 20mL isopropanol with 2mL ammonium hydroxide, QS to 100mL with methylene chloride.

3.2.12 BSTFA + 1% TMCS

3.3 Quality Assurance Materials

3.3.1 Positive Control

NOTE: Positive Control can be prepared with the working solution described below and/or obtained commercially.

3.3.1.1 Positive Control Stock Solution

3.3.1.1.1 Obtain 1mg/mL (1ug/uL) stock drug reference material solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.3.1.2 Positive Control Working Solution

3.3.1.2.1 Add the designated volume of stock solution to 10mL volumetric flask partially filled with methanol. QS with methanol. *Solution is stable for 1 year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.*

Stock Solution (1.0mg/mL)	Volume (µL)	ng/µL
Benzoylecgonine	100	10
Cocaine (optional)	100	10
Ecgonine methyl ester (optional)	100	10

3.3.2 Internal Standard

3.3.2.1 Stock Solution

- 1 mg/mL Mepivacaine

3.3.2.2 Working Internal Standard Solution [10ng/uL]

3.3.2.2.1 Add 100uL Mepivacaine stock solution to 10mL volumetric flask partially filled with methanol. QS with methanol. *Solution is stable for 1 year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.*

3.3.3 Negative Control

3.3.3.1 Commercially obtained or in-house urine verified to be negative for drugs of interest.

3.3.4 Non-extracted Reference Material

3.3.4.1 Reference material not included in extracted positive control should be prepared as necessary.

3.3.4.2 Obtain 1mg/mL stock drug reference material solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.3.4.3 Dilute 1mg/mL drug reference material as necessary. More than one compound may be added to this solution.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 Label extraction tubes (in duplicate) and ALS vials with micro-inserts for Negative Control, Positive Control(s) and with appropriate Laboratory Numbers.

4.1.2 Control Samples

4.1.2.1 Use the same lot of negative urine to prepare both the negative and spiked positive control(s).

4.1.2.2 Positive Control Sample Preparation

4.1.2.2.1 Add 5mL of negative urine to extraction tube.

4.1.2.2.2 Add indicated amount of 10ng/uL working mixed control solution.

Desired ng/mL	µL Working Control
400	200

4.1.2.2.3 Additional concentrations may be used at the discretion of the analyst.

4.1.2.3 Negative Control Sample Preparation

4.1.2.3.1 Add 5mL of negative urine to extraction tube.

4.1.3 Case Sample Preparation

4.1.3.1 Based on enzyme immunoassay screen results, samples may be diluted with negative urine prior to analysis.

4.1.3.2 The total volume of urine or diluted urine should be 5mL.

4.1.3.3 Add 5mL neat or diluted sample to labeled extraction tube.

4.1.3.4 Internal Standard Addition

4.1.3.4.1 Add 250uL of internal standard to controls and case samples. This results in an internal standard concentration of 500ng/mL.

4.1.4 SPE

4.1.4.1 All aspirations must be at ~3 inches Hg to prevent sorbent drying. Ideally, gravity flow should be used.

4.1.4.2 To 5mL prepared Casework and Control samples, add 2mL pH 6 100mM phosphate buffer. Vortex.

4.1.4.3 Check pH. If pH is not 6.0 +/- 0.5, adjust as necessary with 100mM monobasic or dibasic sodium phosphate.

4.1.4.4 Insert labeled CLEAN SCREEN® extraction column into vacuum manifold.

4.1.4.5 Add 3mL of methanol to column.

4.1.4.6 After methanol has flowed through, add 3mL of DI H2O to column.

4.1.4.7 After water has flowed through, add 1mL 100mM phosphate buffer (pH 6.0) to column.

4.1.4.8 After buffer has flowed through, add buffered urine. Load sample onto column at ~2 mL/minute.

4.1.4.9 Wash column with 2mL DI H2O.

4.1.4.10 Wash column with 2mL of 100mM hydrochloric acid.

4.1.4.11 Wash column with 3mL of methanol.

4.1.4.12 Dry column by aspirating at ~ 10 in. Hg for about 5 minutes.

4.1.4.13 Open vacuum manifold, wipe collection tips, and insert collection rack containing collection tubes.

4.1.4.14 Add 3mL of elution solvent to column and allow to gravity-flow through. Once elution appears complete, aspirate slowly, < 3 in. Hg (10kPa), to finish recovery.

4.1.4.15 Remove collection tubes with eluates from rack and place into evaporative concentrator.

4.1.4.16 Evaporate to dryness under a gentle stream of nitrogen at ~37°C.

4.1.5 Derivatization

4.1.5.1 Add 50uL ethyl acetate, vortex.

4.1.5.2 Add 50uL BSTFA + 1% TMCS.

4.1.5.3 Cap and vortex.

4.1.5.4 Heat tubes for 20 minutes at 70°C.

4.1.5.5 Remove tubes from dry heat. Allow to cool to room temperature.

4.1.5.6 Transfer extract to the appropriately labeled ALS vial with microinsert.

4.2 Preparation for Analysis Run

4.2.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.2.2 Load samples, reference material, blanks and controls into the quadrant rack as noted in the sequence table.

4.2.3 GC-MSD Analysis Parameters

4.2.3.1 Refer to instrument METHOD printout for current analysis parameters.

4.2.3.1.1 Instrument Run Parameters

- Inlet at 280 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 80 degrees Celsius, hold 2.5 minutes
- Ramp 25 degrees Celsius
- Final temperature: 300 degrees Celsius, hold at least 7.5 minutes.

4.2.3.2 Current analysis method must be stored centrally as a hard or electronic copy.

4.3 Detection and Identification Criteria

4.3.1 The presence of a drug compound is indicated if the retention time for the sample versus applicable reference material does not differ by more than ±0.1 minutes and there are no significant differences in the mass spectral data.

4.4 Quality Assurance Requirements

4.4.1 Urine samples should be stored frozen or refrigerated prior to analysis.

4.4.2 Urine samples are to be stored under refrigeration or in a freezer while analysis is in process.

4.4.3 Post analysis, urine samples are to be stored frozen until returned to submitting agency.

4.4.4 Refer to relevant sections of Toxicology AM#19 and AM#21 for additional quality assurance and reference material authentication requirements.

4.5 Analysis Documentation

4.5.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.5.2 Original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving.

4.5.3 A copy of control data may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Reference Material

5.1.1 Stock Solutions

- 1mg/mL Benzoylecgonine, Cocaine, Ecgonine Methyl Ester and Mepivacaine.

5.1.2 Working Control Solution

- Add designated volume of Stock Solutions to 10mL Methanol. *Solution is stable for 1 year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.*

Stock Solution	Volume (µL)	ng/µL
Benzoylecgonine	100	10
Cocaine (optional)	100	10
Ecgonine methyl ester (optional)	100	10

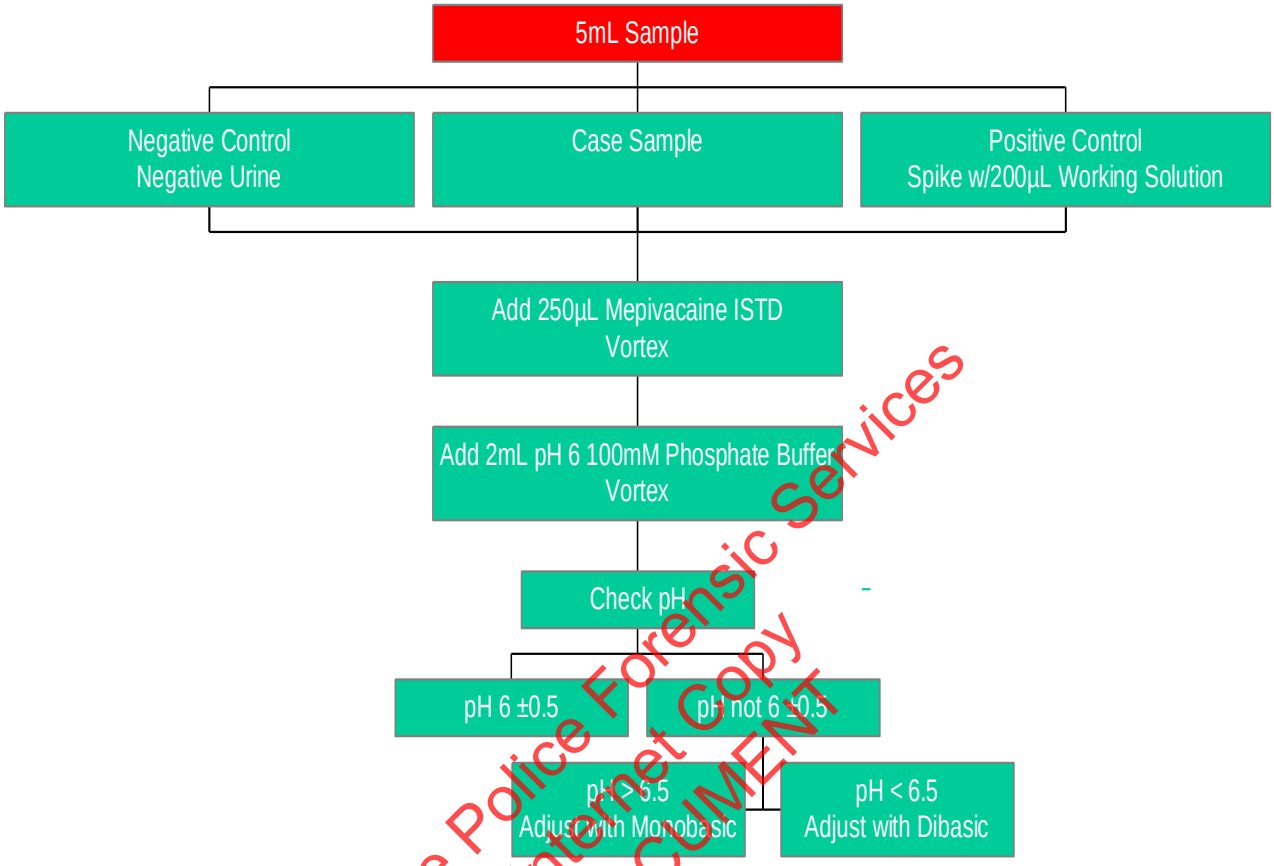
5.1.3 Working Internal Standard Solution

5.1.3.1 Add 100µL Mepivacaine stock solution to 10mL Methanol. Solution is stable for 1 year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.

5.1.4 Elution Solvent

5.1.4.1 Elution solvent must be prepared fresh. Mix 20mL 2-Propanol with 2mL Ammonia Hydroxide in 100mL ball flask. Bring up to volume with Methylene Chloride and mix well.

5.2 Sample Preparation for SPE



Toxicology AM #5: Qualitative Benzodiazepines and Ancillary Compounds in Urine

1.0 Background/References

1.1 Background

Benzodiazepines continue to be the most prescribed group of therapeutic agents. Approximately 20 benzodiazepines are approved for use in the US.[2] Benzodiazepines were first introduced in the 1960s in pursuit of the perfect sedative hypnotic agent, and have replaced barbiturates as the major class of central nervous system (CNS)-depressant drugs.[2] Chlordiazepoxide (Librium) was introduced in 1962 followed by the introduction of Diazepam (Valium) in 1968. There are four main classes of benzodiazepines, the 1,4-benzodiazepines, the triazolobenzodiazepines, the diazolobenzodiazepines, and the 7-nitrobenzodiazepines. Refer to the following chart for a compilation of benzodiazepines currently prescribed in the US or ones that are commonly encountered.

1,4-Benzodiazepines	Trade Name	Major Metabolite(s)
Diazepam	Valium®	Nordiazepam, Oxazepam, Temazepam
Nordiazepam	---	Oxazepam
Oxazepam	Serax®	Glucuronide conjugate
Temazepam	Restoril®	Oxazepam
Clorazepate	Tranxene®	Nordiazepam, Oxazepam
Chlordiazepoxide	Librium®	Demoxepam, Nordiazepam, Oxazepam
Halazepam	Paxipam®	3-Hydroxy-Halazepam, Nordiazepam, Oxazepam
Quazepam	Dormalin®, Doral®	2-Oxoquazepam
Flurazepam	Dalmane®	Desalkylflurazepam
Lorazepam	Ativan®	3-Glucuronide
7-Nitrobenzodiazepines		
Clonazepam	Klonopin®	7-Aminoclonazepam
Flunitrazepam	Rohypnol® Not Prescribed in US	7-Aminoflunitrazepam
Triazolobenzodiazepines		
Alprazolam	Xanax®	α-Hydroxy-alprazolam, 4-Hydroxy-alprazolam
Triazolam	Halcion®	α-Hydroxy-triazolam
Estazolam	ProSom®	---
Diazolobenzodiazepine		
Midazolam	Versed (Parenteral)	α-Hydroxymidazolam

Benzodiazepines are used primarily as anti-epileptics in the treatment of seizure disorders, as anxiolytics for the short-term relief of anxiety disorders, as sedative-hypnotics for the treatment of sleep disorders, and as muscle relaxants to relieve spasticity. The primary side effects that accompany their use include dose-related extensions of the intended actions, including sedation and sleepiness/drowsiness. In addition, other undesired effects that will influence the outcome of field sobriety tests include ataxia, a blocked ability to coordinate movements, a staggering walk and poor balance, lethargy/apathy, indifference or sluggishness, mental confusion, disorientation, slurred speech and amnesia. Impairment of motor abilities, especially a person's ability to drive an automobile, is common. This impairment is compounded by the drug-induced suppression of ones' ability to assess their own level of physical and mental impairment. Alcohol and other CNS depressants (e.g., barbiturates, antidepressants, etc.) will increase CNS depressant effects, such as sedation and impairment of psychomotor function, in an additive manner.[4-6]

The benzodiazepines are lipid soluble and are absorbed well from the GI tract with good distribution to the brain. They are metabolized primarily in the liver. Their CNS active metabolites extend their duration of action. The benzodiazepines work by enhancing, facilitating or potentiating the action of the inhibitory neurotransmitter GABA. They serve to increase the frequency of GABA-mediated chloride ion channel opening.

Benzodiazepines are metabolized primarily in the liver via several different microsomal enzyme systems.[6] Many products of their metabolism are active. Since many of the active metabolites have been marketed as therapeutic agents, it is difficult to ascertain which drug was ingested solely upon the basis of the results of analysis. Current drug therapy will assist in determining the source of a particular compound. The detection of a particular agent is determined partly by whether its metabolism yields active metabolites. Excretion of benzodiazepines is predominantly in the urine. Depending upon the particular benzodiazepine, the urine may contain parent compounds, N-dealkylation and oxidative (hydroxylation) metabolism products, and/or glucuronide conjugates.

1.1.1 At the analyst's discretion, the samples may be extracted with or without derivatizing, and there are two options in the method for derivatization.

1.2 References

1.2.1 Valentine, J.L., Middleton, R., Sparks, C. Identification of Urinary Benzodiazepines and their Metabolites: Comparison of Automated HPLC and GC-MS after Immunoassay Screening of Clinical Specimens. J. Anal. Tox. 20:416-424, 1996.

1.2.2 Levine, B. Central Nervous System Depressants. pp. 191-197. in: Principles of Forensic Toxicology. Levine, B. ed., AACC, 1999.

1.2.3 Huang, W. and Moody, D.E. Immunoassay Detection of Benzodiazepines and Benzodiazepine Metabolites in Blood. J. Anal. Tox. 19:333-342, 1995.

1.2.4 Drug Facts and Comparisons Prescription Drug Information Binder, Updated monthly.

1.2.5 Julien, R.M. A Primer of Drug Action. pp. 95-107, W.H. Freeman and Company: NewYork, 1998.

1.2.6 Hobbs, W.R., Rall, T.W. and Verdoorn, T.A. Hypnotics and Sedatives.. pp. 362-373. in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Hardman, J.G. ed., McGraw-Hill, 1996.

2.0 Scope

2.1 This extraction method is a modification of the method developed by Valentine, et al., for the extraction of benzodiazepines from urine.[1] This method has also been found to be effective in the extraction of opiates and various other drugs such as Zolpidem, Buprenorphine and Trazodone.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Tube Rocker

3.1.2 Laboratory oven or water bath

3.1.3 Laboratory Centrifuge capable of 3500 rpm

3.1.4 Fixed and adjustable volume single channel air displacement pipettes, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.1.5 Dry bath

3.1.6 Evaporative Concentrator equipped with nitrogen tank

3.1.7 Threaded-end 16X100 Round Bottom Glass Tubes and/or 16X114 Tapered Bottom Glass Centrifuge Tubes

3.1.8 Screw caps for 16mm O.D. Tubes

3.1.9 pH Indicator Strips

3.1.10 ALS Vials

3.1.11 ALS Vial Microinserts

3.1.12 Gas Chromatograph equipped with a mass selective detector and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drug compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl).

3.2 Reagents

Refer to Toxicology AM#23 for solution preparation instructions.

3.2.1 BG100 b-Glucuronidase Solution (Kura Biotec)

3.2.2 2M Acetate buffer, pH 4.8

3.2.3 50mM Sodium Bicarbonate, pH 11

3.2.4 Chloroform/Isopropanol 9:1 (Each Certified ACS Grade)

3.2.5 Ethyl Acetate (Certified ACS Grade)

3.2.6 Silylating agent: MSFTA or BSTFA with 1% TMCS

3.3 Qualitative Reference Material and Controls

3.3.1 Positive Control

3.3.1.1 Positive Control can be prepared with single or multi-component working solutions and/or obtained commercially.

3.3.2 Non-extracted Reference Material

3.3.2.1 Run necessary reference material as indicated by examination of GC/MSD data. Reference material mixes may be employed.

3.3.3 Non-extracted Derivatized Reference Material

3.3.3.1 Prepare derivatized reference material as necessary based on current drug therapy and examination of GC/MSD data.

3.3.3.2 Add ~3-5uL (1mg/mL) stock reference material to labeled centrifuge tube. Derivatize as described in 4.1.2.2

3.3.4 Internal Standard

3.3.4.1 Stock Solutions

1 mg/mL Prazepam

3.3.4.2 Working Internal Standard Solution [10ng/uL]

3.3.4.2.1 Add 100uL Prazepam stock solution to 10mL volumetric ball flask. QS with methanol.

NOTE: Working Solution is stable for 1 year (or at the date of the earliest expiring Stock solution) when stored under refrigeration or in a freezer.

3.3.5 Required Extracted Controls (inclusive of all options for method)

3.3.5.1 Extracted Negative Control

NOTE: Commercially obtained or in-house urine verified to be negative for drugs of interest.

3.3.5.2 Positive Control

Positive Control can be prepared with single or multi-component working solutions and/or obtained commercially. The positive control must have at least one compound in it that is appropriate for demonstrating that each chosen extraction and derivatization (if applicable) is working. The preferred concentration range of this control is 300–600ng/mL. Examples of preparation and dilutions for an in-house control are given at the end of this method in Section 6.0: Comments.

3.3.5.3 Positive and Negative Glucuronide Controls.

3.3.5.3.1 These controls may be obtained commercially or prepared in-house by spiking negative urine. The same negative urine must be used to prepare both the positive and negative glucuronide controls. Oxazepam glucuronide, lorazepam glucuronide or morphine glucuronide may be used for these controls and must be at a minimum concentration of 375ng/mL. Examples of preparation are given in Appendix III. Derivatization will be required for the controls prepared in-house, even if there are no case samples requiring derivatization. The positive and negative glucuronide controls are used to demonstrate the glucuronidase was effective (if these samples are run in conjunction with samples that are not derivatized or are derivatized using option 2, one set of glucuronide controls can be used for both.)

4.0 Procedure

4.1 Extraction Procedure

NOTE: This method provides three options for the analyst. The method describes the preparation of an ethyl acetate extract and two options for a derivatized extract. Based on compounds of interest, both extracts need not be prepared and only the corresponding control material must be included.

4.1.1 Non-Derivatized Ethyl Acetate Samples

4.1.1.1 Casework and Control samples

4.1.1.1.1 Transfer 6mL casework samples and controls to screw top extraction tubes.

4.1.1.2 Internal Standard Addition

4.1.1.2.1 To each prepared sample, add 300uL of internal standard (10ng/uL working solution) or 3uL of 1mg/mL stock solution. Vortex to mix.

4.1.1.3 Sample Hydrolysis

4.1.1.3.1 Add 200uL 2M acetate buffer to each tube, vortex.

4.1.1.3.2 To all but the glucuronidase negative, add 150uL BG100 b-Glucuronidase Solution. Cap and vortex gently to mix.

4.1.1.3.3 Place all tubes in 60°C laboratory oven or water bath for one hour.

4.1.1.3.4 Allow samples to cool before proceeding with solvent extraction.

4.1.1.4 Extraction

4.1.1.4.1 Add 2mL 50mM sodium bicarbonate to each sample tube. Vortex.

4.1.1.4.2 Check pH. If necessary, adjust pH to approximately pH 9 with 1N NaOH or KOH.

4.1.1.4.3 Add 4mL of chloroform/isopropanol {9:1}.

4.1.1.4.4 Rock for approximately 15 minutes.

4.1.1.4.5 Centrifuge (~3300-3500 for about 10-15 minutes)

4.1.1.4.6 Transfer lower organic phase from tube into labeled tapered bottom tube.

4.1.1.4.7 Evaporate solvent to dryness under a gentle stream of nitrogen at ~37°C. Proceed to 4.1.1.5 if not derivatizing, or to 4.1.2 if derivatizing before running on GC/MS.

4.1.1.5 Reconstitution with Ethyl Acetate (No Derivatization)

4.1.1.5.1 Add 50uL ethyl acetate. Vortex.

4.1.1.5.2 Transfer extract to labeled ALS vial with micro-insert.

4.1.1.6 Preparation for Analysis Run

4.1.1.6.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.1.1.6.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

4.1.2 Derivatization of Samples: Option 1

4.1.2.1 Follow Ethyl acetate sample preparation steps included in 4.1.1.2 - 4.1.1.4

4.1.2.2 Derivatization

4.1.2.2.1 To the tapered-bottom tubes add 20uL ethyl acetate and 30uL of silylating agent.

4.1.2.2.2 Cap tubes. Vortex.

4.1.2.2.3 Heat tube for 15 minutes in 75°C drybath.

4.1.2.2.4 Remove from heat and allow samples to cool. Transfer derivative to labeled ALS vial with micro-insert.

4.1.2.3 Preparation for Analysis Run

4.1.2.3.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.1.2.3.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

4.1.3 Derivatization of Samples: Option 2

(Complete ethyl acetate sample Extraction Procedure (4.1.1.2-4.1.1.5), run samples on GC/MS, then complete derivatization).

4.1.3.1 Derivatization

4.1.3.1.1 Once the ethyl acetate extracts have run on the GC/MS, add 20uL of silylating agent to remaining extract in the autosampler insert and vortex.

4.1.3.1.2 Heat vials for 15 minutes at 75°C.

4.1.3.1.3 Remove from heat and allow to cool.

4.1.3.2 Preparation for Analysis Run

4.1.3.2.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.1.3.2.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

4.1.3.3 GC-MSD Analysis Parameters

4.1.3.3.1 Refer to instrument method for current analysis parameters.

4.1.3.3.1.1 Instrument Run Parameters

- Inlet at 280 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 80 degrees Celsius, hold 2.5 minutes
- Ramp 25 degrees Celsius
- Final temperature: 300 degrees Celsius, hold at least 7.5 minutes.

4.1.3.3.2 Current analysis method must be stored centrally as a hard or electronic copy.

4.1.4 Detection and Identification Criteria

4.1.4.1 The presence of a drug compound is indicated if the retention time for the sample, versus applicable reference material, does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

4.2 Methods Limitations and Application to Other Analytes

4.2.1 This method is applicable to other compounds, which require an enzymatic hydrolysis to liberate the compound of interest. Both the ethyl acetate extraction and the TMS derivative can be applied toward the identification of these compounds.

4.2.2 This method has proven useful in the identification of opiate class compounds such as codeine, morphine, 6-monoacetylmorphine, hydrocodone, and buprenorphine.

4.2.3 Care should be taken when estazolam is detected, particularly in samples containing alprazolam and/or alpha-hydroxyalprazolam. For samples containing alprazolam and/or alpha-hydroxyalprazolam, estazolam must be detected in both underivatized and derivatized GC/MSD data to be considered reportable. Estazolam shall not be reported if alprazolam and/or alpha-hydroxyalprazolam are detectable in the sample and derivatized estazolam is not detected.

4.3 Quality Assurance Requirements

4.3.1 Refer to Toxicology AM #19 for additional quality assurance and Toxicology AM #21 for reference material authentication requirements.

4.4 Analysis Documentation

4.4.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.4.2 Original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.4.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Reference Material

5.1.1 Benzodiazepine Positive Control

- 5.1.1.1 Stock Solutions
  - 1 mg/mL single component or 250µg/mL multi-component benzodiazepine class reference solutions.
- 5.1.1.2 Direct spiking
  - Spike negative urine with 3µL of each single component stock or 12µL of 250µg/mL stock solution.
- 5.1.1.3 Working Control Solution (10ng/µL)
  - Add 100µL each 1mg/mL or 400µL 250µg/mL Stock Solution to ≅ 9mL MeOH in 10mL ball flask. Bring up to volume with MeOH. Solution is stable for one-year when stored under refrigeration or in a freezer. (Note: A minimum of two benzodiazepine class compounds must be included in the control solution. One of these compounds must form a TMS derivative.)

5.1.2 Conjugated Controls

5.1.2.1 Commercially obtained conjugated control (example: Bio-Rad C3)

5.1.2.2 Stock Solution

5.1.2.2.1 1 mg/mL Oxazepam Glucuronide or 100µg/mL Lorazepam Glucuronide or 100µg/mL Morphine Glucuronide

5.1.2.2.1 Direct spiking

5.1.2.2.1.1 Spike negative urine with 3µL of 1mg/mL stock solution or 30µL of 100µg/mL stock solution.

5.1.2.2.2 Working Glucuronide Solution (10ng/µL)

5.1.2.2.2.1 Add 100µL of 1mg/mL Stock Solution (or 1mL 100µg/mL Stock Solution) to 10mL MeOH. Solution is stable for one year when stored under refrigeration or in a freezer.

5.1.3 Internal Standard

5.1.3.1 Stock Solution

5.1.3.1.1 *1mg/mL* Prazepam.

5.1.3.1.1 Direct addition

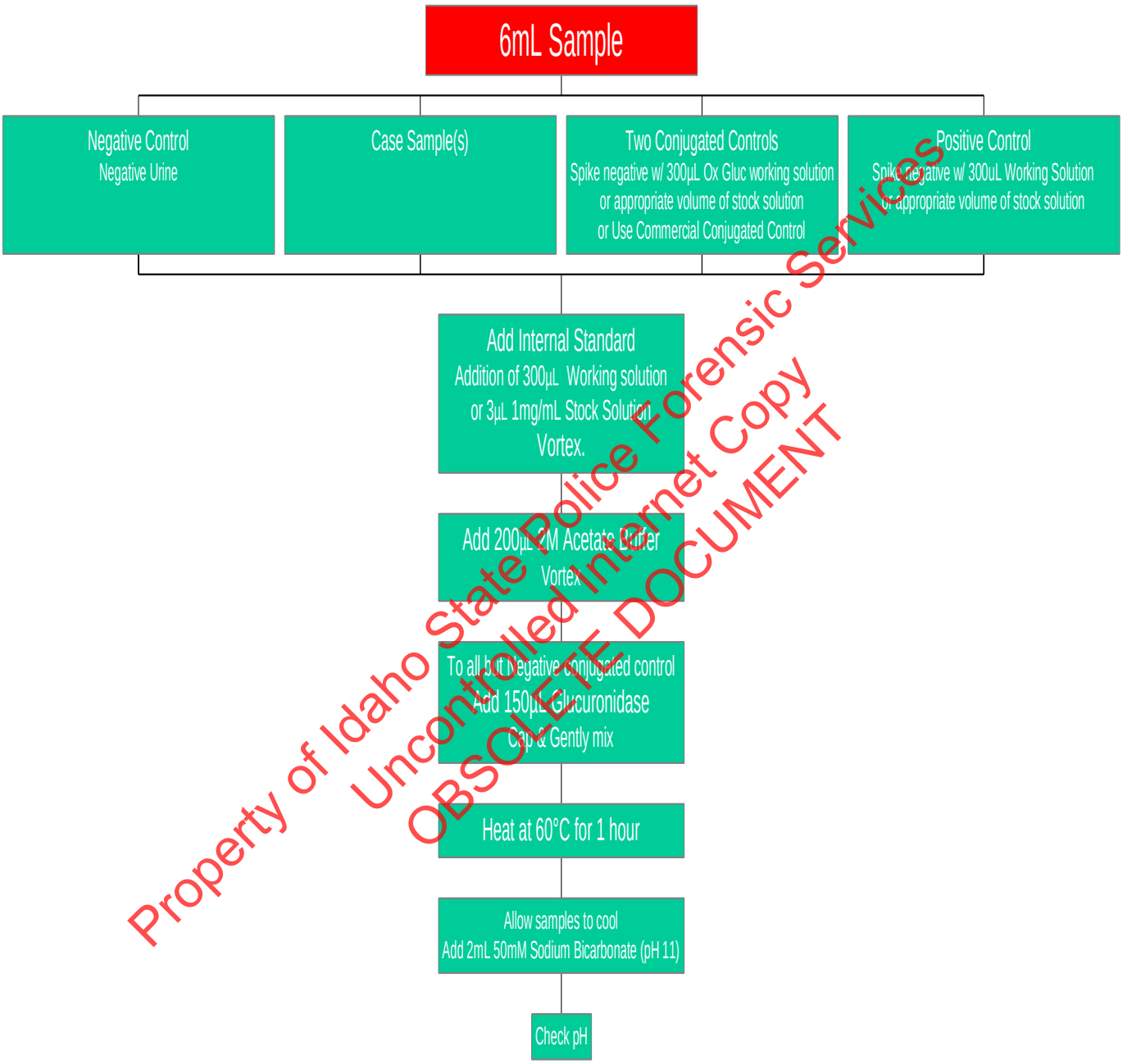
5.1.3.1.1.1 Add 3µL of 1mg/mL stock solution to each 6mL urine sample and control.

5.1.3.1.2 Working Internal Standard Solution

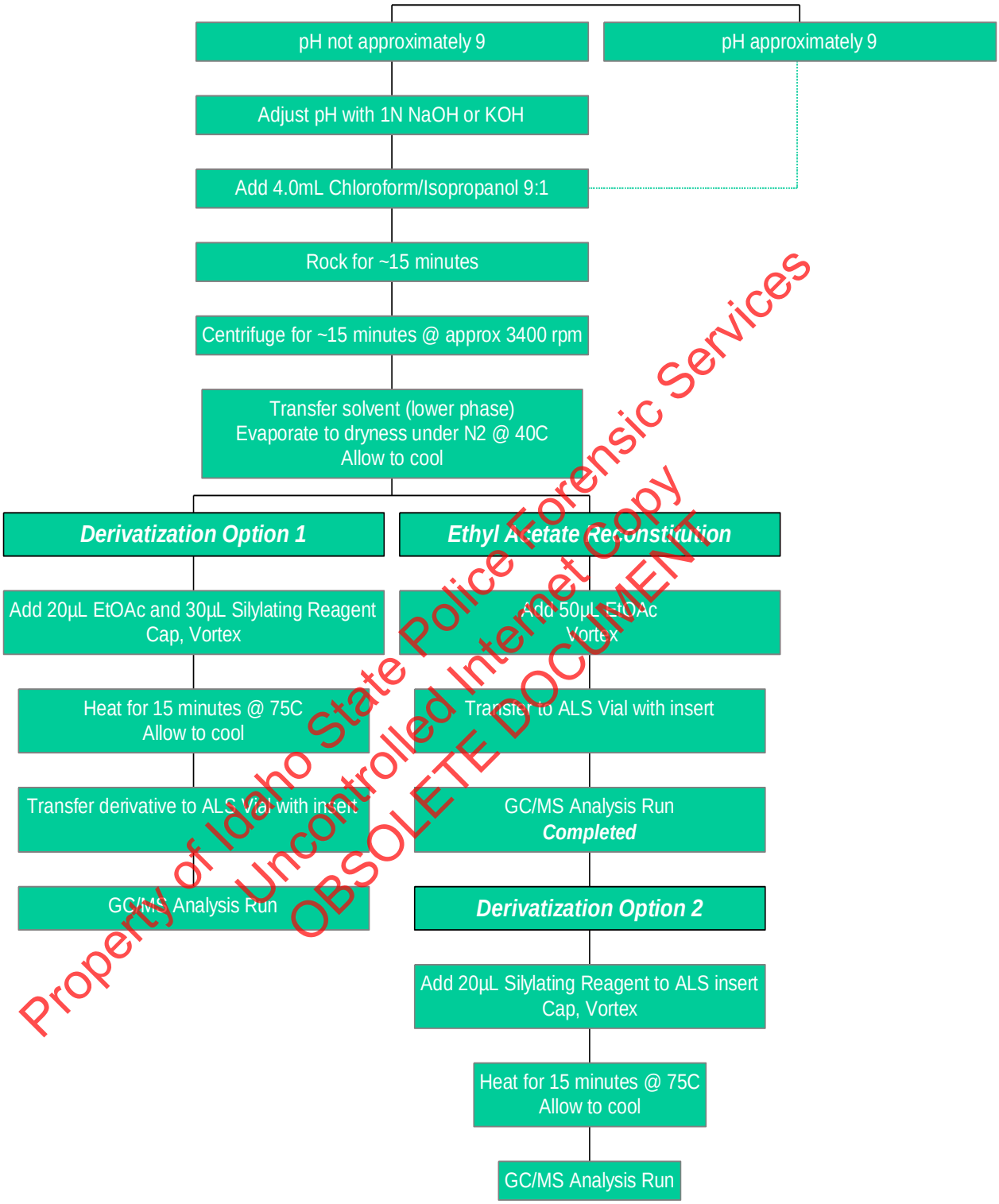
5.1.3.1.2.1 Add 100µL Stock Solution to ≅ 9mL MeOH in 10mL ball flask. Bring up to volume with MeOH. Solution is stable for one year when stored under refrigeration or in a freezer.

5.1.4 Comments: This method has instructions for the preparation of both an underivatized and a TMS-derivatized extract. The analyst has the option of preparing either or both of these extracts.

5.2 Glucuronide Cleaving:



5.3 Extraction Procedure:



6.0 Comments

6.1 Positive Control Sample

Use the lot of negative urine that will be used to prepare negative control.

- Pipette 6mL of commercially obtained positive control or prepare positive control as described below.
- Use the table below as a guide for spiked positive control preparation. Prepare control for a concentration between 300 to 600ng/mL.

Spiking Solution Concentration	Amount to Add (µL)	Resulting ng/mL
100ng/µL (100µg/mL)	30	500
10ng/µL	300	500
1mg/mL	3	500
250ng/µL (250µg/mL)	12	500

Toxicology AM #6: Screening for Gamma-Hydroxybutyrate (GHB) in Urine

1.0 Background/References

1.1 Background:

GHB occurs naturally in minute quantities as a result of the metabolism of the inhibitory neurotransmitter, GABA.[6,8] The drug GHB is a potent tranquilizer that was previously used as an anesthetic and as a treatment for major depressive illnesses, alcohol withdrawal, and narcolepsy.[1-8] Legitimate use is limited due to the side effects associated with the drug. GHB can produce visual disturbances, nausea, vomiting, drowsiness, dizziness, severe respiratory depression, unconsciousness and involuntary muscle spasms.[1,5,6] Overdoses can require emergency medical treatment including intensive care due to the respiratory depression, bradycardia and coma.[5,6,8]

The use of GHB has been increasing since the 1980s and currently is popular among body builders, teenagers and on the club/dance scene. Body builders use the drug due to its alleged role as a growth hormone releasing agent to enhance muscle growth.[5,6,8] GHB has not been proven to possess any anabolic effects.[6] GHB has gained notoriety as a date rape drug due to its ability to produce short-term amnesia and decreased inhibitions.[1-8] The FDA banned the use of GHB in 1990 except for FDA approved physician supervised protocols.[6,8]

A dose of GHB is anywhere from 2.5 to 4.0 grams in approximately 1 teaspoon ("capful") of liquid. The taste of GHB has been described as salty or soapy, the odor is said to be mothball-like.[5,6] Due to the short half-life of GHB (0.3 to 1.0 hours[5, 27] 5 minutes[7]) the person will re-administer every 45 minutes to 1 hour. The onset of effects is 15 to 60 minutes. The effects of the drug will be detectable during a DRE exam for 4 to 6 hours. GHB is classified as a central nervous system depressant. The observed effects include horizontal and vertical nystagmus, lack of convergence, body tremors, and slowed breathing. The person will also exhibit a lowered pulse, blood pressure, and body temperature. In addition, the muscle tone will exhibit flaccidity and the person may be in a trance-like state, the pupils will exhibit a lack of reaction to light.[5] Lower doses will promote an agitated, combative state however their pulse and other vitals will be depressed.[5,6] Combining GHB with alcohol plus a stimulant or marihuana allows the user to remain conscious during use. This allows them to experience the euphoric "buzz" that is the desired effect of its abuse. The desired effect is a state of relaxation and tranquility, a pleasant drowsiness, mild euphoria, hallucinations and a release of inhibitions. Combining GHB with alcohol or other central nervous system depressant will provide an additive depressant effect.[5] GHB is detectable in blood for up to eight hours and in urine for up to 12 hours.[5,6,7] Peak plasma levels are obtained in 20 to 45 minutes.[7] Peak urine GHB concentrations on the order of 1100 ug/mL are observed within the first four hours after a 100 mg/kg oral dose.[3,7]

GHB is manufactured by reacting butyrolactone with sodium hydroxide in an aqueous solution in the presence of a low molecular weight alcohol (methanol, ethanol).[5,6] The average yield of GHB is 70%. If the yield of the manufacturing process is low, there will be significant amounts of the lactone present in the product. Abuse of this compound will continue due to its relatively simple synthesis and the availability and low cost of starting materials.[5] Users can ingest Gamma butyral lactone (GBL), a degreaser and floor stripper, and it is converted in vivo into GHB. GBL is therefore diverted from legitimate sources to either be taken directly or converted to GHB.

This compound has been referred to by a variety of names as listed in the table below.

Street Names	***Marketing Names***
"G"	*Revitalize*
"G" caps	*Rejuvenate*
Liquid X	*Renewtrient*
Soap	*Revivarant*
Easy Lay	*Blue Nitro*
Georgia Home Boy	*Thunder Nectar*
G-riffic	*Rest-Eze*
Grievous Bodily Harm	*Energy Drink*

There are thousands of documented GHB overdoses with numerous deaths. The danger in GHB use stems from its steep dose-response curve. A small increase in dose can create a dramatic difference in adverse effects. This makes the potential of overdosing with GHB very high. This is compounded by the fact that GHB affects users so differently. A dose that one individual uses could adversely affect another, thus word of mouth is a poor determiner of how much of the substance to use.

1.2 References:

1.2.1 Frommhold, S. Gamma-Hydroxybutyrate (GHB): What's "the Scoop?" in: Toxi-News 16(1), 1997; pp. 3-8.

1.2.2 Ferrare, S.D., Tedeschi, L. Frison, G., et. al, Therapeutic gamma-hydroxybutyric acid monitoring in plasma and urine by gas chromatography-mass spectrometry. J Pharm. Biomed Anal, 1993, 11(6):483-487.

1.2.3 Stephens, B. and Baselt, R.C. Driving Under the Influence of GHB? J Anal Tox, 1994, 18:357-358.

1.2.4 ElSohly, M.A. and Salamone, S.J. Prevalence of Drugs used in Cases of Alleged Sexual Assault J Anal Tox, 1999, 23:141-146.

1.2.4 Chase, D.A., Gamma Hydroxy Butyrate, "GHB", Presentation at IACP DRE Conference, Minnesota, 1999.

1.2.5 Good, P.J., Selected Abuse Substances, Presentation at IACP DRE Conference, Portland, Oregon, 1998.

1.2.6 Determination of Gamma-Hydroxybutyric Acid by GC/MS, Dade County Medical Examiner's Toxicology Lab SOP.

1.2.7 Microgram, Volume XXXI, No. 3, March 1998.

1.2.8 Couper, F.J. and Logan, B.K. Determination of gamma-Hydroxybutyrate (GHB) in Biological Specimens by Gas Chromatograph-Mass Spectrometry, J Anal Tox, 2000, 24:1-7.

1.2.9 SOFT/AAFS Forensic Toxicology Laboratory Guidelines, 1997.

1.2.10 Goldberger, B.A., Huestis, M.A., Wilkins, D.G. Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography/Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories, Forensic Sci Rev, 1997, 9(2):59-79.

2.0 Scope

2.1 This method provides an efficient qualitative analysis option for the liquid-liquid extraction of urine samples suspected of containing Gamma-Hydroxybutyrate/ gamma-Hydroxybutyric Acid (GHB). GHB is isolated from an acidified solution into methylene chloride and heptane with zinc chloride to facilitate the extraction process. The extraction is achieved with a De-Tox B extraction tube. The extraction is followed by the creation of a di-TMS derivative of GHB. The derivative is analyzed by full scan GC/MS in EI mode. This method may not provide adequate sensitivity for weaker concentrations of GHB. This method should only be used for driving under the influence of drugs (DUID) situations where GHB is suspected or for screening purposes for drug-facilitated sexual assault (DFSA). Presently DFSA case urine that indicates a positive result by this method must be outsourced for quantitative confirmation.

3.0 Equipment/Reagents

3.1 Equipment

3.1.1 Tube Rocker (Fisher Scientific or equivalent)

3.1.2 Evaporative Concentrator equipped with a nitrogen tank

3.1.3 Laboratory Centrifuge capable of 3000 rpm

3.1.4 Drybath

3.1.5 Fixed and/or adjustable volume pipettes, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.1.6 16X100mm centrifuge tubes

3.1.7 {Optional} 16X144mm tapered-end centrifuge tubes

3.1.8 Caps for 16mm O.D. Tubes

3.1.9 Automated Liquid Sampler (ALS) vials

3.1.10 ALS vial microinserts

3.1.11 Gas Chromatograph equipped with a mass selective detector and a nonpolar capillary column with a phase composition capable of efficiently separating GHB and its analogs in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl)

3.2 Reagents

3.2.1 De-Tox B Tubes (or equivalent Toxi-B tube)

3.2.2 MSFTA or BSTFA with 1% TMCS

3.3 Reference Material

3.3.1 Stock Solution

- 1mg/mL (1000ng/uL) GHB

3.3.2 GHB Spiked Urine Positive Controls (One required)

- Use the same lot of negative urine to prepare both the negative and spiked positive controls.

3.3.2.1 Preparation of 100ug/mL Positive Control

3.3.2.1.1 Add 450uL of GHB 1mg/mL stock to 4050uL negative urine. Vortex.

3.3.2.2 Preparation of 200ug/mL Positive Control

3.3.2.2.1 Add 900uL of GHB 1mg/mL stock to 3600uL negative urine. Vortex.

3.3.3 Non-Extracted GHB Reference Material [10ug]

3.3.3.1 Place 10uL of GHB stock into tube.

3.3.4 Negative Control

3.3.4.1 Negative Urine can be commercially obtained or in-house urine verified to be negative for drugs of interest.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 Label De-Tox B Tubes for positive controls, negative control and case samples.

4.1.1.2 Label tubes and ALS vials for positive controls, negative control, case samples and non-extracted reference material.

4.1.2 Extraction Procedure

4.1.2.1 Extract 4.5 mL of specimen, negative and spiked positive control in De-Tox B Tube (acidic extraction @pH=4.5). Less than 4.5mL may be used if sample is limited.

4.1.2.2 Rock De-Tox tube for ~10 minutes.

4.1.2.3 Centrifuge TOXI-TUBE at ~2500-3000 rpm for ~10 minutes.

4.1.2.4 Transfer solvent from De-Tox Tube into tapered-end centrifuge tube.

4.1.2.5 Evaporate solvent to approximately 50uL with nitrogen at ~40°C. Non-extracted reference material must be evaporated to dryness.

4.1.3 Derivatization Procedure

4.1.3.1 Add 40uL MSFTA or BSTFA with 1%TMCS to evaporated extracted samples, spiked control(s) and non-extracted reference material. Cap tube. Vortex.

4.1.3.2 Place tube in 60°C drybath for 15 minutes.

4.1.3.3 Remove tube from drybath. Allow sample to cool.

4.1.3.4 Transfer derivative to labeled GC/MS ALS vial for analysis.

4.1.4 Gas Chromatography/Mass Spectrometry (GC/MS) Parameters

4.1.4.1 Key parameters are specified below. Parameters not specified are at the discretion of the analyst and should be optimized for both the gas chromatographic and mass spectral characteristics of an instrument. Refer to GC/MS method for current parameters for analysis. Each laboratory shall maintain a centrally stored current method printout or electronic copy.

4.1.4.2 Instrument Run Parameters

- Inlet at 250 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 65 degrees Celsius, hold 1 minute
- Ramp 15 degrees Celsius
- Final temperature: 300 degrees Celsius, hold 2 minutes.

4.1.4.3 Acquisition Mode

4.1.4.3.1 Sample must be analyzed in full scan acquisition mode.

4.1.5 Detection and Identification Criteria

4.1.5.1 Chromatographic Criteria

- The retention time of the analyte should fall within ±2% of the retention time exhibited by GHB reference material and control(s).

4.1.5.2 Mass Spectral Criteria

- Full scan mass spectral data should be compared against within-run GHB reference material and control(s). No significant differences should be apparent.

4.2 Quality Assurance

4.2.1 General

4.2.1.1 Refer to Toxicology AM #19 for additional quality assurance and Toxicology AM #21 for reference material authentication requirements.

4.2.2 Per Analysis Run Control and Reference Material Requirement

- Each run should include, at a minimum, a 100ug/mL and/or 200ug/mL GHB control, a negative control and a non-extracted GHB reference material.

4.3 Analysis Documentation

4.3.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.3.2 Original data for controls will be compiled for each analysis run and stored centrally in the laboratory where the analysis was performed until archiving.

4.3.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Reference Material

5.1.1 GHB Positive Control

- Stock Solutions

  1 mg/mL single component Gamma Hydroyxbutyric Acid (GHB) reference solution.

- Direct spiking

  Minimum of one positive control:

  - Spike one sample containing 4050uL negative urine with 450uL of GHB 1mg/mL.
  - Spike one sample containing 3600uL negative urine with 900uL of GHB 1mg/mL.

5.1.2 Non-extracted GHB Reference Material

- Stock Solution

  1 mg/mL GHB

- Direct spiking

  Spike tapered bottom tube with 10uL, dry and Derivatize.

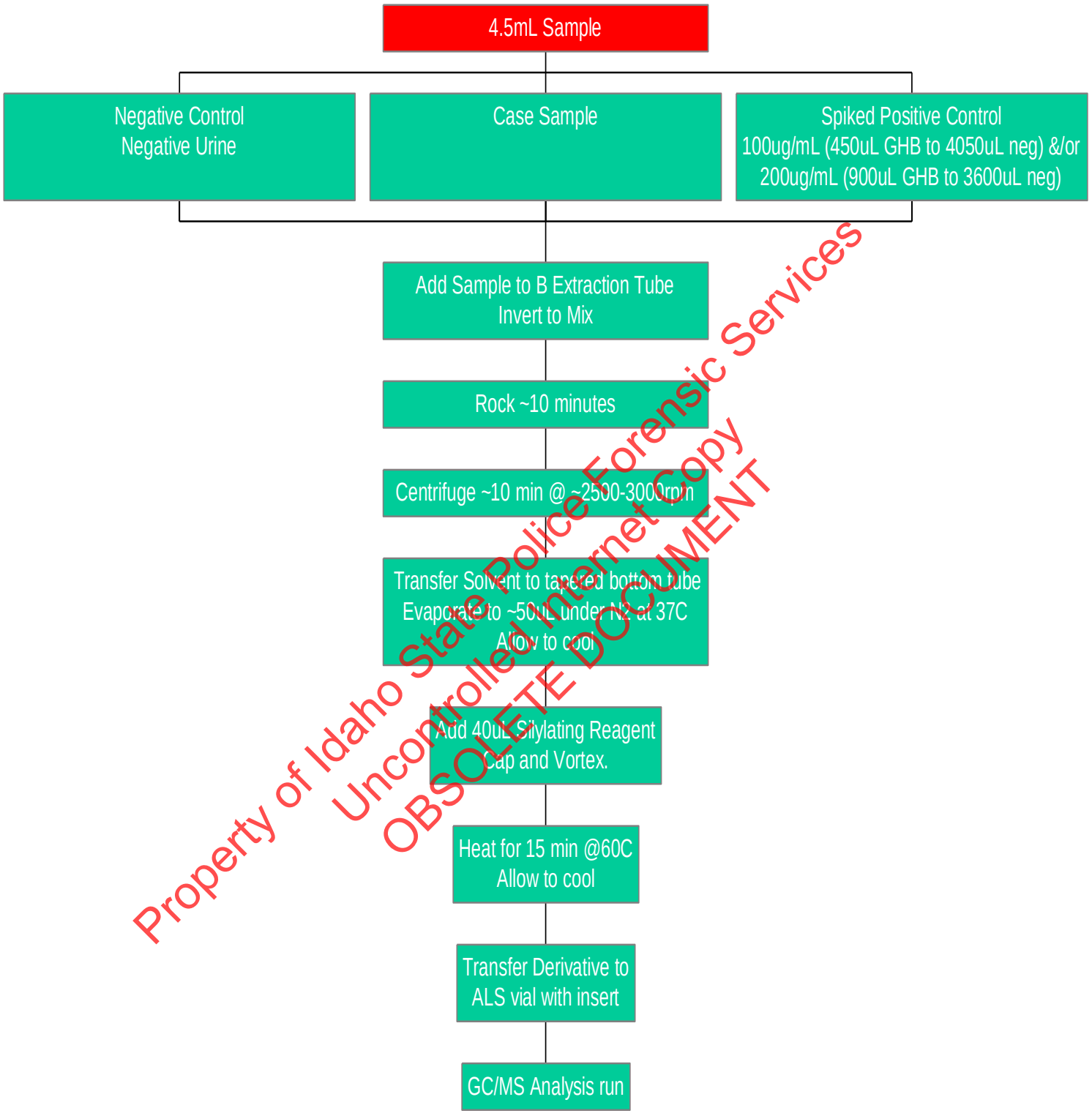
5.2 Reagents

De-Tox B tubes

5.3 Qualitative Controls

Spiked Positive controls

Negative Urine



Toxicology AM #7: Enzyme-Linked Immunosorbent Assay (ELISA) Screening for Drugs of Abuse

1.0 Background/References

1.1 Background

ELISA is an acronym for enzyme-linked immunosorbent assay. An ELISA is an enzyme immunoassay (EIA) in which one reactant is immobilized on a solid phase and the signal generator is an enzyme. The enzyme delivers a signal to indicate to what extent a particular antigen-antibody reaction has occurred. This reaction takes place inside of a polystyrene microtiter plate well. Horseradish peroxidase is an enzyme commonly employed as a signal generator. The small size of HRP, the ease with which small conjugates can be produced through oxidation of its carbohydrate moieties to reactive aldehyde, its rapid kinetics, and reasonable price, make it popular in ELISA.

For the qualitative determination of a specific drug, or class of drugs in blood and urine this method utilized competitive micro-plate immunoassay. Each of the assays requires a predilution step for samples, controls and calibrators. This brings the analytes into an acceptable range for optimum performance of the bound microplate antibodies. Dilutions are either performed manually, with an air displacement pipette or a dilutor, or using the automated dilution capability of the instrument. Samples, calibrators or controls are added to individual wells of the microplate along with the conjugate, which is the drug or hapten labeled with the enzyme horseradish peroxidase (HRP).

There is a competition between the free drug in the matrix sample (blood or urine) and drug bound to enzyme (conjugate) for antibody (sheep or rabbit) fixed on the well. The wells are washed with DI water, the substrate (3,3´,5,5´-tetramethylbenzidine (TMB) with peroxide (H2O2) is added, and a color is produced. HRP catalyzes H2O2 oxidation of the substrate by transferring one electron from the TMB to the peroxide to yield a blue colored product. The reaction is stopped when 2.0N sulfuric acid is added to the well. This acidic environment provides the necessary conditions for the loss of an additional electron to produce the final yellow color. The acidic environment also serves to inactivate the enzymatic activity of the HRP. The resulting absorbance at 450nm is inversely proportional to the amount of drug present in the sample or standard. Consequently, a more intense yellow color results in a greater absorbance and indicates a lower concentration of drug in the sample.

The Micro-Plate EIA utilizes two matrix matched calibrators, one containing no drug (negative calibrator) and one at the concentration corresponding to the accepted cut-off for the drug (cut-off calibrator). In addition, negative and positive controls are used to assess the performance of the kit. An automated microplate analyzer is used for processing on the microplates. The analyzer automatically dispenses samples and all reagents required for ELISA testing. In addition, the analyzer allows for the programming of incubation times and wash steps.

1.2 References

1.2.1 Butler, J.E. Enzyme-Linked Immunosorbent Assay. pp. 759-803 In: "Immunochemistry". Van Oss, C.J.; van Regenmortel, M.H.V., eds., Marcel Dekker, inc., New York, NY: 1994.

1.2.2 DSX Automated ELISA System User's Manual, REV.04-20-05, 2005.

1.2.3 OraSure Technologies DSX Startup Procedure and Setup of a Worklist.

1.2.4 OraSure Technologies Package Inserts for Serum Microplate EIA.

2.0 Scope

2.1 This Micro-plate assay is applied for the qualitative screening for drugs-of-abuse in blood or urine specimens. Appropriate dilutions are made for application to the screening of blood and urine. The outcome of the assay is intended as only a preliminary analytical test result. The presence of a particular drug compound must be verified through analysis with a confirmatory instrument such as a gas chromatograph equipped with a mass selective detector.

As indicated in the table below, each assay in use has an established administrative threshold or cut-off. For this reason, a negative result does not indicate that no drug is present, only that the concentration is less than the administrative cut-off. For this reason there may be situations where confirmation of an analyte may be pursued even if a negative result is indicated for the compound or a class of compounds in question. The exceptions are discussed in section 4.10.2.

2.2

Assay	Compound Used for Calibrator	Urine Cut-off	Blood Cut-off
Benzodiazepine	Oxazepam	300ng/mL	100ng/mL
Cannabinoid	(-)11nor-9-carboxy-Δ-9-THC	50ng/mL	15ng/mL
Cocaine Metabolite	Benzoylecgonine	300ng/mL	50ng/mL
Methadone	Methadone	300ng/mL	50ng/mL
Methamphetamine	S(+)Methamphetamine	500ng/mL	50ng/mL
Opiate	Morphine	300ng/mL	50ng/mL
Barbiturates	Secobarbital	100ng/mL	100ng/mL

3.0 Equipment/Reagents

3.1 Equipment

3.1.1 Sample Diluting Supplies

3.1.1.1 Air-displacement pipettes and appropriate tips.

3.1.1.2 Repeater Pipette and appropriate tips.

3.1.1.3 Automatic Dilutor equipped with appropriate syringes.

3.1.1.4 Screening instrument programmed for automatic dilutions.

3.1.2 Plasticware

3.1.2.1 *2.0*mL control vials with caps

3.1.2.2 *25*mL reagent tubes with caps

3.1.2.3 Disposable 13x75 polypropylene tubes

3.1.2.4 Disposable transfer pipettes

3.1.2.5 Deep-well strips/plates for automated dilution

3.1.3 DSX Automated ELISA Instrument

3.1.4 Tube Rocker

3.1.5 Vortex Mixer

3.2 Reagents

3.2.1 Forensic Specimen Diluent (Remember to check expiration date prior to use.)

3.2.1.1 Assay Kits:

- Micro-plates coated with anti-drug antibodies.
- Enzyme conjugate for specific drug/drug class.
- TMB Substrate reagent (universal).
- 2N H_2SO_4 Stopping reagent (universal).

3.2.2 Processing of New Assay Supplies

3.2.2.1 When a new kit is opened, note the expiration date of all components listed on each assay's specification sheet.

3.2.2.1.1 The manufacturer's kit expiration date may be based on a component not used for the analysis of blood or urine. Only the expiration of the conjugate and plate involve the use of the assay kit since the expiration date of the substrate and stop always far exceeds the expiration date of the conjugate and the plates.

3.2.2.2 Cocaine Assay Conjugate Preparation

3.2.2.2.1 Using a pipette, to the vial containing Benzoylecgonine Lypophilized Stock Enzyme Conjugate, add 2mL Conjugate Diluent.

3.2.2.2.2 Place vial on tube rocker for a minimum of 10 minutes.

3.2.2.2.3 Using a pipette, add the volume of reconstituted Stock Enzyme Conjugate listed on the kit package insert to appropriate volume of Conjugate Diluent. Prepare only necessary volume of conjugate. (Note: The Stock Enzyme Conjugate is lot specific.)

3.2.2.2.4 Gently mix Conjugate Diluent bottle on tube rocker for a minimum of 1 minute.

3.2.2.2.5 Prior to use, allow bottle to equilibrate for a minimum of 30 minutes at room temperature or overnight under refrigeration.

3.3 Reference Material

NOTE: For both urine and blood, the following calibrators and controls must be included in each analysis run. In-house calibrators and controls should be prepared each month, at a minimum. If the method is not utilized during a month, no calibrators or controls need be prepared. If a screen fails, preparation of fresh calibrators/controls is a good troubleshooting step.

3.3.1 Urine

3.3.1.1 Stock Reference Material Solutions

3.3.1.1.1 Obtain Methamphetamine (1mg/mL), Benzoylecgonine (1mg/mL), Methadone (1mg/mL), Morphine (1mg/mL), 11-nor-9-Carboxy-D9-THC (100µg/mL), Oxazepam (1mg/mL), and Secobarbital (1mg/mL) drug reference material from an appropriate vendor. Different vendors should be used to make up the Calibrator and Control Working Solutions whenever possible. If this is not possible, different lots from the same vendor may be used. Certificates of analysis must be stored centrally.

3.3.1.2 Working Standard Solution

3.3.1.2.1 Add ~9mL methanol to 10mL volumetric flask. Add 50uL each of methamphetamine and c-THC stock. Add 30uL each of benzoylecgonine, methadone, morphine and oxazepam stock. Add 10µL of secobarbital stock. QS to 10mL with methanol. Record lot numbers of stock reference material on reagent log.

(Solution is stable for up to 12 months when stored at 4 ℃.)

3.3.1.3 Urine Calibrators:

NOTE: May be commercially obtained or prepared in-house as described in Section 6 of this method.

- Negative Urine Calibrator
- Cut-off Urine Calibrator

3.3.1.4 Platform Urine Controls

NOTE: May be commercially obtained or prepared in-house as described in Section 6 of this method.

- Negative Urine Control
- Positive Urine Control

3.3.1.5 Sample Rack Urine Controls

3.3.1.5.1 Negative Urine Control

- Negative Urine

3.3.1.5.2 Positive Urine Controls

3.3.1.5.2.1 *25%* Above Cut-off Positive Control

- *Each run must include a control at 25% above the cut-off calibrator. To prepare, add 125uL working standard solution to 1mL of negative urine.*

3.3.1.5.2.2 Drugs-of-Abuse Positive Control

- *Each run must include a commercially obtained drugs-of-abuse urine control. The concentration of analytes may be varied.*

3.3.1.5.3 Urine Cannabinoid Cut-off Control

3.3.1.5.3.1 The last sample run on a urine cannabinoid screen will be an aliquot of the cut-off calibrator. This sample will be used for evaluating results.

3.3.2 Blood

3.3.2.1 Stock Reference Material Solutions

3.3.2.1.1 Obtain Methamphetamine (1mg/mL), Benzoylecgonine (1mg/mL), Methadone (1mg/mL), Morphine (1mg/mL), 11-nor-9-Carboxy-D9-THC (100µg/mL), Oxazepam (1mg/mL), and Secobarbital (1mg/mL) drug reference material from an appropriate vendor. Different vendors should be used to make up the Calibrator and Control Working Solutions whenever possible. If this is not possible, different lots from the same vendor may be used. Certificates of analysis must be stored centrally.

3.3.2.2 Working Standard Solution

3.3.2.2.1 Add ~9mL methanol to 10mL volumetric flask. Add 50uL each of stock methamphetamine, benzoylecgonine, methadone and morphine. Add 100uL each of stock oxazepam and secobarbital. Add 150uL of stock c-THC. QS to 10mL with methanol. Record lot numbers of stock reference material on reagent log.

(Solution is stable for up to 12 months when stored at 4 ℃.)

3.3.2.3 Blood Calibrators

NOTE: May be commercially obtained or prepared in-house as described in Section 5 of this Method.

- Negative Blood Calibrator
- Cut-off Blood Calibrator

3.3.2.4 Platform Blood Controls

NOTE: May be commercially obtained or prepared in-house as described in in Section 5 of this Method.

- Negative Blood
- Positive Blood Control

3.3.2.5 Sample Rack Blood Controls

3.3.2.5.1 Negative Blood

- Negative Whole Blood

3.3.2.5.2 Positive Whole Blood Controls

- 25% Above Cut-off Positive Control

  Each run must include a control at 25% above the cut-off calibrator. To prepare, add 25uL working standard solution to 2mL of negative blood.

- Drugs-of-Abuse Positive Control

  Each run must include an in house or commercially obtained drugs-of-abuse blood control. The concentration of analytes may be varied.

4.0 Procedure

4.1 General Rules of Operation for analyzer

4.1.1 Care should be taken to not impede the arm action, as it can be both detrimental to the instrument and dangerous to the user.

4.1.2 It is recommended, but not required, that the instrument run with the top down.

4.2 Initial Processing of Samples

4.2.1 Place laboratory number on each sample container.

4.2.2 Urine samples with an unusually high turbidity may be centrifuged prior to analysis.

4.2.3 Urine samples cannot contain the preservative sodium azide, as this will destroy the conjugate.

4.2.4 If particulates or clots are visible in a blood sample, it may be homogenized with tissue grinder or clarified by centrifuging.

4.3 Sample Dilution

4.3.1 Appropriate Dilution for Each Assay

4.2.3.1.1 Urine

Dilution	Assays
1 in 60	Methamphetamine, Cannabinoids, Opiates, Benzodiazepines, Cocaine Metabolite and Methadone
1 in 5	Barbiturates

4.2.3.1.2 Blood

Dilution	Assays
1 in 5	Methamphetamine, Cannabinoids, Opiates, Benzodiazepines, Cocaine Metabolite, Barbiturates and Methadone

4.3.2 Suggested Volumes for Manual Dilution

4.2.3.2.1 Dilution 1:60

Sample	Forensic Diluent
91µL / 1:5 dilution	1000µL
15µL	885µL

4.2.3.2.2 Dilution 1:5

Sample	Forensic Diluent
160µL	640µL
200µL	800µL
250µL	1000µL

4.4 Preliminary Tasks

4.4.1 Fill wash bottles with deionized water; make sure waste container is empty.

4.4.2 Check pipette tip tray supply. If necessary, fill with appropriate disposable tips.

4.4.3 Check printer paper supply. Refill if necessary.

4.4.4 Remove samples and reagents from refrigerator for a minimum of one hour prior to starting analysis.

4.4.5 Prepare samples for analysis. Dilute as indicated under section 4.3.1.

4.5 Session Preparation

4.5.1 Turn on instrument, then turn on computer.

4.5.2 Double click on the Revelations icon on the desktop. Select "Connect to DSX", then "Do it." The instrument should perform a self-test at this time; make sure ALL TESTS PASSED before proceeding.

4.5.3 From Open Session Screen, select appropriate template from buttons. Then "Add assays using a new batch of samples" and click "OK."

4.5.4 If there is no appropriate template, select "New," then "Worklist." Select "New Plate" and choose the type of assay to run.

4.5.5 Select "Open." Note: If multiple assays are to be tested on one plate, select the first assay then hold the Ctrl button to select the others. If using the Template button, it is still possible to run multiple assays on the same plate by left-clicking the assay immediately to the right of the first plate and selecting "Combined with assay on right." The Cannabinoid assay must be the only assay run on its plate, and the run is limited to 25 blood case samples (run in duplicate) or 52 urine case samples.

4.5.6 Click and drag under the "Test" column to select the assays for the samples. Enter ID numbers for the samples, then click "OK." NOTE: Blood samples are to be run in duplicate. The assays are programmed to do this automatically, depending on the matrix selected.

4.5.7 Select the "PLAY" button (green triangle). The timeline will now be built and displayed. NOTE: To view the timeline, click the down (↓) arrow button; to hide the timeline, click the up (↑) arrow button. To view the *entire* timeline, click the minus (marked as a dash) button.

4.5.8 When ready to load the reagents and consumables, click the "FAST FORWARD" button (two green triangles followed by a green vertical line).

4.5.9 Follow directions on the screen to load all reagents and consumables. Note: when asked to enter "Lot Name," enter the name of the assay (e.g. METHAMPHETAMINE, or METH), followed by the kit lot number and kit expiration date. Click green check mark when done.

4.5.10 Load all samples into sample caddy, as instructed on the screen, then click the green check mark.

4.5.11 Once plate tray is ejected, remove the plate holder, insert assay plate (with the correct number of strips in place) into the plate holder, and place the plate holder back into the tray, as instructed on the screen. Change "Plate Identifier" to the assay being run for that plate, followed by the date (e.g. COC011212). Click green check mark to proceed to next plate. Repeat for each plate being loaded, making sure to change the plate identifier for each.

4.5.12 Load all calibrator and control fluids as instructed on the screen. Make sure to load at least the minimum volumes required for each, as the software instructs. Click green check mark.

4.5.13 Make sure the washer bottle(s) contain sufficient DI water. Click green check mark.

4.5.14 Make sure sufficient sample and reagent tips are loaded. Click green check mark.

4.5.15 Make sure there is sufficient space for waste in tip disposal and waste disposal containers. Click "OK." Note: once you click "OK" the instrument cycle will start, so make sure all objects are out of the way of the arm.

4.5.16 Once all runs are complete, click the "STOP" button (red square). This will cause the plate tray to be ejected. Once the plate tray is ejected, click on the "EJECT" button (a square with an arrow pointing down), to fully eject the tray. Once the tray is fully ejected, the plate holders will be unlocked and easily removed.

4.5.17 Remove the plate holders, dispose of the strips, then return the plate holders to the tray. Push the "IN" button (a square with an arrow pointing up) to make the tray return to its "IN" position.

4.6 Obtaining Results - Post-Run

4.6.1 To display the results, click the blue "UP" arrow. Select the pop-up window displaying assay results.

4.7 Obtaining Archived Results

4.7.1 Results from past runs can be viewed by clicking "Open," then clicking on the "Plates" folder. From here, you can select the appropriate document.

4.7.2 Text files of all runs are also stored. To access the text files, click "Open," then click on the "Text" folder. From here, you can select the appropriate document. These documents can be then be opened in Excel.

4.8 Post-run Tasks

4.8.1 General Clean-up

4.8.1.1 Return conjugates, stop, and diluent reservoirs to refrigerator.

4.8.1.2 Dispose of used calibrators, controls, samples, and tips into appropriate biohazard waste container.

4.8.2 Instrument Shut-down

4.8.2.1 Close out of Revelations, and then shut down computer.

4.8.2.2 Power off the instrument.

4.9 Run Acceptance Criteria

4.9.1 Calibrators and Controls

4.9.1.1 The individual replicates for the absorbance of the *negative calibrator* must be less than 1.2 times the mean *negative calibrator* and greater than 0.8 times the mean *negative calibrator*.

4.9.1.2 The individual replicates of the *cut-off calibrator* must be less than 1.2 times the mean *cut-off calibrator* and greater than 0.8 times the mean *cut-off calibrator*.

4.9.1.3 The mean absorbance for the *negative urine or blood* is greater than the absorbance for the *negative calibrator*.

4.9.1.4 The absorbance for the *negative calibrator* is greater than the mean absorbance for the *cut-off calibrator*.

4.9.1.5 The mean absorbance for the *cut-off calibrator* is greater than the absorbance for the *positive control*.

4.9.1.6 The quality control equations (((KIT1-KIT2)/KIT1)*100)>20 and (((NEG-CUT)/NEG)*100)>30 are default settings allowing tracking of the OraSure immunoassay kits' performance through calculation of percent displacement. These criteria are not critical; they are used for tracking the performance of the specific assay. The default settings may be adjusted as appropriate.

4.9.2 Urine and Blood Sample Controls

4.9.2.1 Matrix matched urine and blood controls, analyzed as samples, should indicate an appropriate positive or negative response.

4.9.2.2 For purposes of this criterion, a significantly depressed absorbance qualifies as a positive result.

4.10 Interpretation of Results

4.10.1 Positive Result

4.10.1.1 A positive result for a sample is indicated by an absorbance less than or equal to the *Cut-off Calibrator*. Urine samples that have an absorbance less than or equal to the cut-off control run at the end of the assay will be considered positive. Blood samples are run in duplicate; if the coefficient of variation is over 10% for a sample, the analyst will use the value with the lowest absorbance for evaluating the sample result.

4.10.2 Depressed Absorbance

4.10.2.1 At the discretion of the analyst, confirmatory techniques may be applied to samples that exhibit depressed absorbance. For purposes of this exception, a depressed absorbance is a response which fall between the value observed for the cut-off calibrator and the negative urine or blood. If data for confirmatory techniques supports the presence of an analyte, it may be reported as present. In addition, samples with compounds that have low cross reactivity may be confirmed and reported with a negative screen result.

4.10.2.2 Blood samples are run in duplicate; if the coefficient of variation is greater than 10% for a sample, the analyst will use the value with the lowest absorbance for evaluating the sample result.

4.10.3 Negative Result

A negative result for a sample is indicated by an absorbance that is greater than the Cut-off Calibrator. Special considerations may apply as outlined above (see 4.10.2).

4.11 Distribution of Assay Information

4.11.1 Assay results are to be recorded in the LIMS system.

4.11.2 The original data printouts will be stored centrally in the laboratory where the analysis was performed.

4.11.3 A copy of assay results may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of the control and standard printouts may be prepared from the centrally stored document.

4.12 DSX Maintenance

4.12.1 When-in-use Daily Maintenance/Tasks

4.12.1.1 Run an optional "Wash" cycle before doing sample runs. It is also recommended that the "Wash" cycle be run after sample runs as well. If any wash ports are clogged, perform the periodic maintenance procedure for unclogging the ports.

4.12.2 Periodic Maintenance

Maintenance to promote cleanliness of the instrument (including washing and air drying the wash and waste tanks and cleaning the tip tray) can be done on an as-needed basis and does not need to be documented. Please note that the tip tray should be disinfected with 70% isopropanol, and not bleach, as bleach can interfere with the assays.

4.12.2.1 Six-Month Maintenance (Approximately every 6 months- does not need to be documented)

4.12.2.1.1 Replace the dispense tubing as needed.

4.12.2.1.2 Replace the aspiration tubing as needed.

4.12.2.2 Preventive Observations (Does not need to be documented)

4.12.2.2.1 During wash cycle, check the washer dispensing apparatus to ensure each port is correctly dispensing water. This may be most effectively accomplished when aided by a flashlight.

4.12.2.2.2 After wash cycle, check plate to make sure most water is removed from wells and remaining water is uniform among the wells.

5.0 Comments

5.1 Appendix I:

5.1.1 Urine Control In-House Preparation Guide

5.1.1.1 Negative Urine Calibrator (1/2x)

5.1.1.1.1 Direct Spiking Preparation

- Add 50µL working standard solution to 1mL negative urine.

5.1.1.1.2 Serial Dilution Preparation

- Prepare 200% of cut-off solution as described in Appendix II, Table 1; then dilute as described in Appendix II, Table 2.

5.1.1.2 Cut-off Urine Calibrator (x)

5.1.1.2.1 Direct Spiking Preparation

- Add 100µL working standard solution to 1mL negative urine.

5.1.1.2.2 Serial Dilution Preparation

- Prepare 200% of cut-off solution as described in Appendix II, Table 1; then dilute as described in Appendix II, Table 2.

5.1.1.3 Positive Urine Calibrator (2x)

- Add 200µL working standard solution to 1mL negative urine.

5.1.1.4 Negative Urine Control (NEG)

- Negative urine only

5.1.1.5 Positive Urine Control (POS)

- Undiluted commercial control (Biorad C3, S3 or similar)

5.1.1.6 Urine Cannabinoid Cut-off Control

- Undiluted aliquot of calibrator run for cannabinoid assay only

5.1.2 Blood Control In-House Preparation Guide

5.1.2.1 Negative Blood Calibrator (1/2x)

5.1.2.1.1 Direct Spiking Preparation

- Add 5µL working standard solution to 1mL negative blood.

5.1.2.1.2 Serial Dilution Preparation

- Prepare 200% of cut-off solution as described in Appendix II, Table 3; then dilute as described in Appendix II, Table 4.

5.1.2.2 Cut-off Blood Calibrator (1x)

5.1.2.2.1 Direct Spiking Preparation

- Add 10µL working standard solution to 1mL negative blood.

5.1.2.2.2 Serial Dilution Preparation

- Prepare 200% of cut-off solution as described in Appendix II, Table 3; then dilute as described in Appendix II, Table 4.

5.1.2.3 Negative Blood (NEG)

5.1.2.3.1 Negative Whole Blood.

5.1.2.4 Positive Blood Control (2x)

5.1.2.4.1 Add 20µL working standard solution to 1mL negative blood.

5.2 Appendix II:

5.2.1 Table 1: Urine Direct Spiking:

To 1mL of negative urine, add working standard solution as indicated below.

Control Type	% of cutoff	Working Standard Solution
Negative	50%	50µL
Positive	200%	200µL

5.2.2 Table 2: Urine Serial Dilution:

Control Type	% of cutoff	Urine Stock	Urine Dilution
Cut-off	100%	500µL of 200%	500µL
Negative	50%	250µL of 200%	750µL

5.2.3 Table 3: Whole Blood Direct Spiking:

To 1mL of negative blood, add working standard solution as indicated below.

Control Type	% of cutoff	Working Standard Solution
Negative	50%	5µL
Positive	200%	20µL

5.2.4 Table 4: Whole Blood Serial Dilution:

Control Type	% of cutoff	Whole Blood Stock	Whole Blood Dilution
Negative	50%	250µL of 200%	750µL
Cut-off	100%	500µL of 200%	500µL

Toxicology AM #8: Basic and Neutrals Drugs in Blood

1.0 Background/References

1.1 Background

This method is a general blood extraction procedure for a variety of commonly encountered neutral and basic drugs along with their metabolites. The method is based upon the principle of liquid/liquid extraction. Positive controls are spiked for a resulting concentration of 200ng/mL or 500ng/mL of drugs of interest. The sample pH is adjusted with a pH 9.2 borate buffer and extracted with n-butyl chloride. An optional back extraction procedure removes most frequently encountered interfering substances. Two internal standards are used to monitor extraction efficiency and chromatographic performance. Gas chromatography in conjunction with full scan mass spectrometry is used to confirm the presence of basic and neutral analytes of interest.

1.2 References

1.2.1 Procedure for Basic Drug Analysis, Courtesy of Jim Hutchison, Montana Department of Justice, Forensic Services Division, 2005.

1.2.2 Procedure for Back Extraction, Courtesy of Jim Hutchison, Montana Department of Justice, Forensic Services Division, 2006.

1.2.3 Strong Bases Extractions - Screening SOP, Courtesy of Dr. Graham Jones, Office of the Chief Medical Examiner, Edmonton, Canada, 2003.

1.2.4 Jones, G., Postmortem Toxicology. pp. 98-102, in: Clarke's Analysis of Drugs and Poisons, 3rd Edition, Moffat, A.C, Osselton, M.D. and Widdop, B. eds., Pharmaceutical Press, 2004.

1.2.5 Hearn, W.L. and Walls, H.C., Strategies for Postmortem Toxicology Investigation. pp. 937-939. in: Drug Abuse Handbook, S.B. Karch, ed., CRC Press, Boca Raton, FL, 1998.

2.0 Scope

2.1 This method is a general blood extraction procedure for a variety of commonly encountered neutral and basic drugs, along with their metabolites. This method has also been shown capable of extracting some acidic drugs (e.g. butalbital) if sufficient quantity is present in the sample. This method does not efficiently extract some basic compounds, such as morphine and hydromorphone, due to pKa/pH considerations. The method allows for the analyst to use either methanol or ethyl acetate as a reconstitution solvent. Some benzodiazepines are more efficiently extracted using ethyl acetate than methanol as a reconstitution solvent. In addition, samples reconstituted in ethyl acetate can also be derivatized to increase sensitivity and detection of some compounds. Some drugs are more efficiently extracted using methanol as a reconstitution solvent. It is at the analyst's discretion to determine which solvent to use.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Tube Rocker

3.1.2 Vortex Mixer

3.1.3 Evaporative concentrator equipped with nitrogen tank.

3.1.4 Laboratory centrifuge capable of 3400rpm

3.1.5 Fixed and adjustable single channel pipettes, and appropriate tips

3.1.6 16X100mm round bottom glass screw-top tubes

3.1.7 Screw Cap for 16mm O.D. tubes

3.1.8 GC/MS Automated Liquid Sampler (ALS) vials

3.1.9 GC/MS Vial Microinsert

3.1.10 Gas Chromatograph equipped with a Mass Selective Detector

3.1.11 5%-Diphenyl-95%-Dimethyl-siloxane copolymer capillary GC column, 12.5 to 30M.

3.2 Reagents

Refer to Toxicology AM #23 for Solution Preparation instructions.

3.2.1 Methanol (ACS Grade)

3.2.2 n-Butyl chloride (ACS Grade)

3.2.3 Borate Buffer (pH 9.2)

3.2.4 Deionized/Distilled (DI) Water

3.2.5 1% Hydrochloric Acid in Methanol

3.2.6 100mM Hydrochloric Acid

3.2.7 Ethyl Acetate (ACS Grade)

3.2.8 Silylating Agent (select from):

- BSTFA/1% TMCS
- MSTFA

3.3 Reference Materials

3.3.1 Positive Control

NOTE: Positive Control can be prepared with the working solution described below and/or obtained commercially.

3.3.1.1 Positive Control Stock Solution

3.3.1.1.1 Obtain 1mg/mL stock drug standard solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.3.1.2 Positive Control Working Solution

3.3.1.2.1 Add the designated volume of stock solution to 10mL methanol. A minimum of four compounds must be used.

NOTE: Solution is stable for 6-months when stored at room temperature or 12-months when stored under refrigeration or in a freezer. Re-make solution if deterioration is noted.

Stock Solution (1.0mg/mL)	Volume (µL)
Amitriptyline	20
Caffeine	20
Codeine	20
Diphenhydramine	20
Lidocaine	20
Meperidine	20
Methadone	20
Nicotine	20
PCP	20
Trazodone	50
Methamphetamine	20
Phentermine	20

3.3.2 Internal Standard

3.3.2.1 Stock Solutions

- 1 mg/mL Benzphetamine
- 1mg/mL Papaverine

3.3.2.2 Working Internal Standard Solution [10ng/uL]

3.3.2.2.1 Add 100uL Benzphetamine and Papaverine stock solutions to 10mL volumetric ball flask. QS with methanol.

NOTE: Solution is stable for three months when stored at room temperature.

3.3.3 Negative Control

- Negative Whole Blood

3.3.4 Reference Standards

3.3.4.1 Stock Standard Solution

3.3.4.1.1 Obtain 1mg/mL stock drug standard solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.3.4.2 Working Drug Standard Solution

3.3.4.2.1 Add the designated volume found in 3.3.1.2.1 of stock solution to 10mL methanol.

4.0 Procedure

4.1 Extracted Procedure

4.1.1 Initial set-up

4.1.1.1 For each control and case sample, label two screw-top extraction tubes and one ALS vial with micro-insert.

4.1.2 Positive Control Samples

NOTE: The same lot of negative blood must be used for the preparation of both negative and positive spiked controls.

4.1.2.1 Prepare control sample by adding 200uL mixed working control solution to 2mL negative whole blood or pipette a 2mL sample of commercially-obtained whole blood positive control.

4.1.2.2 When the optional back extraction is used, prepare an additional positive control to parallel the back extraction process.

4.1.2.3 When some samples in a batch are going to be reconstituted with methanol and others with ethyl acetate, a separate positive control must be run for each reconstitution solvent.

4.1.3 Casework Samples

4.1.3.1 Transfer 2mL casework sample to screw top extraction tube.

4.1.4 Negative Control Sample

4.1.4.1 Transfer 2mL negative whole blood to extraction tube. If some samples are going to be run with ethyl acetate and some with methanol as a reconstitution solvent, a negative control must be prepared and run for each reconstitution solvent. If the optional back extraction is used, prepare an additional negative control to parallel the back extraction process.

4.1.5 Internal Standard

4.1.5.1 Add 20uL of internal standard mixture and vortex. If benzodiazepines are of interest, 3uL of Prazepam (1mg/mL) may also be spiked into the sample.

NOTE: If the analyst has reason to suspect the sample may contain one or more of these internal standard compounds, it is permissible for the analyst to eliminate one of the internal standard compounds (e.g. papaverine) or replace one of the internal standard compounds with an appropriate alternative (prazepam may be used). Clear notation of the replacement, along with justification, must be included in the analysis notes. If the analyst prefers to use a different internal standard than those listed here, s/he must confer with the toxicology discipline leader in the selection of said internal standard.

4.1.5.2 Allow sample to stand 10 minutes.

4.1.6 Initial Extraction

4.1.6.1 Add 2mL borate buffer. Vortex.

4.1.6.2 Pipet 4mL n-butyl chloride into each tube, cap.

4.1.6.3 Place tube on rocker for 10 minutes.

4.1.6.4 Centrifuge for 10 minutes at 3400 rpm.

4.1.6.5 Transfer the n-butyl chloride layer (upper) to second screw-top tube.

4.1.6.6 Add 50uL 1% HCl in Methanol.

4.1.6.7 Evaporate to dryness under N2 at ≤37°C.

4.1.6.7.1 If no clean-up proceed to 4.1.8.

4.1.7 Optional Sample Clean-up

4.1.7.1 Reconstitute with 50uL 100mM HCl.

4.1.7.2 Add 1mL of n-Butyl Chloride. Vortex.

4.1.7.3 Rock for 5 minutes.

4.1.7.4 Centrifuge for 5 minutes at 3400 rpm.

4.1.7.5 Discard upper n-Butyl Chloride layer.

4.1.7.6 Add 2mL of pH 9.2 borate solution. Vortex

4.1.7.7 Add 4 mL of n-Butyl Chloride.

4.1.7.8 Rock for 5 minutes.

4.1.7.9 Centrifuge for 5 minutes at 3400 rpm.

4.1.7.10 Transfer upper n-Butyl Chloride layer into screw-top tube.

4.1.7.11 Evaporate to just dryness under N_2 at ≤45°C.

4.1.8 Reconstitution

4.1.8.1 Add 50uL Methanol or Ethyl Acetate to the residue, vortex.

4.1.8.2 Transfer extract to labeled ALS vial with micro-insert.

4.1.9 Preparation for Analysis Run

4.1.9.1 Into Sequence log table, enter the case samples, blanks and controls.

4.1.9.2 Load samples, standards, blank and controls into the quadrant rack as noted in the sequence table.

4.1.10 Optional Derivatization *(samples that are extracted with ethyl acetate may be derivatized at the analyst's discretion)*

4.1.10.1 After ethyl acetate extraction samples have run on the GC-MSD, add 20uL silylating agent to the sample. In addition to the case samples, the extracted positive and negative controls must also be derivatized and run.

4.1.10.2 Heat at about 75°C for ~15 minutes.

4.1.10.3 Allow samples to cool, run on GC-MSD.

4.1.11 GC-MSD Acquisition Parameters

4.1.11.1 Refer to instrument method printout for current acquisition parameters.

4.1.11.1.1 Instrument Run Parameters (Short Run)

- Inlet at 250 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 70 degrees Celsius, hold 2 minutes
- Ramp 35 degrees Celsius to 290 degrees Celsius, hold 3 minutes
- Ramp 40 degrees Celsius to 300 degrees Celsius, hold at least 6 minutes
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.1.11.1.2 Instrument Run Parameters (Longer Run)

- Inlet at 250 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 90 degrees Celsius, hold 4 minutes
- Ramp 15 degrees Celsius
- Final temperature: 290 degrees Celsius, hold at least 10.667 minutes
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.1.11.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.1.12 GC-MSD Qualitative Detection and Identification Criteria

4.1.12.1 For the identification of compounds not included in positive control, analyze appropriate non-extracted reference materials.

4.1.12.2 The presence of a drug compound is indicated if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data. NOTE: early eluting drugs, as well as drugs known to have similar retention times and mass spectral fragmentation patterns (e.g. phentermine and methamphetamine), may not differ from the retention time of the applicable reference material by more than ±0.1 minutes.

4.2 Quality Assurance Requirements

4.2.1 General

4.2.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

4.2.1.2 Refer to Toxicology AM #17, AM #19 and AM #21 for quality assurance and reference material authentication requirements.

4.3 Analysis Documentation

4.3.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.3.2 A packet containing original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.3.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Reference Material

5.1.1 Mixed Standard Solution

- Add designated volume of each drug compound (minimum of 4).
- Total volume is 10mL.
- Solution is stable for12-months under refrigeration or in a freezer.
- Record Preparation on standard log.

Stock Solution	Volume (µL) 1mg/mL Stock
Amitriptyline	20
Caffeine	20
Codeine	20
Diphenhydramine	20
Lidocaine	20
Meperidine	20
Methadone	20
Methamphetamine	20
Nicotine	20
PCP	20
Trazodone	50

5.1.2 Internal Standard Mix

5.1.2.1 Working Internal Standard Solution [10ng/mL]

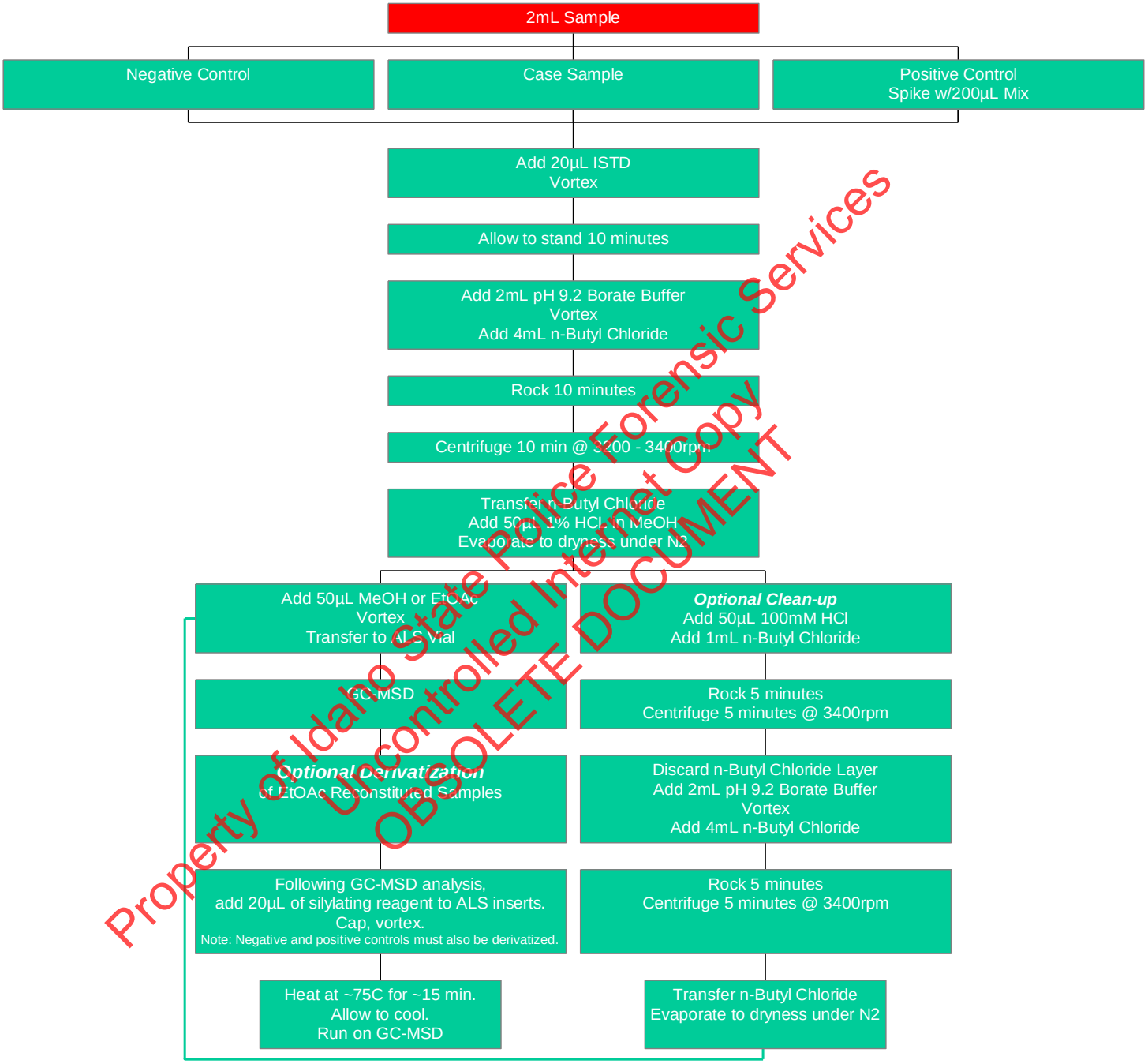
Benzphetamine and Papaverine Stock solutions {1mg/mL (1µg/µL)}

- Add 100µL Benzphetamine and Papaverine stock solutions to 10mL volumetric ball flask. QS with methanol.

Solution is stable for three months when stored at room temperature.

5.1.3 Comments: This method allows for analyst discretion when determining whether to reconstitute the sample with methanol or ethyl acetate. When reconstituted with ethyl acetate, instructions for the preparation of both an underivatized and a TMS-derivatized extract are provided. The analyst has the option of preparing either or both of these extracts.

5.2 Extraction Work Instructions:



Toxicology AM #9: Acidic and Neutral Drugs in Blood

1.0 Background/References

1.1 This method is a general liquid-liquid procedure to extract a variety of commonly encountered acidic and neutral drugs along with their metabolites from blood. Drug compounds are extracted from blood by a liquid-liquid extraction process. Blood pH is adjusted with saturated ammonium chloride followed by extraction with ethyl acetate. After evaporation and a hexane wash, the final extract is subjected to analysis by GC-MSD. Two internal standards are used to monitor extraction efficiency and chromatographic performance. This extraction yields excellent recovery of most acidic and neutral drugs, and can generally be accomplished in under one hour. The extraction is designed to yield fewer and lower levels of endogenous compounds that can interfere with drug detection.

1.2 Reference

1.2.1 Procedure for Acid/Neutral Drug Analysis, Courtesy of Jim Hutchison, Montana Department of Justice, Forensic Services Division, 2005.

1.2.2 Foerster, E.H., Dempsey, J., and Garriott, J.D., A Gas Chromatography Screening Procedure for Acid and Neutral Drugs in Blood. J Anal Tox, 3:87-91, 1979.

1.2.3 Jones, G., Postmortem Toxicology. pp. 98-102, in: Clarke's Analysis of Drugs and Poisons, 3rd Edition, Moffat, A.C, Osselton, M.D. and Widdop, B., eds., Pharmaceutical Press, 2004.

1.2.4 Hearn, W.L. and Walls, H.C. Strategies for Postmortem Toxicology Investigation. pp. 937-939. In: "Drug Abuse Handbook" S.B. Karch, ed., CRC Press, Boca Raton, FL: 1998.

2.0 Scope

2.1 This method is used to extract a variety of commonly encountered acidic and neutral drugs, and their metabolites, from blood. Some basic drug compounds (e.g. diazepam), if in sufficient quantity in the sample, have been successfully extracted with this method.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Tube rocker

3.1.2 Evaporative concentrator equipped with nitrogen tank.

3.1.3 Vortex mixer

3.1.4 Laboratory centrifuge capable of 3400rpm.

3.1.5 Fixed and adjustable volume single channel pipettes, and appropriate tips

3.1.6 16X100mm round bottom glass screw-top tubes

3.1.7 Screw Cap for 16mm O.D. tubes

3.1.8 GC/MS Automated Liquid Sample (ALS) vials

3.1.9 GC/MS Vial Microinsert

3.1.10 Gas Chromatograph equipped with a Mass Selective Detector

3.1.11 5%-Diphenyl-95%-Dimethyl-siloxane copolymer capillary GC column, 12.5 to 30M.

3.2 Reagents

Refer to Toxicology AM #23 for Solution Preparation instructions.

3.2.1 Methanol (Certified ACS Grade)

3.2.2 Hexane (Certified ACS Grade)

3.2.3 Ethyl acetate (Certified ACS Grade)

3.2.4 Acetonitrile (Certified ACS Grade)

3.2.5 2N Sodium Hydroxide

3.2.6 Saturated Ammonium Chloride

3.3 Quality Assurance Material

3.3.1 Positive Control Working Solution

Note: Positive Control can be prepared with the working solution described below and/or obtained commercially.

3.3.1.1 Obtain 1mg/mL stock drug standard solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.3.1.2 Add the designated volume of stock solution to 10mL methanol. A minimum of four compounds must be used.

Stock Solution	Volume (µL)
Acetaminophen	20
Butalbital	20
Carbamazepine	20
Carisoprodol	20
Meprobamate	20
Phenobarbital	20
Secobarbital	20

3.3.1.3 Solution is stable for 6 months when stored at room temperature. Remake solution if deterioration is noted.

3.3.2 Internal Standard Mix

3.3.2.1 Stock Solution

3.3.2.1.1- 1mg/mL Aprobarbital

3.3.2.2 Working Internal Standard Solution [50ng/uL]

3.3.2.2.1 Add 500uL Aprobarbital stock solution to 10mL volumetric ball flask. QS with methanol.

3.3.2.2.2 Solution is stable for 3 months when stored at room temperature.

3.3.3 Negative Control

3.3.3.1 Negative Whole Blood

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 Label ALS vials, with micro-inserts, and two extraction tubes for each control and case sample.

4.1.2 Positive Control

Note: The same lot of negative blood must be used for the preparation of both negative and positive spiked controls.

4.1.2.1 Prepare or use commercially obtained positive control. To prepare: add 100uL mixed working control solution to 1mL negative whole blood.

4.1.2.2 Positive control may be run in duplicate.

4.1.3 Negative Control

4.1.3.1 Transfer 1mL negative whole blood to screw top extraction tube.

4.1.4 Casework Samples

4.1.4.1 Transfer 1mL casework samples to screw top extraction tube.

4.1.4.2 To all samples, including controls, add 20uL of internal standard working solution.

4.1.4.3 Vortex.

4.1.4.4 Add 1mL saturated ammonium chloride and vortex.

4.1.5 Extraction

4.1.5.1 Pipet 4mL ethyl acetate into each tube, cap.

4.1.5.2 Place tube on rocker for 10 minutes.

4.1.5.3 Centrifuge for 10 minutes at 3400rpm.

4.1.5.4 Transfer the ethyl acetate (top) layer to second tube.

4.1.5.5 *If necessary, this is potential overnight stopping point. Tubes must be capped and refrigerated.*

4.1.6 Evaporation

4.1.6.1 Evaporate to dryness under a gentle stream of nitrogen at approximately 37°C.

4.1.7 Hexane Wash

4.1.7.1 Pipet 500uL hexane into each tube and vortex.

4.1.7.2 Place tube on rocker for 5 minutes.

4.1.7.3 Pipet 50uL Acetonitrile. Vortex briefly.

4.1.7.4 Centrifuge for 5 minutes at 3400rpm

4.1.7.5 Discard the hexane (top) layer.

4.1.7.6 Transfer acetonitrile extract to labeled ALS vial with micro-insert.

4.1.8 Preparation for Analysis Run

4.1.8.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

4.1.8.2 Load samples, standards, blank and controls into the quadrant rack as noted in the sequence table.

4.1.9 Acquisition Parameters

4.1.9.1 Refer to instrument method printouts for acquisition parameters.

4.1.9.1.1 Instrument Run Parameters

- Inlet at 250 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 120 degrees Celsius, hold 2 minutes
- Ramp 35 degrees Celsius, hold 3 minutes
- Final temperature: 290 degrees Celsius, hold at least 9.143 minutes
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.1.9.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.1.10 GC-MSD Qualitative Detection and Identification Criteria

4.1.10.1 For the identification of compounds not included in positive control, analyze appropriate non-extracted reference materials.

4.1.10.2 The presence of a drug compound is indicated if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

4.2 Quality Assurance Requirements

4.2.1 General

4.2.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

4.2.1.2 Refer to Toxicology AM #17, AM #19and AM #21 for quality assurance and reference material authentication requirements.

4.3 Analysis Documentation

4.3.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.3.2 A packet containing original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.3.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Reference Material

5.1.1 Mixed Standard Solution

- Add 20µL of each compound (minimum of 4).
- Total volume is 10mL.
- Solution is stable for 6-months @ room temperature.
- Record Preparation on standard log.

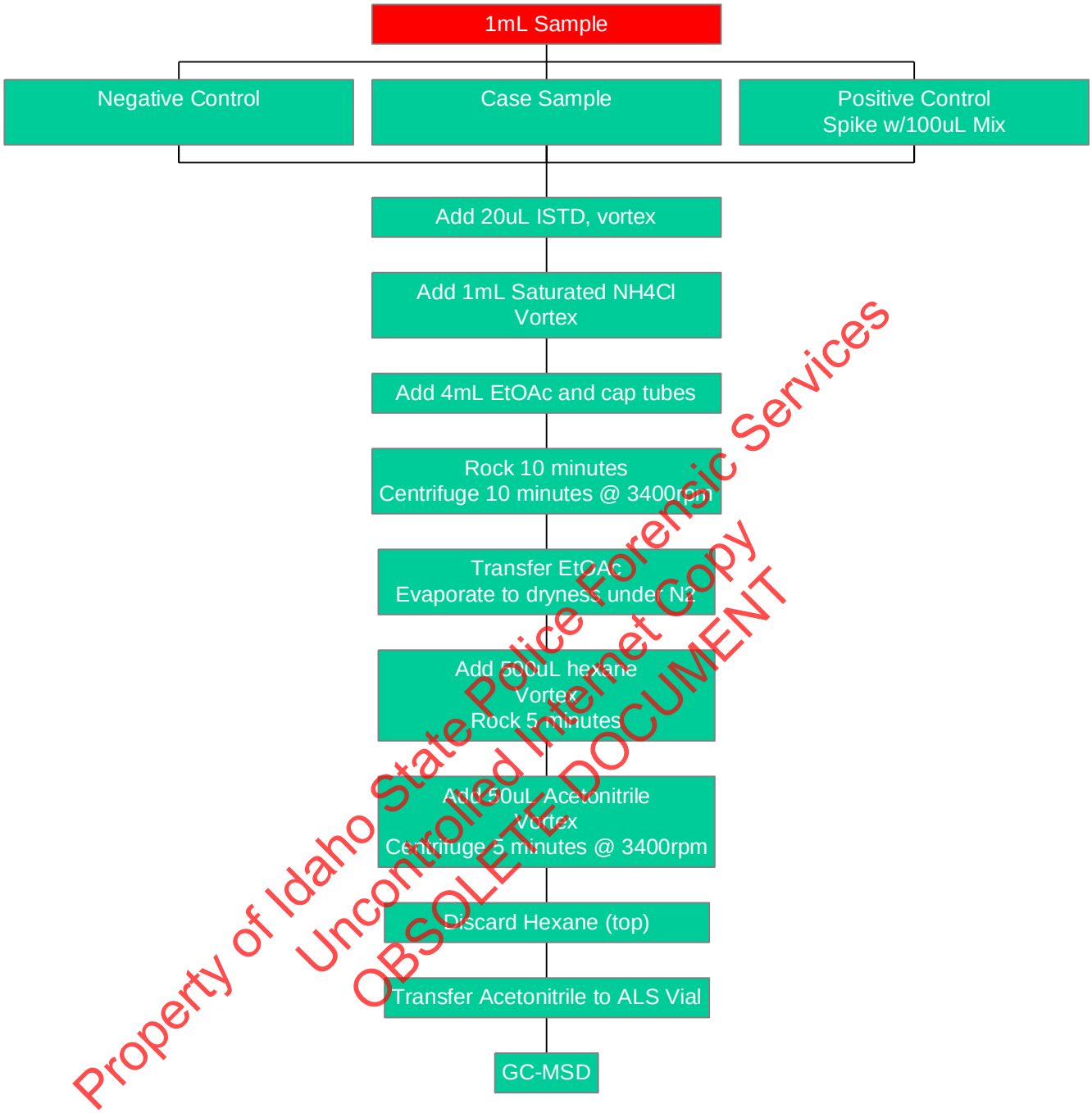
1mg/mL Stock Solution
Acetaminophen
Butalbital
Carbamazepine
Carisoprodol
Meprobamate
Phenobarbital
Secobarbital

5.1.2 Working Internal Standard Solution [50ng/µL]:

5.1.2.1 Add 500µL 1mg/mL Aprobarbital stock solution to 10mL volumetric ball flask. QS with DI water.

Solution is stable for three months when stored at room temperature.

5.2 Extraction Procedure



Toxicology AM #10: Methamphetamine and Amphetamine in Blood

1.0 Background/References

1.1 Background

The drug amphetamine dates back to 1887. It was used freely as a nasal decongestant, appetite suppressant, and to treat disorders such as narcolepsy in the early part of the 20th century, until its potential for abuse was fully realized.[4,5,6] The use of amphetamine and methamphetamine to treat narcolepsy, attention deficit disorder and obesity continues in a more regulated environment. Amphetamine (figure 1) and Methamphetamine (figure 2) are phenethylamines structurally related to norepinephrine and epinephrine, respectively.

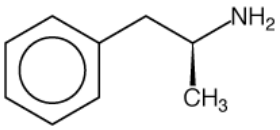


figure 1

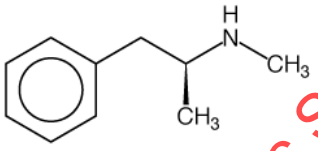


figure 2

The blood concentrations of methamphetamine and amphetamine should be considered in conjunction with all available information to determine the degree and nature of an individual's impairment.[2,3] Therapeutic levels for legitimate methamphetamine and amphetamine use are one to two orders of magnitude less than abuse and toxic levels.[6]

Consult provided references for additional information regarding the pharmacology of these compounds.

1.2 References and Recommended Reading

1.2.1 Chaturevidi, A.K., Cardona, P.S., Soper, J.W. and Canfield, D.V., Distribution and Optical Purity of Methamphetamine Found in Toxic Concentration in a Civil Aviation Accident Pilot Fatality, U.S. Department of Transportation Federal Aviation Administration Technical Report, December 2004.

1.2.2 Logan, B.K., Methamphetamine - Effects on Human Performance and Behavior, Forensic Science Rev. 14(1/2): 133-151, 2002.

1.2.3 Logan, B.K., Methamphetamine and Driving Impairment. J Forensic Sci, 1996, 41(3):457-464.

1.2.4 Drummer, O.H., Stimulants, pp. 49-96. in: The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.5 Moore, K.A., Amphetamine/Sympathomimetic Amines. pp. 245-264. in: Principles of Forensic Toxicology. Levine, B. ed., AACC, 2003.

1.2.6 Baselt, R.C., d-Methamphetamine, pp. 683-685. and Amphetamine, pp. 66-69. in: Disposition of Toxic Drugs and Chemicals in Man, Seventh ed., 2004.

2.0 Scope

2.1 Methamphetamine and amphetamine are recovered through the application of the Varian Bond Elut Certify® solid phase extraction (SPE) cartridge. The Bond Elut Certify® SPE cartridge contains a sorbent which utilizes cation exchange and non-polar mechanisms to recover methamphetamine and amphetamine from blood. Following the addition of deuterated internal standard mixture, the blood proteins are precipitated with cold acetonitrile. Following centrifugation, the supernatant is decanted and the pH adjusted with a 100mM phosphate buffer (pH 6). The sample is loaded onto the SPE cartridge that has been conditioned with methanol and a 100mM phosphate buffer (pH 6). The methanol conditioning opens up the coiled hydrophobic portion of the sorbent so that it interacts with the polar, buffered blood matrix. The addition of the buffer removes excess methanol and creates an environment similar to the matrix thus allowing for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the cationic functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent.

The cartridge is subsequently washed with 1M acetic acid followed by methanol, to selectively remove matrix components and interfering substances from the cartridge. The wash also disrupts hydrophobic and adsorption interactions, leaving behind the ionically bound material. Next, the sorbent is thoroughly dried to remove traces of aqueous and organic solvents which could adversely affect the analyte recovery. When the sorbent is dry, the analytes of interest are recovered from the cartridge with alkaline ethyl acetate. The alkaline environment serves to disrupt the ionic interactions of the analyte with the sorbent and the ethyl acetate disrupts the hydrophobic interactions. Following elution from the SPE cartridge, the evaporated extract is acylated for confirmation on the GC/MSD. The quantitation is accomplished through the use of a deuterated internal standard and a five-point calibration curve. This method is based on the method utilized by the Bioaeronautical Sciences Research laboratory.[1] This method can also be used to confirm drugs in full scan mode. If this is the intention, no calibrators need be used. However, the run must still contain a negative control and at least one positive control.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Varian Bond Elute Certify® SPE Cartridge or UCT Clean Screen DAU or equivalent
Sorbent type: Mixed mode octyl (C8) and benzenesulfonic acid (SCX), Sorbent mass: 130mg, Particle size: 40um

3.1.2 Disposable inserts for SPE manifold ports

3.1.3 Drybath or laboratory oven capable of 70°C

3.1.4 Evaporative concentrator equipped with nitrogen tank.

3.1.5 Vacuum manifold/pump

3.1.6 Tube rocker

3.1.7 Vortex mixer

3.1.8 Laboratory centrifuge capable of 3400- 3500rpm

3.1.9 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.1.10 *16 x 100mm* round bottom glass tube

3.1.11 Screw Cap for 16mm O.D. tube

3.1.12 GC/MS Automated Liquid Sample (ALS) vials

3.1.13 GC/MS Vial Microinsert

3.1.14 GC equipped with a mass selective detector and a nonpolar capillary column with a phase composition comparable to 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.2 Reagents

Refer to Toxicology AM #23 for solution preparation instructions.

3.2.1 Deionized/distilled (DI) water

3.2.2 Methanol (Certified ACS grade or better)

3.2.3 Hexane (Certified ACS grade or better)

3.2.4 Ethyl Acetate (Certified ACS grade or better)

3.2.5 Acetonitrile (Certified ACS grade or better)

3.2.6 Ammonium Hydroxide (Certified ACS grade or better)

3.2.7 Concentrated HCl (Certified ACS grade or better)

3.2.8 *1%* HCl in Methanol

3.2.9 *100mM* Phosphate Buffer (pH 6.0)

3.2.10 *1M* Acetic Acid

3.2.11 Pentafluoropropionic acid anhydride (PFAA)

3.3 Quality Assurance Material

3.3.1 Calibrator and Control Solutions

Note: Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available.

3.3.1.1 Reference Material Stock Solutions

3.3.1.1.1 Concentration: 1 mg/mL

- (±)-Methamphetamine
- (±)-Amphetamine
- Phentermine
- MDPV

- Alpha-PVP
- Alpha-PHP

3.3.1.1.2 Store remaining stock solution as recommended by manufacturer.

3.3.1.2 Reference Material Working Solutions

Working solutions are stable for 6 months when stored under refrigeration or in a freezer.

3.3.1.2.1 ***Concentration: 10ng/uL***

- Add 100uL each 1mg/mL Amphetamine and Methamphetamine Stock Solution to ~9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.1.2.2 ***Concentration: 1ng/uL***

- Add 1mL 10ng/uL working drug solution to ~5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.2 Internal Standard Stock Solutions

3.3.2.1 Stock Solutions

Concentration: 1mg/mL

- (±)-Methamphetamine-D8
- (±)-Amphetamine-D8

Store remaining stock solution as recommended by manufacturer.

3.3.2.2 Working Internal Standard Solution

Note: Working internal standard solution is stable for 6 months when stored under refrigeration or in a freezer.

3.3.2.2.1 ***Concentration: 10ng/uL***

Add 100uL each 1mg/mL Amphetamine-D8 and Methamphetamine-D8 Stock Solution to ~9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.3 Commercial Whole Blood Controls

3.3.3.1 Negative Whole Blood

3.3.3.2 Optional: Positive Whole Blood

3.3.3.2.1 Control containing Amphetamine and Methamphetamine each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized.

3.3.3.3 Optional: Positive Whole Blood

3.3.3.3.1 Control containing additional compounds (e.g. phentermine, alpha-PVP, alpha-PHP, MDPV) each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized. (NOTE: This control is required if additional compounds are to be confirmed.)

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 Label extraction tubes (x3), SPE columns (x1), and GC/MSD vials with microinserts (x1) for calibrators, controls and case samples.

4.1.2 Calibrator Preparation

NOTE: To prepare calibrators, use the same lot of negative blood used to prepare the negative control.

4.1.2.1 Add 2mL of negative whole blood to five screw-top extraction tubes.

4.1.2.2 Add the volume of 1ng/uL Amphetamine and Methamphetamine working solution as indicated in the chart below.

Level	Desired ng/mL	µL Working Reference material
1	25	50
2	50	100

4.1.2.3 Add the volume of 10ng/uL Amphetamine and Methamphetamine working solution as indicated in the chart below.

Level	Desired ng/mL	µL Working Reference material
3	100	20
4	250	50
5	500	100

4.1.3 Positive Control Sample Preparation

NOTE: To prepare positive controls, use the same lot of negative blood used to prepare the negative control.

4.1.3.1 Add 2mL of negative whole blood to two screw top tubes.

4.1.3.2 Add indicated amount of 10ng/uL working solution.

Desired ng/mL	µL Working Control
75	15
300	60

4.1.3.3 Additional or alternative concentrations at the discretion of the analyst may be used as long as the requirements in 4.5.2 are met.

4.1.4 Negative Control Sample Preparation

4.1.4.1 Add 2mL of negative whole blood to screw top tube.

4.1.5 Case Sample Preparation

4.1.5.1 Based on enzyme immunoassay screen results, samples may be diluted with negative whole blood prior to analysis.

4.1.5.2 Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary.

4.1.5.3 Transfer 2mL neat or diluted sample to labeled screw top tube.

4.1.5.4 If there is a low-volume sample, analyst may halve the volume of sample (1mL). Internal standard added should also be halved. One additional low and one high control must also be added to mirror the low-volume extraction.

4.1.6 Internal Standard Addition

4.1.6.1 To calibrators, controls and case samples, add 20uL of internal standard mix (10ng/uL).

4.1.6.2Vortex tube briefly and let stand 15 to 30 minutes for sample equilibration.

4.1.7 Protein Precipitation

4.1.7.1 While vortexing, add 5mL cold acetonitrile to case, calibrator and control samples.

4.1.7.2 Cap tubes and rock samples for approximately 15 minutes. Remove from rocker and place samples into centrifuge and let stand for 5 minutes.

4.1.7.3 Centrifuge at 3400 – 3500 rpm for 10 minutes.

4.1.7.4 Transfer organic supernatant into second labeled tapered bottom centrifuge tube.

4.1.7.5 Transfer tube to Evaporative Concentrator. Evaporate sample to approximately 1mL under nitrogen at approximately 40°C. Do not allow extract to go to dryness.

4.1.7.6 To concentrated extract, add 2mL 100mM phosphate buffer (pH 6). Vortex to mix.

4.1.7.7 If needed, centrifuge an additional 5 minutes to remove blood fragments or foam.

4.1.8 SPE Column Preparation

4.1.8.1 Insert valve liners and labeled SPE columns into appropriate location on vacuum manifold. For each following SPE step, allow to gravity flow or aspirate at ≤3 in. Hg to prevent sorbent drying

4.1.8.2 Add 2mL methanol to the column.

4.1.8.3 Add 2mL 100mM Phosphate buffer (pH 6.00) to the column.

4.1.9 Blood Extract Loading

4.1.9 Decant buffered blood extract onto the SPE column.

4.1.10 Column Clean-up

4.1.10.1 Add 1mL 1M Acetic Acid.

4.1.10.2 Turn on/increase vacuum to ~10 in. Hg (34 kPa) for ~5 minutes.

4.1.10.3 Add 6mL methanol.

4.1.11 Pre-Elution Dry Disc

4.1.11.1 Turn on/increase vacuum to ~10 in. Hg (34 kPa) for ~5 minutes.

4.1.12 Compound Elution

4.1.12.1 Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered bottom centrifuge tubes.

4.1.12.2 Add 4mL of 2% NH4OH in ethyl acetate elution solvent to the column. Collect with gravity flow or apply minimal vacuum.

4.1.12.3 Add 50uL 1% HCl in Methanol into each tube to minimize analyte loss.

4.1.13 Eluate Evaporation

4.1.13.1 Transfer centrifuge tube to evaporative concentrator. Take solvent to dryness, under a gentle stream of nitrogen at approximately 40°C.

4.1.14 Derivatization

4.1.14.1 Add 50uL ethyl acetate. Vortex for ~15 seconds.

4.1.14.2 Add 50uL PFAA.

4.1.14.3 Cap tubes and vortex briefly.

4.1.14.4 Heat tubes at 70°C for 20 minutes.

4.1.14.5 Remove from heat and allow to cool to room temperature.

4.1.14.6 Return tubes to evaporative concentrator and evaporate to dryness under nitrogen at approximately 40°C. Never inject PFAA extract directly into GC/MSD.

4.1.14.7 Reconstitute extract with 50uL ethyl acetate.

4.1.14.8 Transfer reconstituted extract to labeled GC/MSD ALS vial with microinsert.

4.1.15 Preparation for GC-MS Run

4.1.15.1 Into Sequence log table, enter the case sample, calibrators, blanks and control information.

4.1.15.2 Load samples, calibrators, blank and controls into the quadrant rack as noted in the sequence table.

4.1.16 GC-MS Calibration Curve

4.1.16.1 The calibration curve should be established with a minimum of four data points.

4.1.16.2 Calibrators should be analyzed in order of increasing concentration.

4.1.16.3 The least squares line resulting from the analysis of calibrators must have a coefficient of correlation of ≥0.98.

4.1.16.4 If calibration reference materials are run in duplicate, it is not required that duplicate calibration points are included as long as the linearity requirement is met.

4.2 GC and MSD Acquisition Parameters

4.2.1 Acquisition Parameters

4.2.1.1 Refer to instrument method printouts for acquisition parameters.

4.2.1.1.1 Instrument Run Parameters

- Inlet at 250 degrees Celsius, 25:1 split injection, injection volume: 1ul
- Oven at 90 degrees Celsius, hold 1 minute
- Ramp 36.5 degrees Celsius
- Final temperature: 290 degrees Celsius, hold at least 1 minute
- Additional sample runs may be done with adjustments to these parameters, for example if there is a co-eluting peak or a late eluting compound.

4.2.1.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.2.1.3 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Amphetamine	190	118	91
Amphetamine-D8	193	126	96
Methamphetamine	204	160	118
Methamphetamine-D8	211	163	123

4.3 Reporting Criteria

4.3.1 Qualitative Chromatographic Criteria

4.3.1.1 Acceptable retention time window established by calibrators is ±0.1 minutes.

4.3.2 Qualitative Mass Spectral SIM Criteria

4.3.2.1 Ion ratios for the analyte and its corresponding internal standard, established by calibrators for target and qualifier ions, must not differ by more than ±20% (relative). Refer to section 4.3.4.4 for administrative cutoff criteria.

4.3.3 Qualitative Mass Spectral Full Scan Criteria

4.3.3.1 Analytes may be confirmed from full scan data if the retention time for the sample versus applicable reference material does not differ by more than ±0.1 minutes and there are no significant differences in the mass spectral data.

4.3.4 Quantitative Mass Spectral and Control Criteria

4.3.4.1 Refer to Section 4.4.1 for determination of when this method may be used for quantitative purposes.

4.3.4.1 Quantitative results can be accepted if the calculated concentrations of all calibrator and control samples are within ±20% of their respective concentrations.

4.3.4.2 Quantitation is achieved through the plotting of the target ion response ratio versus the concentration for each calibrator.

4.3.4.3 Quantitative values for case samples, calibrators and controls will be truncated for reporting purposes.

4.3.4.4 Administrative limit of detection (LOD) for Amphetamine and Methamphetamine is 25ng/mL. Results ≤ this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

4.3.4.5 If the concentration exceeds the calibration range, the sample needs to be appropriately diluted with negative whole blood for reanalysis. Alternatively, the analyte(s) may be reported using full scan data; refer to section 4.3.3 for criteria.

4.4 Reporting of Results

4.4.1 Currently, this method is **only approved for the qualitative identification** of drugs. Quantitative values are not to be reported or expressed. They are currently being used to establish an administrative cut off. Once the uncertainty of measurement is established for this method it will be evaluated for quantitative reporting.

4.5 Quality Assurance Requirements

4.5.1 General

4.5.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

4.5.1.2 Refer to Toxicology AM #15 or AM #16, AM #17, AM #19 and AM #21 for quality assurance and reference material authentication requirements.

4.5.2 Per Analysis Run Quality Requirements

4.5.2.1 A solvent blank must follow the highest calibrator, as well as precede each case sample.

4.5.2.2 A minimum of the spiked blood controls described in section 4.1.3 must be run per batch of samples. Controls should not be grouped at the beginning of the acquisition sequence. Rather, controls should be interspersed throughout the sequence.

4.5.2.3 If the number of case samples exceeds 10, in addition to the two spiked controls described in 4.1.3, one spiked or commercially-obtained blood control must be run for each additional 10 case samples. Additional concentrations may be used.

4.5.2.4 Analysts may combine their samples into a single run to conserve supplies. However, each analyst with samples in the run must independently comply with the control requirements in section 4.5.2.2. A third-party reviewer must independently review the central file packet for compliance to method requirements.

4.5.2.5 If a drug other than Amphetamine or Methamphetamine is to be identified in full scan acquisition mode, one additional in-run control verifying the extraction of that compound is required. Multiple compounds may be extracted simultaneously.

4.5.3 Monitoring of Control Values

4.5.3.1 Once the method has been approved for quantitative purposes, the following is required: upon the completion of analysis, input blood control values on spreadsheet used to assess uncertainty for this method.

4.6 Analysis Documentation

4.6.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.6.2 A packet containing original data for controls and calibrators will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.6.3 A copy of controls and calibrators need not be included in individual case files. When necessary, a copy of the control and calibrator printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Quality Assurance Material

5.1.1 Working Reference Material Solutions

NOTE: Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. Working reference material solutions are stable for 6 months when stored under refrigeration or in a freezer.

5.1.1.1 *10ng/µL*

- Add 100.0µL each Amphetamine and Methamphetamine Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

5.1.1 2 *1ng/µL*

- Add 1.0mL of 10ng/µL working drug solution to ≅5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

5.1.2 Calibrator Preparation

5.1.2.1 Add the volume of working Amphetamine-Methamphetamine mixed reference material to appropriate tube as indicated below.

Level	ng/mL	Working Solution (ng/µL)	Volume to add (µL)
1	25	1	50
2	50	1	100
3	100	10	20
4	250	10	50
5	500	10	100

5.1.3 Spiked Positive Control Preparation

5.1.3.1 Add the volume of working Amphetamine-Methamphetamine mixed reference material to appropriate tube as indicated below.

ng/mL	Working Solution (ng/µL)	Volume to add (µL)
75	10	15
300	10	60

5.1.4 *10ng/µL* Working Internal Standard Solution

5.1.4.1 Add 100.0µL each 1mg/mL Amphetamine-D8 and Methamphetamine-D8 Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. *Solution is stable for 6 months when stored under refrigeration or in a freezer.*

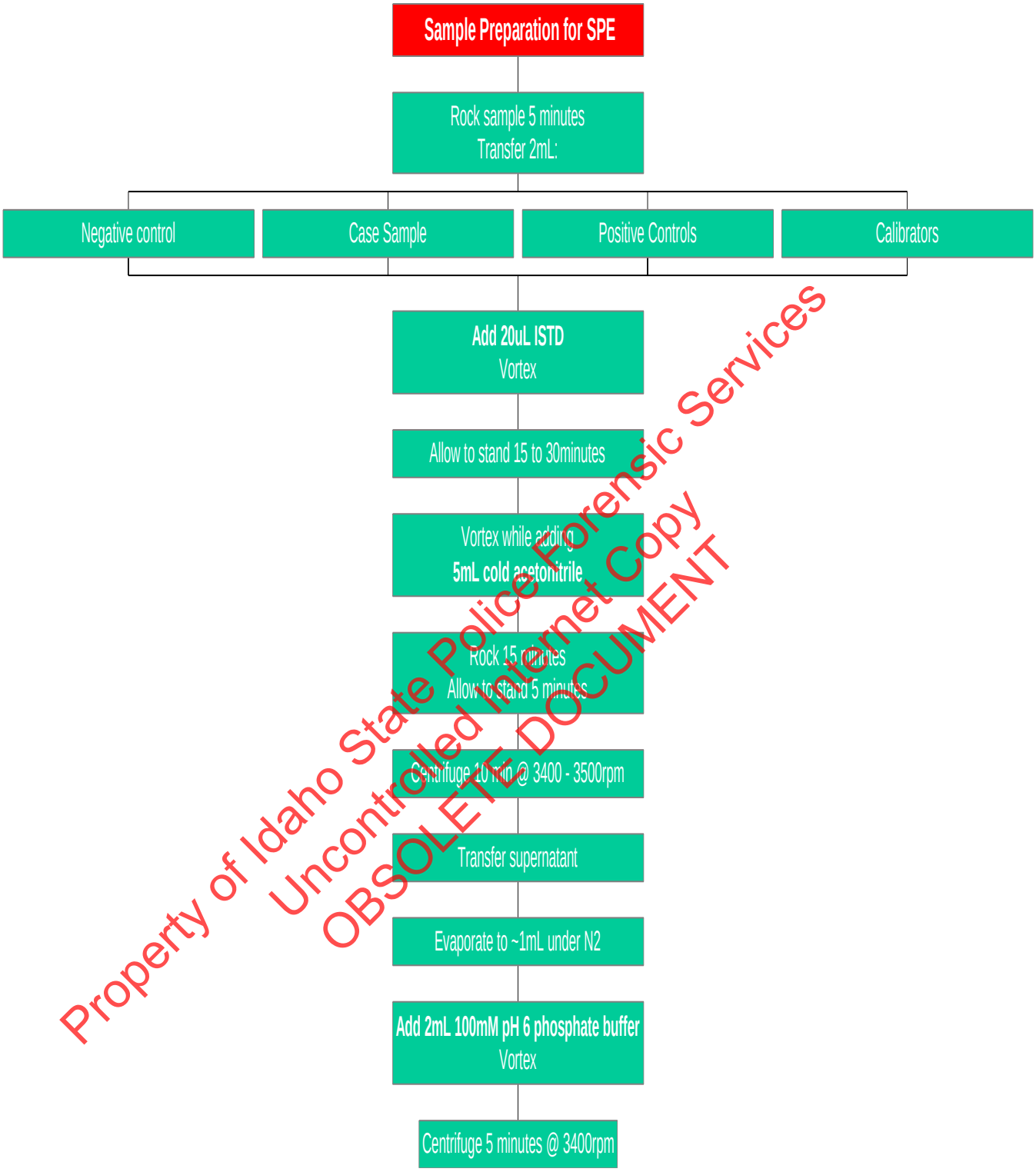
5.1.5 Optional: Whole Blood Positive Control

5.1.5.1 Control containing Amphetamine and Methamphetamine each at a specified target concentration. Refer to package insert for verified value and expected range.

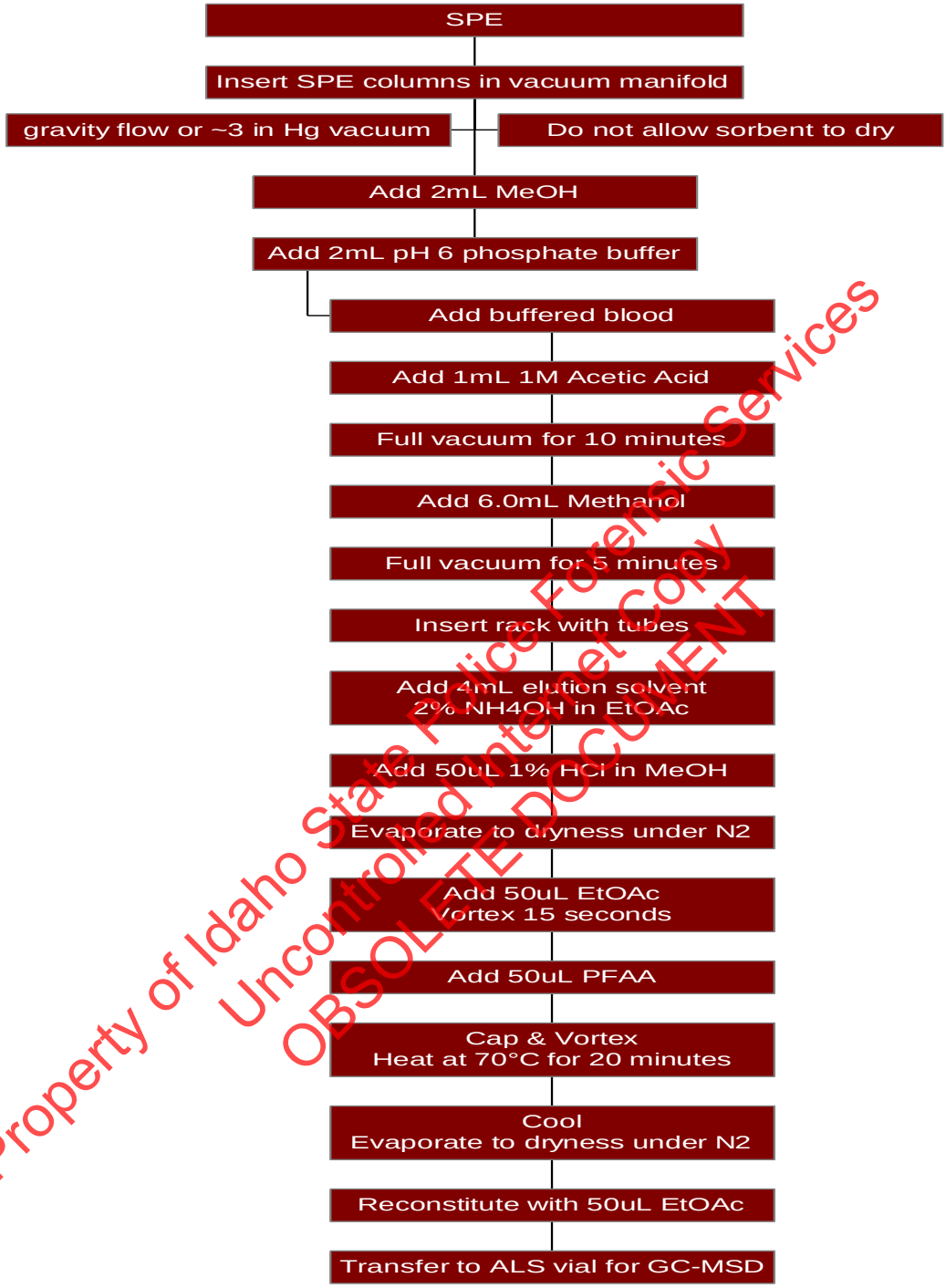
5.1.6 Optional: Additional Compounds Whole Blood Positive Control

5.1.6.1 Control containing additional compounds (e.g. phentermine, MDPV, etc). Required for any additional compounds to be qualitatively confirmed.

5.2 Sample Extraction



5.3 SPE Instructions



Toxicology AM #11: Opiates in Blood

1.0 Background/References

1.1 Background

Refer to provided references and current literature for information regarding the background and pharmacology of Codeine (figure 1) and Morphine (figure 2).[2-5]

Figure 1

Figure 2

1.2 References

1.2.1 Telepchak, M.J., August, T.F. and Chaney, G., Drug Methods for the Toxicology Lab, pp. 227 - 230. in: Forensic and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

1.2.2 Stout, P.R. and Farrell, L.J., Opioids - Effects on Human Performance and Behavior, Forensic Science Rev. 15(1): 29 - 60, 2003.

1.2.3 Drummer, O.H., Opioids pp. 219 - 265. in: The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.4 Kerrigan, S. and Goldberger, B.A., Opioids. Refer to index for page numbers, in: Principles of Forensic Toxicology. Levine, B. ed., AACC, Third ed., 2010 or more recent version.

1.2.5 Baselt, R.C., Codeine, pp. 355 - 360 and Morphine, pp. 1057 - 1061. in: Disposition of Toxic Drugs and Chemicals in Man, Biomedical Publications: Foster City, CA. Eighth ed., 2008 or more recent version.

2.0 Scope

2.1 This procedure is based on a method developed by United Chemical Technology (UCT) which applies the UCT 200 mg CLEAN SCREEN® extraction column for the extraction of opiates from blood.[1] The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the blood sample. The cation exchanger utilizes an anionic sorbent to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption.

For the extraction of opiate-class drugs, the blood sample is diluted and adjusted to pH 6 with a phosphate buffer. After centrifugation, the sample is loaded onto a pre-conditioned SPE column. The blood pH is adjusted to maximize the ionic character of the analyte. Column conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the amine functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water, a weak aqueous buffer and methanol to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions, but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. When the column is dry the analytes of interest are eluted from the column with a basic organic solvent mixture. Following elution from the SPE column and evaporation, the extract is derivatized for confirmation on the GC/MSD. Quantitation is accomplished using the corresponding deuterated standard to establish the response factor.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 200mg CLEAN SCREEN® Extraction Column (ZSDAU020 or ZCDAU020 or equivalent)

3.1.2 Disposable inserts for SPE manifold ports

3.1.3 Laboratory oven or drybath capable of 70°C

3.1.4 Evaporative concentrator equipped with nitrogen tank

3.1.5 Tube Rocker

3.1.6 Vortex Mixer

3.1.7 Laboratory centrifuge capable of 3400 - 3500rpm

3.1.8 Vacuum Manifold pump

3.1.9 Fixed and adjustable volume single channel air displacement pipettes, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.1.10 pH indicator strips

3.1.11 16 x 100mm silanized glass tubes

3.1.12 Screw Cap for 16mm O.D. tube

3.1.13 GC/MS Automated Liquid Sample (ALS) vials

3.1.14 Silanized GC/MS Vial Microinsert

3.1.15 Gas Chromatograph (GC) equipped with a mass selective detector (MSD) and a nonpolar capillary column with a phase composition comparable to 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.2 Reagents

NOTE: Refer to Toxicology AM #23 for solution preparation instructions.

3.2.1 Deionized/distilled (DI) water

3.2.2 Methanol (Certified ACS Grade)

3.2.3 Methylene Chloride (Certified ACS Grade)

3.2.4 Ethyl Acetate (Certified ACS Grade)

3.2.5 Isopropanol (Certified ACS Grade)

3.2.6 Ammonium Hydroxide (Certified ACS Grade)

3.2.7 100mM Phosphate Buffer (pH 6.0)

3.2.8 100mM Acetate Buffer (pH 4.5)

3.2.9 100mM Monobasic sodium phosphate

3.2.10 100mM Dibasic sodium phosphate

3.2.11 Elution Solvent

3.2.11.1 Mix 20mL Isopropanol and 2mL Ammonium Hydroxide. QS to 100mL with methylene chloride. pH should be 11-12. Make fresh.

3.2.12 BSTFA + 1% TMCS

3.3 Quality Assurance Material

3.3.1 Control Solutions

3.3.1.1 Reference Material Stock Solutions

Compound	Concentration
Codeine	1 mg/mL
Morphine	1 mg/mL

Store remaining stock solution as recommended by manufacturer.

3.3.1.2 Reference Material Working Solutions

Working solutions are stable for 6 months when stored under refrigeration or in a freezer.

3.3.1.2.1 - 10ng/µL

- Add 100µL each Codeine and Morphine Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.1.2.2 - 1ng/µL

- Add 1mL 10ng/µL working drug solution to ≅5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.2 Internal Standard Solutions

3.3.2.1 Stock Solutions

Compound	Concentration
Codeine-D_3 or -D_6	1 mg/mL or 100µL/mL
Morphine-D_3 or -D_6	1 mg/mL or 100µL/mL

Store remaining stock solution as recommended by manufacturer.

3.3.2.2 Working Internal Standard Solution <u>1ng/µL</u>

3.3.2.2.1 Add 10µL each 1mg/mL or 100µL each 100µL/mL Codeine-D_3 or -D_6 and Morphine-D_3 or -D_6 Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. *Working solution is stable for 6 months when stored under refrigeration or in a freezer.*

3.3.3 Commercial Whole Blood Controls

3.3.3.1 Negative Whole Blood

3.3.3.2 Optional: Positive Whole Blood

3.3.3.2.1 Positive control must contain Codeine and Morphine each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 Label extraction tubes (x2), SPE columns (x1), and GC/MSD vials with microinserts (x1), for controls and case samples.

4.1.2 Positive Control Sample Preparation

NOTE: To prepare positive controls, use the same lot of negative blood used to prepare the negative control.

4.1.2.1 Add 1mL of negative whole blood to screw-top tubes.

4.1.2.2 Add indicated amount of 10ng/µL working mixed control solution.

ng/mL	µL Working Control
100	10
750	75

4.1.2.3 Additional or alternative concentrations at the discretion of the analyst may be used as long as the requirements in 4.4.2 are met.

4.1.3 Negative Control Sample Preparation

4.1.3.1 Add 1mL of negative whole blood to a screw top tube.

4.1.4 Case Sample Preparation

4.1.4.1 Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary.

4.1.4.2 Add 1mL of sample to labeled screw top tube.

4.1.5 Internal Standard Addition

4.1.5.1 To prepare 100ng/mL internal standard add 10µL of 1mg/mL (1000ng/µL) or 100µL of (100ng/µL) 1ng/µL of internal standard mix to controls and casework samples.

4.1.5.2 Vortex tube briefly and allow to stand 15 - 30 minutes for sample equilibration.

4.1.6 Sample Preparation

4.1.6.1 Add 4mL DI water, vortex.

4.1.6.2 Add 2mL 100mM phosphate buffer (pH 6.0), vortex, allow sample to stand for 5-10 minutes.

4.1.6.3 Check pH. Sample pH should be 6.0 ± 0.5. Adjust as necessary with 100mM monobasic sodium phosphate or 100mM dibasic sodium phosphate.

4.1.6.4 Centrifuge for about 10 minutes at approximately 3400 – 3500 rpm.

4.1.7 SPE Column Preparation

4.1.7.1 Insert valve liners and labeled SPE columns into appropriate location on vacuum manifold. For each following SPE step, allow to gravity flow or aspirate at ≤ 3 in. Hg to prevent sorbent drying.

4.1.7.2 Add 3mL methanol to the column.

4.1.7.3 Add 3mL DI water to the column.

4.1.7.4 Add 1mL 100mM phosphate buffer (pH 6.00) to the column.

4.1.8 Blood Extract Loading

4.1.8.1 Decant buffered blood extract onto the SPE column. Care should be taken that very little solid matter (from centrifugation of whole blood) is applied to the SPE column.

4.1.9 Column Clean-up

4.1.9.1 Add 2mL DI water to the column.

4.1.9.2 Add 2mL 100mM acetate buffer (pH 4.5) to the column.

4.1.9.3 Add 3mL methanol.

4.1.19.4 Increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥5 minutes (disc should be dry).

4.1.10 Compound Elution

4.1.10.1 Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled centrifuge tubes.

4.1.10.2 Add 3mL elution solvent (3.2.11.1) to the column. Collect eluate with gravity flow or apply minimal vacuum.

4.1.11 Eluate Evaporation

4.1.11.1 Transfer centrifuge tube to Evaporative Concentrator. Evaporate solvent to dryness under a gentle stream of nitrogen at approximately 40°C.

4.1.12 Derivatization

4.1.12.1 Add 50µL ethyl acetate. Vortex for ≅15 seconds.

4.1.12.2 Add 50µL BSTFA + 1% TMCS.

4.1.12.3 Cap tubes and vortex briefly.

4.1.12.4 Heat tubes at 70°C for 20 minutes.

4.1.12.5 Remove from heat and allow to cool to room temperature.

4.1.12.6 Transfer derivative to labeled GC/MSD ALS vial with microinsert.

4.1.13 Preparation for GC-MS Run

4.1.13.1 Into Sequence log table, enter the case sample, calibrators, blanks and control information.

4.1.13.2 Load samples, blank and controls into the sample rack(s) as noted in the sequence table.

4.2 GC and MSD Acquisition Parameters

4.2.1 Acquisition Parameters

4.2.1.1 Refer to instrument method printouts for acquisition parameters.

4.2.1.1.1 Instrument Run Parameters

- Inlet at 250 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 90 degrees Celsius, hold 1 minute
- Ramp 30 degrees Celsius
- Final temperature: 290 degrees Celsius, hold at least 5 minutes
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.2.1.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.3 Reporting Criteria

4.3.1 Qualitative Mass Spectral Full Scan Criteria

4.3.1.1 The retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

4.4 Quality Assurance Requirements

4.4.1 General

4.4.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

4.4.1.2 Refer to Toxicology AM #15, AM #16, AM #19 and AM #21 for quality assurance and reference material authentication requirements.

4.4.2 Per Analysis Run Quality Requirements

4.4.2.1 Minimally, a solvent blank must precede each case sample.

4.4.2.2 A minimum of the spiked blood controls described in section 4.1.2 must be run per batch of samples. Controls should not be grouped at the beginning of the acquisition sequence. Rather, controls should be interspersed throughout the sequence.

4.5 Analysis Documentation

4.5.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.5.2 Original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.5.3 A copy of data for controls and standards may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of the control and standard printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Quality Assurance Materials

5.1.1 Spiked Positive Control Preparation

5.1.1.1 Add the volume of working Codeine-Morphine mixed RM to appropriate tube as indicated:

ng/mL	Working Solution (ng/µL)	Volume to add (µL)
100	1	100
750	10	75

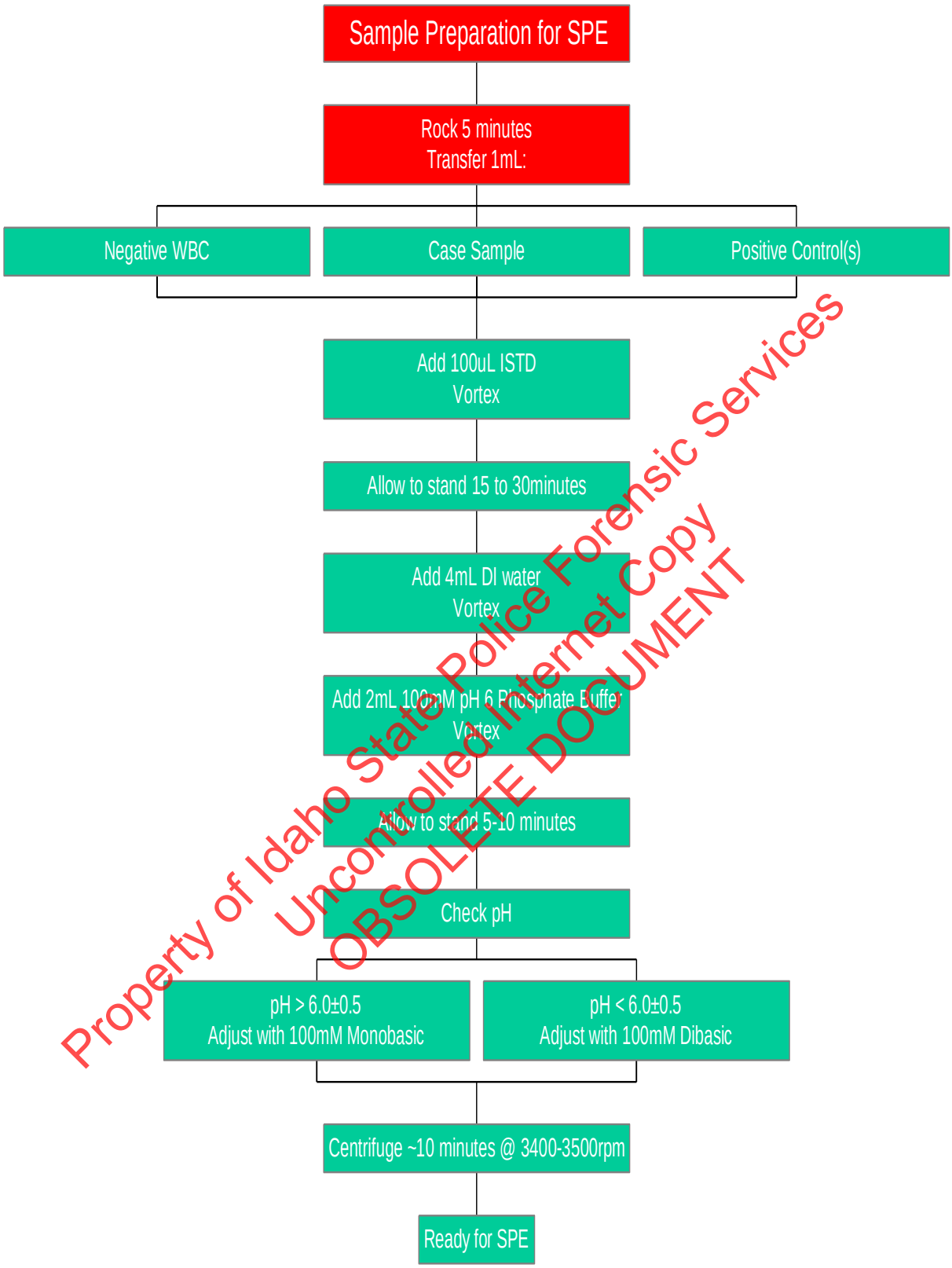
5.1.2 *1.0ng/µL* Working Internal Standard Solution

5.1.2.1 Add 10.0µL each 1mg/mL (1000ng/µL) or 100µL each 100µg/mL (0.1µg/µL) Codeine-D3 or Codeine-D6 and Morphine-D3 or Morphine-D6 Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. *Solution is stable for 6 months when stored under refrigeration or in a freezer.*

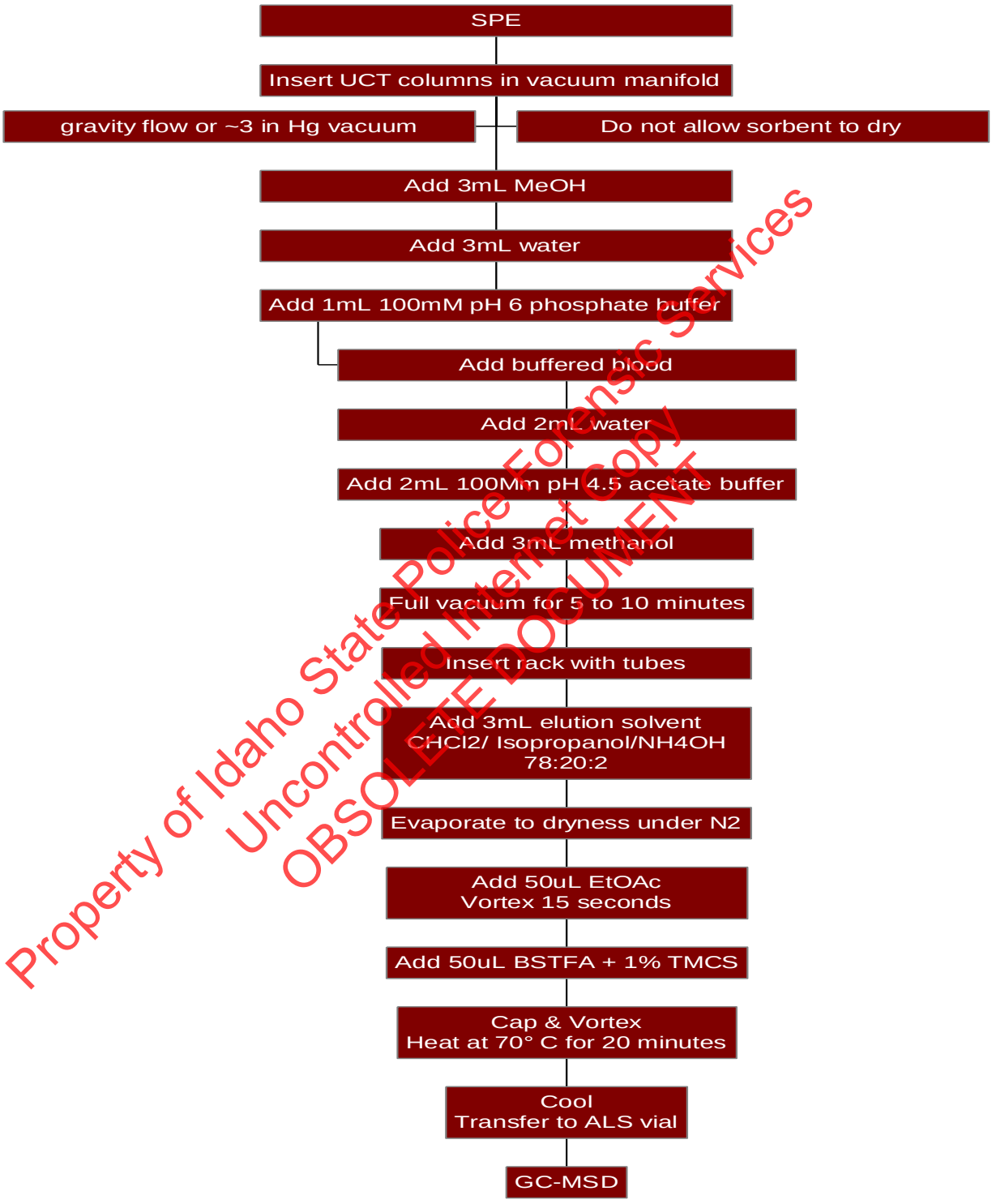
5.1.3 Optional: Commercial Blood Positive Control

5.1.3.1 Commercial controls must contain Codeine and Morphine each at a target concentration of 100ng/mL. Refer to package insert for verified value and expected range.

5.2 Sample Preparation



5.3 – SPE Extraction



Toxicology AM #12: Cocaine and Cocaine Metabolites in Blood

1.0 Background/References

1.1 Background

The major metabolites of Cocaine (Methylbenzoylecgonine (Figure 1)), are benzoylecgonine, ecgonine and ecgonine methyl ester, all of which are inactive. When cocaine is ingested with ethanol, the methyl ester portion undergoes transesterification to form the active compound Cocaethylene (ethyl benzoylecgonine) that in turn adds the inactive metabolite, ecgonine ethyl ester. Refer to qualitative urine cocaine analytical method 2.3.6 and provided references and current literature for information regarding the background and pharmacology of these compounds.[2-8]

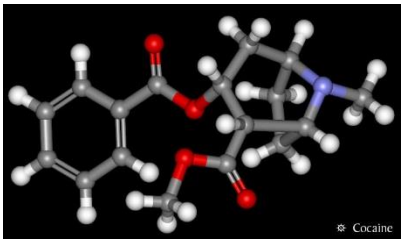


Figure 1.

1.2 References

1.2.1 Telepchak, M.J., August, T.F. and Chaney, G. Drug Methods for the Toxicology Lab, pp. 209-211. *in:* Forensic and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

1.2.2 Crouch, D.J., Alburges, M.E., Spanbauer, A.C., Rollins, D.E. and Moody; D.E., Analysis of Cocaine and Its Metabolites from Biological Specimens Using Solid-Phase Extraction and Positive Ion Chemical Ionization Mass Spectrometry, J. Anal. Toxicol. 19(6): 352-358, 1995.

1.2.3 Cone, E.J., Hillsgrove, M. and Darwin, W.D., Simultaneous Measurement of Cocaine, Cocaethylene, Their Metabolites, and "Crack" Pyrolysis Products by Gas Chromatography - Mass Spectrometry, Clin Chem 40(7):1299-1305, 1994.

1.2.4 Isenschmid, D.S., *Cocaine - Effects on Human Performance and Behavior,* Forensic Science Rev. 14(1&2): 62-100, 2002.

1.2.5 Drummer, O.H., *Stimulants* pp. 49-96. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.6 Isenschmid, D.S., *Cocaine,* pp. 207-228. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 2nd ed, 2003.

1.2.7 Baselt, R.C., *Cocaine*, pp. 256-262. *in:* Disposition of Toxic Drugs and Chemicals in Man, Biomedical Publications: Foster City, CA. 7th ed., 2004.

1.2.8 *Cocaine*, pp. 842-845. *in:* Clarke's Analysis of Drugs and Poisons. Pharmaceutical Press: London, 3rd ed., 2004.

2.0 Scope

2.1 This procedure is based on a method developed by United Chemical Technology (UCT) which applies the UCT 200 mg CLEAN SCREEN® extraction column for the extraction of blood for cocaine and cocaine metabolites.[1] The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the blood sample. The cation exchanger utilizes an anionic sorbent to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption.

For this extraction method, the blood sample is diluted and adjusted with a pH 6 phosphate buffer. After centrifugation, the sample is loaded onto a pre-conditioned SPE column. The blood pH is adjusted to maximize the ionic character of the analyte. Column conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the amine functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water, 100mM hydrochloric acid, and methanol to selectively remove matrix components and interfering substances from the column.

The wash also disrupts the hydrophobic and adsorption interactions, but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. After drying the sorbent, the analytes of interest are eluted from the column with a basic organic solvent mixture. Following the elution and evaporation of the solvent, the extract is derivatized for confirmation on the GC/MSD. Quantitation is accomplished using the corresponding deuterated internal standard to establish a response factor. This method can also be used to confirm drugs in full scan. If this is the intention, no calibrators need be used. However, the run must still contain a negative control and at least one positive control.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 200mg CLEAN SCREEN® Extraction Column (ZSDAU020 or ZCDAU020 or equivalent)

3.1.2 Disposable inserts for SPE manifold ports

3.1.3 Drybath or laboratory oven

3.1.4 Evaporative concentrator equipped with nitrogen tank.

3.1.5 Vortex mixer

3.1.6 Vacuum manifold/pump

3.1.7 Laboratory centrifuge capable of 3400rpm

3.1.8 Fixed and/or adjustable volume pipettes, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.1.9 pH indicator strips

3.1.10 16 x 100mm round bottom glass tube

3.1.11 Screw Cap for 16mm O.D. tube

3.1.12 GC/MS Automated Liquid Sample (ALS) vials

3.1.13 GC/MS Vial Microinsert

3.1.14 Gas Chromatograph (GC) equipped with a mass selective detector (MSD) (HP 6890 GC/5973 MSD or equivalent) and a nonpolar capillary column with a phase composition comparable to 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.2 Reagents

NOTE: Refer to Toxicology AM #23 for solution preparation instructions.

3.2.1 Deionized/distilled (DI) water

3.2.2 Methanol (Certified ACS Grade)

3.2.3 Methylene Chloride (Certified ACS Grade)

3.2.4 Ethyl Acetate (Certified ACS Grade)

3.2.5 Isopropanol (Certified ACS Grade)

3.2.6 Ammonium Hydroxide (Certified ACS Grade)

3.2.7 100mM Phosphate Buffer (pH 6.0)

3.2.8 100mM HCl

3.2.9 100mM Monobasic sodium phosphate

3.2.10 100mM Dibasic sodium phosphate

3.2.11 Elution Solvent

3.2.11.1 Mix 20mL Isopropanol and 2mL Ammonium Hydroxide. QS to 100mL with methylene chloride. pH should be 11-12. Make fresh.

3.2.12 BSTFA + 1% TMCS

3.3 Quality Assurance Materials

3.3.1 Calibrator and Control Solutions

NOTE: Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. The addition of Cocaethylene is optional.

3.3.1.1 Reference Material Stock Solutions

Compound	Concentration
Benzoylecgonine	1 mg/mL
Cocaine	1 mg/mL
Cocaethylene (optional)	1 mg/mL

Store remaining stock solution as recommended by manufacturer.

3.3.1.2 Reference Material Working Solutions

Working solutions are stable for 6 months when stored under refrigeration or in a freezer.

3.3.1.2.1 *10ng/µL*

- Add 100µL each Benzoylecgonine, Cocaine and Cocaethylene (optional) Stock Solutions to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.1.2.2 *1ng/µL*

- Add 1mL 10ng/µL working drug solution to ≅5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.3.2 Internal Standard Stock Solutions

Compound	Concentration
Benzoylecgonine–D_3 or –D_6	100µg/mL (100ng/µL)
Cocaine–D3	100µg/mL
Cocaethylene–D3*	100µg/mL

**Use if Cocaethylene will be included.*

Store remaining stock solution as recommended by manufacturer.

3.3.3 *1ng/µL* Working Internal Standard Solution

3.3.3.1 Add 100µL Benzoylecgonine–D_3 or –D_6, Cocaine–D_3, and Cocaethylene–D_3 (optional) stock solutions to 9800µL Methanol. *Working solution is stable for 6 months when stored under refrigeration or in a freezer.*

3.3.4 Commercial Whole Blood Controls

3.3.4.1 Negative Whole Blood

3.3.4.2 Optional: Positive Whole Blood

3.3.4.2.1 Positive control must contain a minimum of Benzoylecgonine and Cocaine each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Initial set-up

4.1.1.1 For calibrators, controls and case samples label extraction tubes (two per sample), an SPE extraction column, and a GC/MSD vial with microinsert.

4.1.2 Calibrator Preparation

NOTE: To prepare calibrators, use the same lot of negative blood used to prepare the negative control.

4.1.2.1 Add 1mL of negative whole blood to screw-top tubes.

4.1.2.2 Add the volume of 1ng/µL Benzoylecgonine, Cocaethylene and Cocaine working solution as indicated in the following table.

Level	ng/mL	µL Working Reference Material
1	25	25
2	50	50
3	100	100

4.1.2.3 Add the volume of 10ng/µL Benzoylecgonine, Cocaethylene and Cocaine working solution as indicated in the following table.

Level	ng/mL	µL Working Reference Material
4	250	25
5	500	50
6	1000	100

4.1.2.4 Additional or alternative concentrations may be used as necessary as long as the requirements in 4.1.15 are met.

4.1.3 Positive Control Sample Preparation

NOTE: To prepare positive controls, use the same lot of negative blood used to prepare the negative control.

4.1.3.1 Add 1mL of negative whole blood to screw top tubes.

4.1.3.2 Add indicated amount of 1ng/µL working mixed control solution.

Desired ng/mL	µL Working Control
75	75

4.1.3.3 Add indicated amount of 10ng/µL working mixed control solution.

Desired ng/mL	µL Working Control
750	75

4.1.3.4 Additional or alternative concentrations may be used at the discretion of the analyst as long as the requirements in 4.2 are met.

4.1.4 Negative Control Sample Preparation

4.1.4.1 Add 1mL of negative whole blood to screw top tube.

4.1.5 Case Sample Preparation

4.1.5.1 Based on enzyme immunoassay screen results, samples may be diluted with negative whole blood prior to analysis.

4.1.5.2 The total volume of blood or diluted blood should be 1mL.

4.1.5.3 Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary.

4.1.5.4 Add 1mL neat or diluted sample to labeled extraction tube.

4.1.6 Internal Standard Addition

4.1.6.1 Add 100µL of internal standard mix to calibrators, controls and case samples. This results in an internal standard concentration of 100ng/mL.

4.1.6.2 Vortex and allow tubes to stand 15 - 30 minutes for sample equilibration.

4.1.7 Sample Preparation

4.1.7.1 Add 4mL DI water, vortex.

4.1.7.2 Add 2mL 100mM phosphate buffer (pH 6.0), vortex, allow to stand for 5-10 minutes.

4.1.7.3 Check pH. Sample pH should be 6.0 ± 0.5. Adjust as necessary with 100mM Monobasic sodium phosphate or 100mM Dibasic sodium phosphate.

4.1.7.4 Centrifuge for about 10 minutes at approximately 3400 - 3500rpm.

4.1.8 SPE Column Preparation

4.1.8.1 Insert valve liners and labeled SPE columns into appropriate location on vacuum manifold. For each following SPE step, allow to gravity flow or aspirate at ≤ 3 in. Hg to prevent sorbent drying.

4.1.8.2 Add 3mL methanol to the SPE column.

4.1.8.3 Add 3mL DI water to the SPE column.

4.1.8.4 Add 1mL 100mM Phosphate buffer (pH 6.00) to the SPE column.

4.1.9 Blood Extract Loading

4.1.9.1 Decant buffered blood extract onto the SPE column. Care should be taken that very little solid matter (from centrifugation of whole blood) is applied to the SPE column.

4.1.10 Column Clean-up

4.1.10.1 Add 2mL DI water to the column.

4.1.10.2 Add 2mL 100mM HCl to the column.

4.1.10.3 Add 3mL Methanol.

4.1.10.4 Increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥5 minutes (disc should be dry).

4.1.11 Compound Elution

4.1.11.1 Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled centrifuge tubes.

4.1.11.2 Add 3mL elution solvent (3.2.11) to the column.

4.1.11.3 Collect eluate with gravity flow or apply minimal vacuum.

4.1.12 Eluate Evaporation

4.1.12.1 Transfer centrifuge tube to Evaporative Concentrator. Evaporate solvent to dryness under a gentle stream of nitrogen at approximately 40°C.

4.1.13 Derivatization

4.1.13.1 Add 50µL ethyl acetate. Vortex for ≅15 seconds.

4.1.13.2 Add 50µL BSTFA + 1% TMCS.

4.1.13.3 Cap tubes and vortex briefly.

4.1.13.4 Heat tubes at 70°C for 20 minutes.

4.1.13.5 Remove from heat and allow to cool to room temperature.

4.1.13.6 Transfer derivative to labeled GC/MSD ALS vial with microinsert.

4.1.14 Preparation for GC-MS Run

4.1.14.1 Into Sequence log table, enter the case sample, calibrators, blanks and control information.

4.1.14.2 Load samples, calibrators, blank and controls into the quadrant rack as noted in the sequence table.

4.1.15 GC-MS Calibration Curve

4.1.15.1 The calibration curve must be established with a minimum of four data points.

4.1.15.2 Calibrators should be analyzed in order of increasing concentration.

4.1.15.3 The least squares line resulting from the analysis of calibrators must have a coefficient of correlation of ≥0.98.

4.1.15.4 If calibrators are run in duplicate, it is not required that duplicate calibration points are included as long as the linearity requirement is met.

4.2 GC and MSD Acquisition Parameters

4.2.1 Acquisition Parameters

4.2.1.1 Refer to instrument method printouts for acquisition parameters.

4.2.1.1.1 Instrument Run Parameters

- Inlet at 260 degrees Celsius, splitless injection, injection volume: 1ul
- Oven at 120 degrees Celsius, hold 0.5 minutes
- Ramp 40 degrees Celsius to 210 degrees Celsius
- Ramp 10 degrees Celsius to 290 degrees Celsius
- Final temperature: 290 degrees Celsius, hold at least 1.25 minutes
- Additional sample runs may be done with adjustments to these parameters, for example if there is a coeluting peak or a late eluting compound.

4.2.1.2 Current acquisition method must be stored centrally as a hard or electronic copy.

4.2.3 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Benzoylecgonine-TMS	240	256	361
Benzoylecgonine-TMS-D3	243	259	364
Benzoylecgonine-TMS-D6	243	354	369
Cocaine	182	198	303
Cocaine-D3	185	201	306
Cocaethylene	196	212	317
Cocaethylene-D3	199	215	320

4.3 Reporting Criteria

4.3.1 Qualitative Chromatographic Criteria

4.3.1.1 Acceptable retention time window established by calibrator is ± 0.2 minutes.

4.3.2 Qualitative Mass Spectral SIM Criteria

4.3.2.1 Ion ratios for the analyte and its corresponding internal standard, established by calibrators for target and qualifier ions, must not differ by more than ±20% (relative). Refer to section 4.3.3.5 for cutoff criteria.

4.3.2.2 Qualitative Mass Spectral Full Scan Criteria

4.3.2.2.1 Analytes may be confirmed from full scan data if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

4.3.3 Quantitative Mass Spectral Criteria (NOT APPROVED FOR REPORTING)

4.3.3.1 Refer to Section 4.4.2 for determination of when this method may be used for quantitative purposes.

4.3.3.2 Quantitative results can be accepted if the calculated concentration of all calibrator and control samples are within ±20% of their respective concentrations (relative).

4.3.3.3 Quantitation is achieved through the plotting of the target ion response ratio versus the concentration for each calibrator.

4.3.3.4 Quantitative values for case samples, calibrators and controls will be truncated for reporting purposes.

4.3.3.5 Administrative limit of detection (LOD) for Benzoylecgonine, Cocaine and Cocaethylene is 25ng/mL. Results < this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

4.3.3.6 If the concentration exceeds the calibration range, the sample must be appropriately diluted with negative whole blood for reanalysis. Alternatively, the analyte(s) may be reported using full scan data; refer to section 4.3.2 for criteria.

4.4 Reporting of Results

4.4.1 Qualitative Confirmation

4.4.1.1 If Cocaine, Benzoylecgonine and Cocaethylene meet confirmation criteria, they may be reported. The administrative cut-off of 25ng/mL, or the lowest calibrator meeting quality assurance requirements, will be used to determine if the analyte is detected.

4.4.2 Quantitative Value

4.4.2.1 Currently, this method is only approved for the qualitative identification of drugs. Quantitative values are not to be reported or expressed. They are currently being used to establish an administrative cut off. Once the uncertainty of measurement is established for this method, it will be evaluated for quantitative reporting.

4.5 Quality Assurance Requirements

4.5.1 General

4.5.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

4.5.1.2 Refer to Toxicology AM #15, AM #16, AM #19and AM #21 for quality assurance and reference material authentication requirements.

4.5.2 Per Analysis Run Quality Requirements

4.5.2.1 A solvent blank must follow the highest calibrator, as well as precede each case sample.

4.5.2.2 A minimum of the spiked blood controls described in section 3.10.4.6.3 must be run per batch of samples. Controls should not be grouped at the beginning of the acquisition sequence. Rather, controls should be interspersed throughout the sequence.

4.5.2.3 If the number of case samples exceeds 10, in addition to the two spiked described in 3.10.4.6.3, one spiked or commercially obtained blood control must be run for each additional 10 case samples. Additional concentrations may be used.

4.5.2.4 Analysts may combine their samples into a single run to conserve supplies. However, each analyst with samples in the run must independently comply with the control requirements in section 4.5.2. A third-party reviewer must independently review the central file packet for compliance to method requirements.

4.5.2.5 If a drug other than Cocaine, Benzoylecgonine, or Cocaethylene is to be identified in full scan acquisition mode, one additional in-run control verifying the extraction of that compound is required. Multiple compounds may be extracted simultaneously.

4.5.3 Monitoring of Control Values (Currently Not a Requirement)

4.5.3.1 Once the method has been approved for quantitative purposes, the following is required: upon the completion of analysis, input blood control values on a spreadsheet used to assess uncertainty for this method.

4.6 Analysis Documentation

4.6.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.6.2 Original data for controls and standards will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

4.6.3 A copy of controls and standards may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of the control and standard printouts can be prepared from the centrally stored document.

5.0 Work Instructions

5.1 Quality Assurance Material

5.1.1 Working Reference Material Solutions

NOTE: Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. Working reference material solutions are stable for 6 months when stored under refrigeration or in a freezer.

5.1.1.1 *10ng/µL*

5.1.1.1.1 Add 100.0µL each 1mg/mL Benzoylecgonine, Cocaine and Cocaethylene (optional) Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

5.1.1.2 *1ng/µL*

5.1.1.2.1 Add 1.0mL 10ng/µL working solution to ≅8mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

5.1.2 Calibrator Preparation

5.1.2.1 Add the volume of working Benzoylecgonine, Cocaine and Cocaethylene (optional) mixed RM to appropriate tube as indicated below.

Level	ng/mL	Working Solution (ng/µL)	Volume to add (µL)
1	25	1	25
2	50	1	50
3	100	1	100
4	250	10	25
5	500	10	50
6	1000	10	100

5.1.3 Spiked Positive Control Preparation

5.1.3.1 Add the volume of working Benzoylecgonine, Cocaine and Cocaethylene (optional) mixed RM to appropriate tube as indicated below.

ng/mL	Working Solution (ng/µL)	Volume to add (µL)
75	1	75
750	10	75

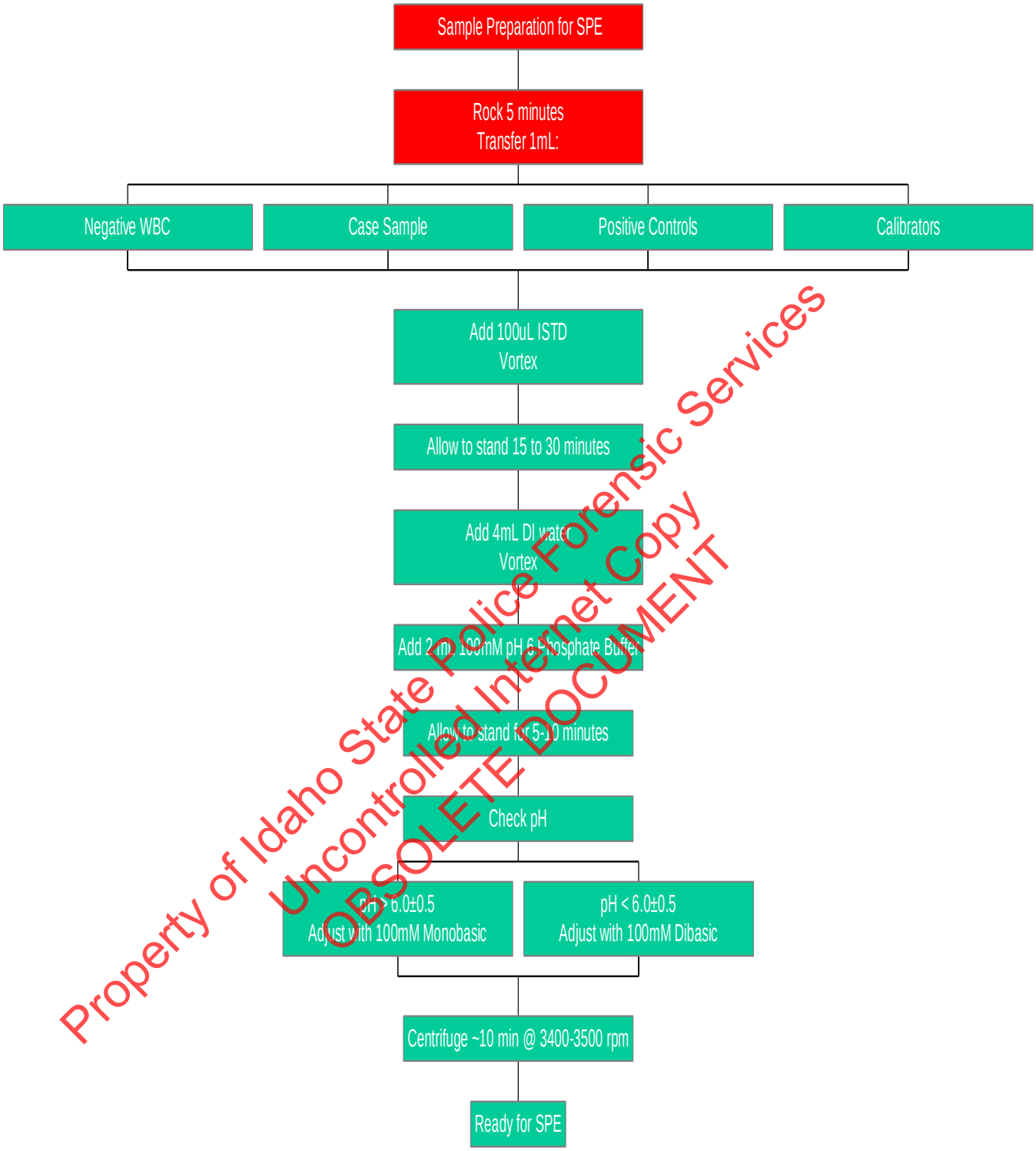
5.1.4 1ng/µL Working Internal Standard Solution

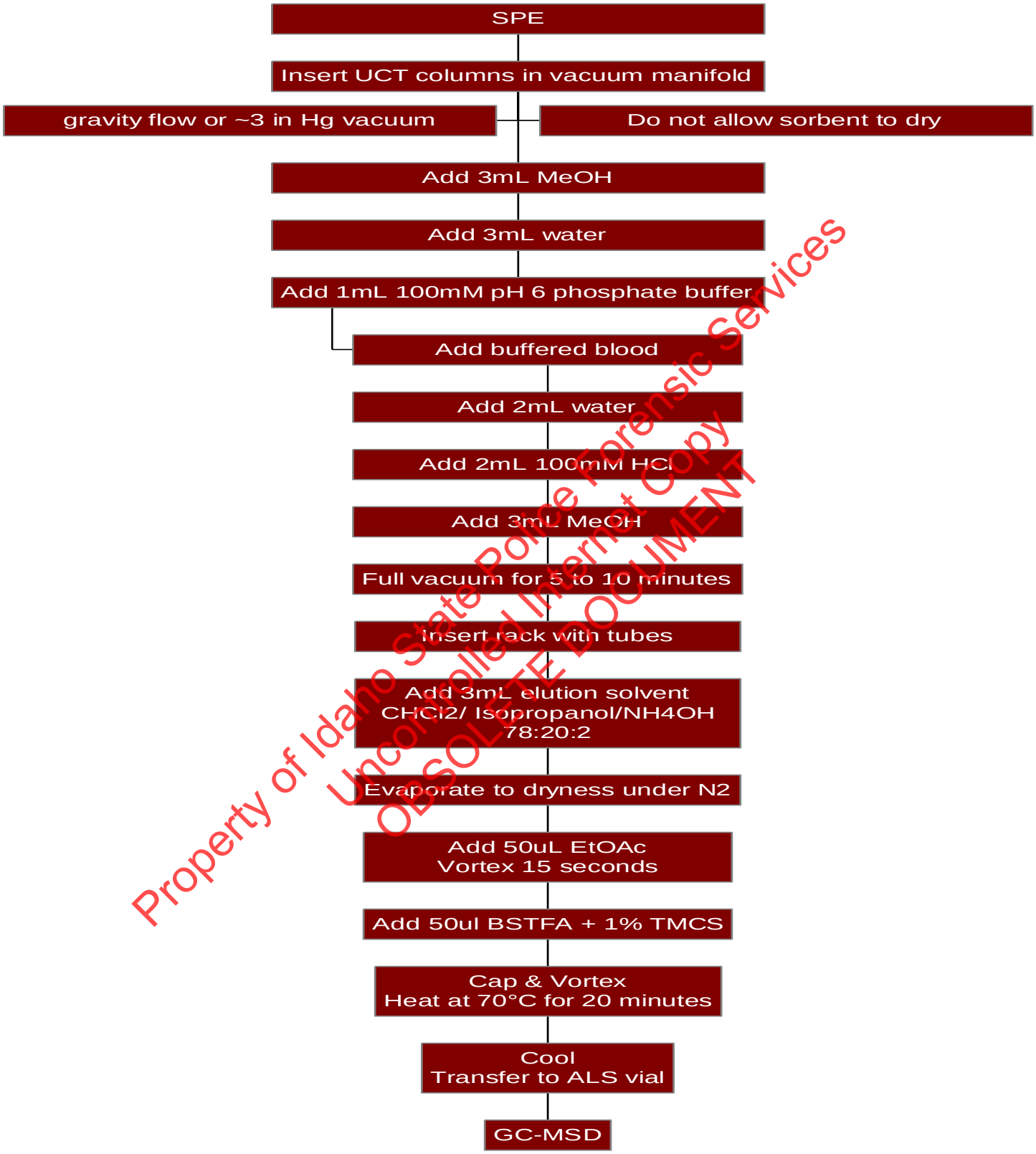
5.1.4.1 Add 100.0µL each 100µg/mL (100ng/µL) Stock Solution of Benzoylecgonine-D3 or –D6, Cocaine-D3 and Cocaethylene-D3 (add if included above) to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. *Solution is stable for 6 months when stored under refrigeration or in a freezer.*

5.1.5 Optional: Commercial Positive Control

5.1.5.1 Control must contain a minimum of Benzoylecgonine and Cocaine each at a target concentration of 100ng/mL. Refer to package insert for verified value and expected range.

5.2 Extraction Method





Toxicology AM #13: LCMS-QQQ Confirmation of Benzodiazepines and Z drugs in Blood and Urine

1.0 Background/References

1.1 Background

Benzodiazepines continue to be the most prescribed group of therapeutic agents. Approximately 20 benzodiazepines are approved for use in the US.[2] Benzodiazepines were first introduced in the 1960s in pursuit of the perfect sedative hypnotic agent, and have replaced barbiturates as the major class of central nervous system (CNS)-depressant drugs.[2] In 1962, Chlordiazepoxide (Librium®) was introduced, followed by the introduction of Diazepam (Valium®) in 1968. There are four main classes of benzodiazepines, the 1,4-benzodiazepines, the triazolobenzodiazepines, the diazolobenzodiazepines, and the 7-nitrobenzodiazepines.

Benzodiazepines are used primarily as anti-epileptics in the treatment of seizure disorders, as anxiolytics for the short-term relief of anxiety disorders, as sedative-hypnotics for the treatment of sleep disorders, and as muscle relaxants to relieve spasticity. The primary side effects that accompany their use include dose-related extensions of the intended actions, including sedation and sleepiness/drowsiness. In addition, other undesired effects that will influence the outcome of field sobriety tests include ataxia, a blocked ability to coordinate movements, a staggering walk and/or poor balance, lethargy/apathy, indifference or sluggishness, mental confusion, disorientation, slurred speech, and amnesia. Impairment of motor abilities, especially a person's ability to drive an automobile, is common. This impairment is compounded by the drug-induced suppression of one's' ability to assess their own level of physical and mental impairment. Alcohol combined with other CNS depressants (e.g., barbiturates antidepressants, etc.) will increase CNS depressant effects, such as impairment of psychomotor function and sedation, in an additive manner.[4-6]

Z drugs (zolpidem, zopiclone), prescribed as sleep aids, and quetiapine which is used in the treatment of mental disorders act in a similar manner to benzodiazepines, but are not included in that particular class of drugs.

The benzodiazepines are lipid soluble and are absorbed well from the GI tract with good distribution to the brain. They are metabolized primarily in the liver. Their CNS active metabolites extend their duration of action. The benzodiazepines work by enhancing, facilitating or potentiating the action of the inhibitory neurotransmitter GABA. They serve to increase the frequency of GABA-mediated chloride ion channel opening.

Benzodiazepines are metabolized primarily in the liver via several different microsomal enzyme systems.[6] Many products of their metabolism are active. Since many of the active metabolites have been marketed as therapeutic agents, it may be difficult to ascertain which drug was ingested based solely upon the results of analysis. Current drug therapy will assist in determining the source of a particular compound. The detection of a particular agent is determined partly by whether its metabolism yields active metabolites. Excretion of the benzodiazepines is predominantly in the urine. Depending upon the particular benzodiazepine, the urine may contain parent compounds, N-dealkylation and oxidative (hydroxylation) metabolism products and/or glucuronide conjugates.

1.2 References

1.2.1 This method was developed in conjunction with Agilent. Patrick Friel from Agilent came to the Idaho State Police Forensic lab located in Coeur d'Alene and provided application training July 23-26, 2012.

1.2.2 Williamson S.C, ISP Toxicology Analytical Method 2.4.3 (Archived- See Toxicology AM

1.2.3 Levine, B. *Central Nervous System Depressants*. pp. 191-197. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 1999.

1.2.4 Huang, W. and Moody, D.E. *Immunoassay Detection of Benzodiazepines and Benzodiazepine Metabolites in Blood.* J. Anal. Tox. **19**:333-342, 1995.

1.2.5 Fu, S. Molnar, A. Bowen, P. Lewis J. Wang H. *Reduction of Temazepam to Diazepam and Lorazepam to Delorazepam During Enzyme Hydrolysis.* Anal Bioanal Chem 400: 153-164, 2011.

1.2.6 Julien, R.M. *A Primer of Drug Action*. pp. 95-107. W.H. Freeman and Company: NewYork, 1998.

1.2.7 Hobbs, W.R., Rall, T.W. and Verdoorn, T.A. *Hypnotics and Sedatives*. pp. 362-373. *in:* Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Hardman, J.G. ed., McGraw-Hill, 1996.

2.0 Scope

2.1 This method is used for the confirmation of 7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam in blood and urine. The words *calibrator* and *calibration* are used to coincide with the terminology in instrument software and manufacturer manuals. The manufacturer's term *calibrator* refers to what is considered by ISP-FS as reference material that has a certified concentration of drug present.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B or equivalent LC/MS/MS system and MassHunter software

3.1.2 De-Tox A Tubes (or equivalent Toxi A tubes)

3.1.3 Tapered glass tubes for evaporation and reconstitution

3.1.4 Transfer pipettes

3.1.5 Pipettes for accurate dispensing of volumes 10 µL to 4 mL

3.1.6 Auto-sampler vials with snap-caps for Agilent 1260 ALS

3.1.7 Test tube rocker or rotator

3.1.8 Centrifuge capable of 3000 rpm

3.1.9 Oven capable of 60°C

3.2 Reagents

Refer to AM#23 for Solution Preparation instructions.

3.2.1 BG100 β-Glucuronidase Solution (Kura Biotec)

3.2.2 2M Acetate buffer, pH 4.8

3.2.3 0.1% formic acid in water (mobile phase A)

3.2.4 0.1% formic acid in acetonitrile (mobile phase B)

3.2.5 Deionized water

3.2.6 LC/MS grade water

3.2.7 LC/MS grade acetonitrile

3.2.8 LC/MS grade methanol

3.2.9 LC/MS grade formic acid

3.2.10 Extract reconstitution solvent: 9:1 mobile phase A to mobile phase B

3.3 Qualitative Assurance: Reference Materials and Controls

3.3.1 Calibrator and Control Solutions

NOTE: Corresponding calibrator and control reference material must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available.

NOTE: Stock solution concentrations other than those listed here may be obtained, but appropriate addition volume adjustments must be made when direct spiking or preparing working solutions. Stock solutions should be stored as recommended by vendor.

3.3.1.1 Reference Material Stock Solutions

3.3.1.1.1 *1mg/mL* single component benzodiazepine-class reference solutions. A multi-component benzodiazepines mix (250 µg/mL) may be obtained for use in controls.

3.3.1.2 Reference Material Working Solutions

3.3.1.2.1 Refer to Appendix 1 for the preparation instructions and stability of the working solutions.

3.3.1.3 Internal Standard Solutions

3.3.1.3.1 Stock Solution (100 µg/mL)

- 7-Aminoflunitrazepam-D7
- Alphahydroxyalprazolam-D5
- Oxazepam-D5
- Nordiazepam-D5

- Clonazepam-D4
- Temazepam-D5
- Diazepam-D5

3.3.1.3.2 Working Solution

- Refer to Appendix 1 for the preparation instructions and stability of the working solution.

Sections 3.3.2 – 3.3.4 include Required Extracted Controls for all options contained in this method:

3.3.2 Extracted Negative Control

An extracted negative control will be run for each matrix that is included in the run. The controls may be commercially obtained or in-house urine or blood verified to be negative for drugs of interest.

3.3.3 Extracted Positive Control

An extracted positive control will be run for each matrix that is included in a run. Positive Controls can be prepared with single or multi-component working solutions and/or obtained commercially. The positive control must have at least two compounds in it that are included in the scope of the method. Controls should contain an approximate concentration between 75 ng/mL and 400 ng/mL. *The compounds in the controls **cannot** be the same lots as were used for the calibrators*. For the control to be considered passing, it should give a response greater than 50 ng/mL for each intended analyte.

3.3.4 Extracted Glucuronide Controls (URINE ONLY).

Positive and negative glucuronide controls are required for any run that includes urine samples. These controls may be obtained commercially or prepared in-house by spiking negative urine. The same lot of negative urine must be used to prepare both the positive and negative glucuronide controls. Oxazepam-glucuronide or Lorazepam-glucuronide may be used; approximate concentration of controls should be 300ng/mL.

3.3.4.1 Stock Solution

100µg/mL Oxazepam- or Lorazepam-Glucuronide

3.3.4.2 Direct spiking

3.3.4.2.1 Spike negative urine with 3µL of 100µg/mL stock solution or 30 µL working solution.

3.3.4.3 Working Glucuronide Solution (10ng/µL)

3.3.4.3.1 Add 1mL 100µg/mL Stock Solution, Q.S. to 10mL with MeOH (LC Grade). Solution is stable for one year when stored under refrigeration or in a freezer.

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Calibrator preparation (calibrators may be prepared in advanced and re-run if they were prepared with the same internal standard as the samples)

4.1.1.1 Label a conical glass tube for each calibrator. Add 100 µL of 1.0 µg/mL ISTD mix to each tube, as well as the following volumes of reference material. Evaporate to dryness.

Sample Type	1.0 µg/mL Target mix
Blank	-
10 ng/mL Cal 2	10 µL
25 ng/mL Cal 3	25 µL
50 ng/mL Cal 4	50 µL
100 ng/mL Cal 5	100 µL

Sample type	10.0 µg/mL Target mix
500 ng/mL Cal 6	50 µL
1000 ng/mL Cal 7	100 µL
3000 ng/mL Cal 8 (Urine ONLY)	300 µL

4.1.1.2 Reconstitute in 100 µL 9:1 mobile phase A to mobile phase B.

4.1.1.3 Label autosampler vials to correspond to the evaporation tubes.

4.1.1.4 Transfer most of the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap the vials.

4.1.2 Casework Sample and Control Preparation

4.1.2.1 Casework and Control Samples (Blood or Urine)

4.1.2.1.1 Transfer 1.0 mL casework and controls to labeled conical tubes. (Not required for blood samples- these may be added directly into the De-Tox extraction tube A)

4.1.2.2 Internal Standard Addition

4.1.2.2.1 Add 100 µL of 1.0 µg/mL ISTD mix to labeled conical glass tube for each blank, QC and case sample. Vortex to mix.

4.1.2.3 Sample Hydrolysis (Urine Samples Only)

4.1.2.3.1 Enzyme hydrolysis: add 20µL 2M acetate buffer to all controls and case samples, and 76µl BG100 β-glucuronidase to calibrators, controls and casework samples (except the negative glucuronidase control sample). Cap and gently vortex the samples. Incubate at approximately 60°C in an oven for 30 minutes. Remove from oven and allow to cool.

4.1.2.4 Extraction

4.1.2.4.1 Label a De-Tox Tube A for each QC, blank, and case sample.

4.1.2.4.2 To the De-Tox Tubes, add ~4 mL of deionized water to each tube (or add the 4 mLs to the conical tubes with the samples).

4.1.2.4.3 Transfer the casework and control samples with added ISTD from the labeled conical tube to the corresponding De-Tox Tube (*for blood samples, the ISTD and sample may be added directly to the De-Tox tube. There is no requirement to place it in a conical tube first.*)

4.1.2.4.4 Cap the De-Tox Tubes and mix by inverting.

4.1.2.4.5 Rotate or rock the tubes gently for ~5 minutes.

4.1.2.4.6 Centrifuge the tubes at approximately 2000-2500 rpm for ~ 5 minutes.
NOTE: If an emulsion occurs, it may be broken up with a disposable transfer pipette and the tube re-centrifuged at approximately 3000 rpm for ~5 minutes. Care should be taken that no solvent is lost to the disposable pipette when the emulsion is broken up.

4.1.2.4.7 Transfer most (~2 mL) of the upper organic layer from each De- Tox Tube to the corresponding labeled evaporation tube. Avoid transferring any solids.

4.1.2.4.8 Evaporate to dryness under nitrogen at ~ 40 degrees Celsius. It is critical that the extracts are evaporated completely to dryness.

4.1.2.5 Reconstitution

4.1.3.5.1 Reconstitute in 100 µL 9:1 mobile phase A to mobile phase B.

4.1.3.5.2 Transfer the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap.

4.1.3 Instrument/Run set up

4.1.3.1 Refer to Toxicology AM #24 for general LCMS instrument operation and maintenance.

4.1.3.2 Cycle time for the method is approximately 13 minutes.

4.1.3.3 Instrument acquisition method will be printed and stored near the instrument (this may also be done electronically and stored in a central location)

4.1.3.3.1 Acquisition Required Settings

- Column Temperature - 40*C
- Injection volume – 2uL - 5uL
- Mobile Phase Flow rate - 0.5 mL/min
- Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0.00	90	10
4.00	70	30
8.00	60	40
8.50	5	95
10.50	5	95
11.0	90	10

4.1.4 Data Analysis

4.1.4.1 Refer to Toxicology AM #24 for general instructions on instrument operation, Data Analysis and report generation.

4.1.5 Analytical Method Specific Batch Review:

4.1.5.1 The lab criterion for acceptable calibration curve R^2 is $\geq$0.975. A minimum of four calibration points are required for a valid curve. If the confirmation decision point (25 ng/mL) is removed from the curve, the new administrative cutoff will be the lowest calibrator that meets quality assurance requirements (excluding the 10 ng/mL data point). If the 10 ng/mL calibration point for a compound is removed from the curve, no results of "inconclusive" may be reported for that compound in that batch.

4.1.5.2 The default criterion for Accuracy is that each calibrator result should agree with the target value $\pm$ 20%. For values below 10ng/mL the results should be within $\pm$ 30% of the target value.

4.1.5.3 The default criteria for a positive result are:

4.1.5.3.1 The sample must have a concentration greater than the 25ng/mL calibrator; samples that meet all other criteria for identification but fall between the 10 ng/mL calibrator and 25 ng/mL calibrator can be reported as inconclusive. (See section 4.4.3 for the exceptions regarding Nitrazepam reporting). Samples with concentrations exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met.

4.1.5.3.2 Inconclusive samples are those that meet all other criteria for identification but fall between the 10ng/mL calibrator and the administrative cutoff (See section 4.1.6.2.1 for cutoff requirements and 4.4.1 and 4.4.3 for the exceptions regarding Diazepam and Nitrazepam). Samples with concentrations exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met.

4.2 Quality Assurance Requirements:

4.2.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication requirements.

4.3 Analysis Documentation

4.3.1 The printed results for each case sample will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.3.2 Reports for the batch, including calibration curves and controls, if printed, will be stored centrally in the lab in which the analysis was performed. A copy of data for controls may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

4.3.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

4.4 Limitations of Method

4.4.1 The hydrolysis process for glucuronides in urine has limited efficiency; based on the validation study, the estimated conversion is about 60-70 percent. There is potential for a small amount of Temazepam to convert to diazepam in the hydrolysis process. If both diazepam and Temazepam are detected in a urine sample, the diazepam will not be reported unless it has a response that is greater than 5% of the Temazepam response.

4.4.2 This method is only approved for qualitative identification of the listed compounds in urine and blood. *The uncertainty associated with the quantitative values has not been established; therefore, no values shall be referenced or reported.*

4.4.3 Nitrazepam has been found to have significant variability in concentration responses with this method, though no false positives have been observed. If a case sample gives a nitrazepam response that is >5 ng/mL and <50 ng/mL, it will be reported as "inconclusive for nitrazepam due to method limitations."

5.0 Work Instructions

5.1 Quality Assurance Material

5.1.1 Working Reference Material Solutions

NOTE: Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. Working reference material solutions are stable for 12 months when stored under refrigeration or in a freezer.

5.1.1.1 Calibrators and Controls

Stock Solutions

- 1mg/mL or 250ug/mL single component benzodiazepine-class reference solutions.

5.1.1.1.1 Calibrator Solutions

The calibrator solutions must contain 7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam

5.1.1.1.2 1.0 µg/mL Target mix in methanol

- Add 10uL each 1mg/mL or 40uL 250µg/mL Stock Solution to ~ 6mL MeOH (LCMS grade) in 10mL ball flask. QS with LC/MS grade MeOH.

5.1.1.1.3 10.0 µg/mL Target mix in methanol

- Add 250uL each 1mg/mL Stock Solution to ~20mL MeOH (LCMS grade) in 25mL ball flask. QS with LC/MS grade MeOH.

5.1.2 Calibrator Preparation

5.1.2.1 Add the volume of working calibrator working solution to appropriate tube as indicated below.

Level	ng/mL	Working Solution (ng/µL)	Volume to add (µL)
Blank	-	-	-
2	10	1	10
3	25	1	25
4	50	1	50
5	100	1	100
6	500	10	50
7	1000	10	100
8	3000 (Urine ONLY)	10	300

5.1.3 Positive Control

5.1.3.1 At minimum, the control must contain two compounds included in the scope of the method and fall between the approximate concentrations of 75 and 400 ng/mL.

5.1.4 Conjugated Controls (Urine samples only)

5.1.4.1 Spiked Negative urine (must be the same lot for both positive and negative Glucuronide controls)

5.1.4.2 Stock Solution

- 100µg/mL Oxazepam Glucuronide or Lorazepam Glucuronide

5.1.4.2.1 Direct spiking

- Spike negative urine with 3µL of 100µg/mL stock solution or 30µL working solution.

5.1.4.2.2 Working Glucuronide Solution (10ng/µL)

- Add 1mL 100µg/mL Stock Solution to 10mL MeOH, adjustments to the total volume may made (for example: 250ul stock to 2.5mL MeOH.) Solution is stable for one year when stored under refrigeration or in a freezer.

5.1.5 Internal Standard

5.1.5.1 Stock Solution

- 100 µg/mL 7-aminoflunitrazepam-D7, alphahydroxyalprazolam-D5, oxazepam-D5, nordiazepam-D5, clonazepam-D4, temazepam-D5, diazepam-D5

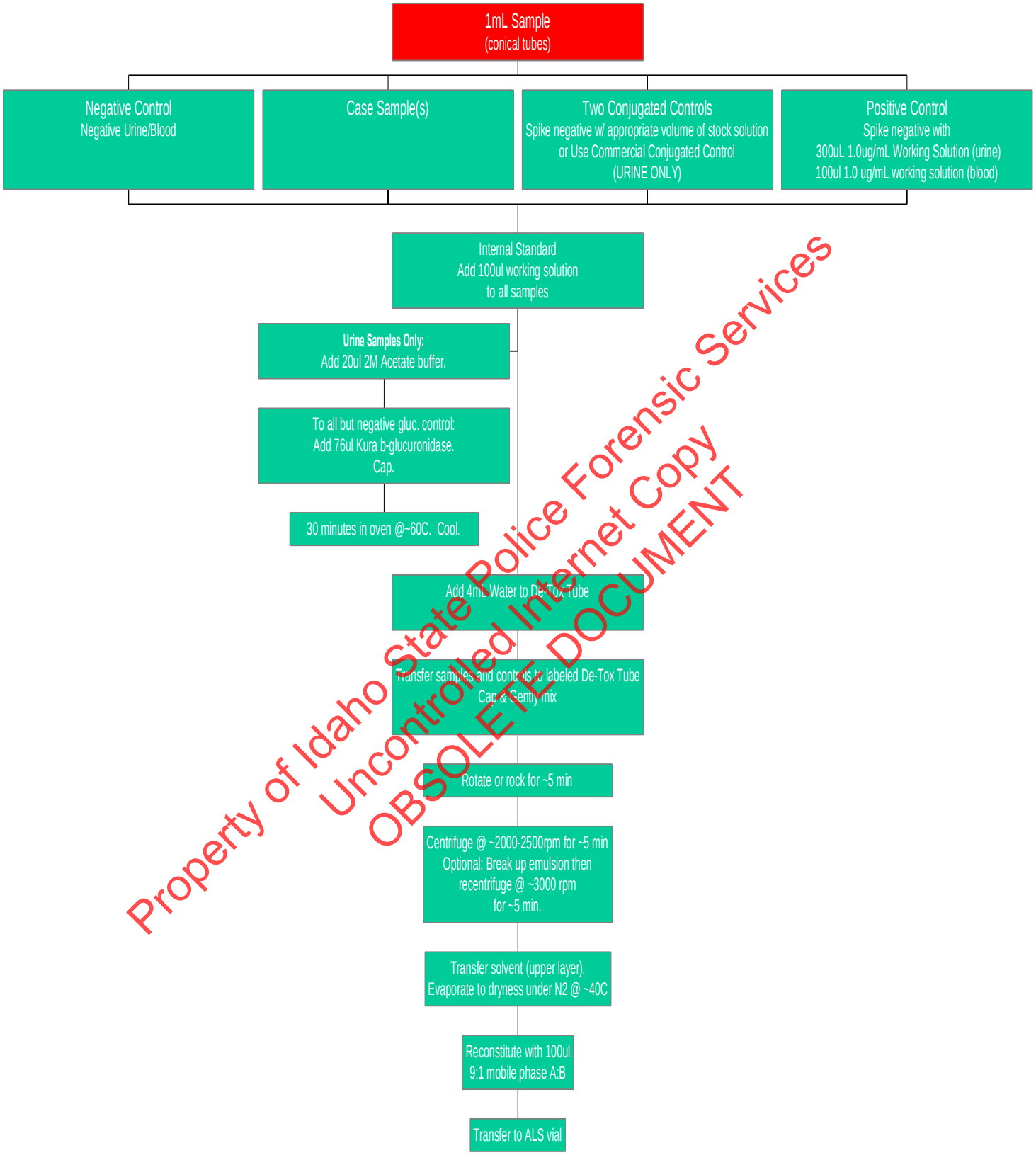
5.1.5.2 Working Internal Standard Solution (1.0 µg/mL ISTD mix in methanol)

- Add 100µl of 100µg/mL stock solution to ~9mL MeOH in 10mL ball flask. QS with LCMS grade MeOH. Solution is stable for one year when stored under refrigeration or in a freezer.

5.2 Comments

This method has instructions for the preparation of both urine and blood casework samples.

5.3 Sample Preparation:



6.0 Comments

6.1 Appendix IV:

6.1.1 - 1µg/mL Target mix in methanol

(Document on a prep sheet with an expiration of the earliest expiring reference material or one year whichever is first, store under refrigeration or in a freezer)

- In a 10 mL volumetric flask fill the flask about half full with methanol, add 10 µL of 1mg/mL stock solution of the following compounds. (If the stock solution is a different concentration, you will need to adjust addition volumes.)
- 7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam
- QS with methanol and ensure it is thoroughly mixed.

6.1.2 -10µg/mL Target mix in methanol

(Document on a prep sheet with an expiration of one year, store under refrigeration or in a freezer)

- In a 25 mL volumetric flask fill the flask about half full with methanol add 250 µl of 1mg/mL stock solution of the following compounds.
- 7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam
- QS with methanol and ensure it is thoroughly mixed.

6.1.3 - 1µg/mL ISTD mix in methanol

(Document on a prep sheet with an expiration of one year, store under refrigeration or in a freezer)

- Fill a 10 mL volumetric flask about half full with methanol, add 100 µl of 100ug/mL stock solution of the following compounds. (If the stock solution is a different concentration you will need to adjust addition volumes.)
- 7-aminoflunitrazepam-D7, alphahydroxyalprazolam-D5, oxazepam-D5, nordiazepam-D5, clonazepam-D4, temazepam-D5, diazepam-D5
- QS with methanol and ensure it is thoroughly mixed.

6.1.4 - 1µg/mL Control mix in methanol

(Document on a prep sheet with an expiration of the earliest expiring reference material or one year whichever is first, store under refrigeration or in a freezer)

- Add 10µL each 1mg/mL or 40µL 250µg/mL Stock Solution to ≅ 6mL MeOH (LCMS grade) in 10mL ball flask. QS with LC/MS grade MeOH.

Toxicology AM #14: LCMS-QQQ Confirmation of Cannabinoids in Blood and Urine

1.0 Background/References

1.1 Background

Δ^9-THC (Figure 1) is the chief psychoactive cannabinoid resulting from exposure to marijuana. Δ^9-THC has a peak blood concentration within 5 to 15 minutes following smoking of a marijuana cigarette.[4,5,6] This blood concentration drops rapidly after cessation of smoking.[4,5] The level may fall to less than 5ng within 30 to 60 minutes, although longer detection times have been reported.[4,5] Detection of low dose (1.75%) post-smoking Δ^9-THC has been reported to vary from 3 to 12 hours.[5] This detection window was based on a limit of quantitation of 0.5ng/mL. The number, duration, and spacing of puffs, hold time, and inhalation volume all impact the degree of drug exposure and thus bioavailability.[5] Longer detection times have been observed for frequent users. Δ^9-THC is metabolized to an active metabolite, 11-hydroxy-tetrahydrocannabinol(Hydroxy-THC, THC-OH), and an inactive metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (carboxy-THC). The concentration of the Δ^9-THC inactive metabolite, carboxy-THC, gradually increases and may plateau for several hours.[5] There is poor correlation between blood Δ^9-THC and psychoactive effects since the Δ^9-THC concentrations begin to decline prior to the time of peak effects.[4,5,6] The detection window for the active analytes is much shorter than that of carboxy-THC.[7] Cannabinol and cannabidiol are minor cannabinoids also found in marijuana.[8,9] Due to their low abundance, rapid clearance from blood, and similar pharmacokinetic profiles in chronic versus occasional smokers, the presence of cannabidiol and cannabinol in blood has been suggested to be a sufficient but unnecessary marker for recent marijuana use.[8]

Negative behavioral effects reported from exposure to marijuana include altered time perception, lack of concentration, impaired learning and memory which can lead to impairment of cognitive and performance tasks.[5] Establishing impairment in an individual is based on evaluation of all available information in conjunction with quantitative blood levels.

For additional background, refer to Toxicology AM#3 and obsolete analytical methods 3.10.1, as well as provided references.

Figure 1.

1.2 References

1.2.1 This method was obtained independently from Agilent and Washington State Patrol (WSP) Toxicology Laboratory. Patrick Friel from Agilent came to the Idaho State Police Forensic lab located in Coeur d'Alene and provided application training July 23-26, 2012. Amanda Black (Quality Manager at WSP) provided copies of their validation documents to assist with the validation of this method in the ISP Forensic Services laboratory system.

1.2.2 Dr. Ryan van Wagoner with Sports Medicine Research and Testing Laboratory (SMRTL), provided consultation services for the validation of this project. This was funded by the National Institute of Justice under the 2013 Paul Coverdell Forensic Science Improvement Grants Program. The grant number is 70048 13NFSI00.

1.2.3 Williamson S.C, ISP Toxicology Analytical Methods 2.4.4 and 3.10.1.

1.2.4 Standard Operating Procedure for Blood SPE THC and Carboxy-THC GC/MSD Assay, Edmonton, Canada Office of the Chief Medical Examiners, 2003.

1.2.5 Huestis, M.A., *Cannabis (Marijuana) - Effects on Human Behavior and Performance,* Forensic Science Rev. 14(1/2): 16-60, 2002.

1.2.6 Drummer, O.H., *Cannabis*, pp. 178-212. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.7 Huestis, M. *Marijuana*. pp. 229-244. *in:* Principles of Forensic Toxicology, Second Edition. Levine, B. ed., AACC, 2003.

1.2.8 Desrosiers, N.A.; Himes, S.K.; Scheidweiler, K.B.; Concheiro-Guisan, M.; Huestis, M.A. *Phase I and II Cannbinoid Disposition in Blood and Plasma of Occasional and Frequent Smokers Following Controlled Smoked Cannabis*. Clinical Chemistry, 60:4, pp. 631-643, 2014.

1.2.9 Nadulski, T., et al. *Simultaneous and Sensitive Analysis of THC, 11-OH0THC, THC-COOH, CBD, and CBN by GC-MS in Plasma after Oral Application of Small Doses of THC and Cannabis Extract*. Journal of Analytical Toxicology, Vol 29, pp. 782-789, November/December 2005.

2.0 Scope

2.1 This method is used for the confirmation of Δ^9-THC, 11-nor-Δ^9-THC-9-COOH (Carboxy-THC), 11-hydroxy-Δ^9-THC (Hydroxy-THC), and cannabidiol (optional) in blood and urine. The words *calibrator* and *calibration* are used to coincide with the terminology in instrument software and manufacturer manuals. The manufacturer's term *calibrator* refers to what is considered by ISP-FS as reference material that has a certified concentration of drug present.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B or equivalent LC/MS/MS system and MassHunter software

3.1.2 16x100mm silanized extraction tubes & caps

3.1.3 Tapered glass tubes for evaporation and reconstitution

3.1.4 Glass transfer pipettes

3.1.5 Pipettes for accurate dispensing of volumes 10 µL to 4 mL

3.1.6 Auto-sampler vials with snap-caps for Agilent 1200 and/or 1260 ALS

3.1.7 Flat-bottomed inserts compatible with the ALS vials in 3.1.6

3.1.8 Test tube rocker or rotator

3.1.9 Centrifuge

3.1.10 Oven or Waterbath

3.1.11 Evaporative concentrator

3.2 Reagents

Refer to Toxicology AM #23 for Solution Preparation instructions.

3.2.1 Glacial Acetic Acid

3.2.2 10N NaOH

3.2.3 10% Acetic Acid in Water

3.2.4 Hexane

3.2.5 Ethyl Acetate

3.2.6 LC/MS grade Water

3.2.7 Deionized Water

3.2.8 LC/MS grade Acetonitrile

3.2.9 Extract reconstitution solvent: 1:1 Water: Acetonitrile (LC/MS grade only)

3.2.10 LC/MS grade formic acid (optional)

3.2.11 0.1% formic acid in water (mobile phase A)

3.2.12 0.1% formic acid in acetonitrile (mobile phase B)

3.3 Qualitative Assurance: Reference Materials and Controls

3.3.1 Calibrator and Control Solutions

NOTE: Corresponding calibrator and control reference material shall be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. NOTE: Stock solution concentrations other than those listed here may be obtained, but appropriate addition volume adjustments must be made when direct spiking or preparing working solutions. Stock solutions should be stored as recommended by vendor. Appropriate vendors for the stock solutions include Cerilliant and Grace. Additional vendors may be used provided that the standards are run in addition to ones known to work and provide comparable responses and ion ratios

3.3.1.1 Reference Material Stock Solutions

3.3.1.1.1 Single component cannabinoid-class reference solutions

- Δ^9-THC
- 11-nor-Δ^9-THC-9-COOH
- 11-hydroxy-Δ^9-THC
- Cannabidiol (optional)

3.3.1.1.2 Reference Material Working Solutions

- Refer to Appendix 1 for the preparation instructions and stability of the working solutions.

3.3.1.2 Internal Standard Solutions

3.3.1.2.1 Stock Solution (100 µg/mL)

- Δ^9-THC-D3
- 11-nor-Δ^9-THC-9-COOH-D3
- 11-hydroxy-Δ^9-THC –D3
- Cannabidiol-D3 (optional)

3.3.1.2.2 Working Solution

- Refer to Appendix 1 for the preparation instructions and stability of the working solution.

Sections 3.3.2 thru 3.3.4 are the required Extracted Controls for all options contained in this method:

3.3.2 Extracted Negative Control

3.3.2.1 An extracted negative control will be run for each matrix that is included in the run. The controls may be commercially obtained or in-house urine or blood verified to be negative for drugs of interest. The same lot of negative urine or blood should be used for extracted calibrators and all controls made in house.

3.3.3 Extracted Positive Control

NOTE: Extracted positive controls will be run for each matrix that is included in a run. Positive Controls can be prepared with single or multi-component working solutions and/or obtained commercially.

3.3.3.1 Urine

3.3.3.1.1 The positive control must have at least two compounds in it that are included in the scope of the method, at an approximate concentration between 5 and 25 ng/mL for all compounds except carboxy-THC. The response for carboxy-THC should be between 25 ng/mL and 125 ng/mL. (Note: spike with 50-250 µl of the low control working solution). *The compounds in the controls **cannot** be the same lots as were used for the calibrators*. For the control to be considered passing, it should give a response greater than the LOD for each intended analyte. *NOTE: carboxy-THC-glucuronide may be one of the compounds included in this control. If included in the positive control, a separate glucuronide control is not required. The concentration of the glucuronide compound shall meet criteria specified in section 6.1.2.5.4*

3.3.3.2 Blood

3.3.3.2.1 In blood, two positive controls should be run. One control shall be a low control, with a concentration between 5 ng/mL and 10 ng/mL for all compounds except carboxy-THC. The response for carboxy-THC should be between 25 ng/mL and 50 ng/mL. The second control shall be either a mid- or high-concentration control at an approximate concentration between 25 ng/mL and 100 ng/mL for all compounds except carboxy-THC. The response for the carboxy-THC should be between 125 ng/mL and 500 ng/mL. (Note: for the low control, spike with 50-100µl of the low control working solution and for the mid to high control, spike with 25-100 µl of the high control working solution).

3.3.3.2.2 Both positive controls must minimally contain two compounds included in the scope of the method as follows: any analytes being quantitated, and at least one other analyte. For the control to be considered passing, it should give a response within 20% of the target concentration for the analyte being reported quantitatively. For analytes being reported qualitatively, a response greater than the LOD is considered passing.

3.3.4 Extracted Glucuronide Controls (URINE ONLY)

NOTE: A positive glucuronide control is required for any run that includes urine samples. This control may be obtained commercially or prepared in house by spiking negative urine. Carboxy-THC-glucuronide should be used, and the approximate concentration should be between 10 and 200 ng/mL.

3.3.4.1 Stock Solution

3.3.4.1.1 *100µg/mL* Carboxy-THC-Glucuronide

3.3.4.2 Working Glucuronide Solution (10ng/µL)

3.3.4.2.1 Add 10µL of 100µg/mL Stock Solution to 990µL of MeOH or Acetonitrile. *Solution is stable for one week when stored under refrigeration or in a freezer.*

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Calibrator Preparation (calibrators will be prepared and extracted with each run; calibrators must be prepared and extracted for each matrix included in the analysis run).

4.1.1.1 Label an extraction tube for each calibrator. Add 1 mL of matrix (blood or urine), then spike each with 10 µL of 1.0 µg/mL ISTD mix, as well as the following volumes of reference material. Extract as described in section 4.1.2. *NOTE: A minimum of 4 calibration points are required to establish a valid calibration curve.*

Sample Type	1.0/5.0 µg/mL Target Mix (Containing c-THC)	1.0/5.0 µg/mL Target Mix (Containing c-THC)	Or 1.0 µg/mL Target Mix (Without c-THC)	And 100 µg/mL c-THC stock
Blank	-	-	-	-
1/5 ng/mL - Cal 1	10 µL	-	-	-
2/10 ng/mL - Cal 2	20 µL	-	-	-
5/25 ng/mL - Cal 3	50 µL	-	-	-
10/50 ng/mL - Cal 4	100 µL	-	-	-
25/125 ng/mL - Cal 5	250 µL	25 µL	-	-
50/250 ng/mL - Cal 6	-	50 µL	50 µL	2.5 µL
100/500 ng/mL - Cal 7 (Optional for blood)	-	100 µL	100 µL	5 µL

4.1.2.1 Casework and Control Samples (Blood or Urine)

4.1.2.1.1 Transfer 1.0 mL casework and controls to extraction tubes.

4.1.2.2 Internal Standard Addition

4.1.2.2.1 Add 10 µL of 1.0 µg/mL ISTD mix to each blank, QC and case sample. Vortex to mix.

NOTE: Since the negative control can be used as a matrix blank (run before each sample), a larger reconstituted volume may be used for larger batches. Additional internal standard may be spiked into the negative control provided that the concurrent reconstitution volume maintains the correct ratios of 10µL ISTD to 100µL of reconstitution solvent. (E.g. if 40µL of ISTD is spiked into the negative control, it should be reconstituted in 400µL of reconstitution solvent in step 4.1.2.5.1 of the Extraction Procedure.)

4.1.2.3 Sample Hydrolysis (***Urine ONLY***)

4.1.2.3.1 Enzyme hydrolysis: add 40µL 10N NaOH to each calibrator, control and case sample. Vortex and verify that pH >10.

4.1.2.3.2 Verify water bath or oven temperature. Cap and incubate at 60°C for 20 minutes. Remove from heat and allow to cool.

4.1.2.3.3 Add 25 µL of Glacial Acetic Acid to each calibrator, control and case sample. Vortex and verify that pH is ~5.5 (pH 5-6 is acceptable). Proceed with extraction at section 4.1.3.4.3.

4.1.2.4 Extraction

4.1.2.4.1 Add 2mL of deionized water to each tube containing blood. Vortex to mix.

4.1.2.4.2 Add 0.8mL of 10% Acetic Acid in Water to each tube containing blood. Vortex to mix.

4.1.2.4.3 Add 8mL of 10% Ethyl Acetate in Hexane to each tube (blood) or 3mL of 10% Ethyl Acetate in Hexane to each tube (urine).

4.1.2.4.4 Rock or rotate tubes gently for no less than 10 minutes.

4.1.2.4.5 Centrifuge the tubes at approximately 2500-3500 rpm for 5 minutes.

4.1.3.4.6 Using a glass transfer pipette, transfer most of the upper organic layer from each tube to the corresponding labeled conical evaporation tube. Avoid transferring any solids.

4.1.2.4.7 Evaporate the organic phase to dryness under nitrogen at ~ 40 degrees Celsius. Start the airflow slowly (~5-10 psi) to avoid splashing tube contents. It is critical that the extracts are evaporated completely to dryness, but DO NOT over-dry.

4.1.2.5 Reconstitution

4.1.2.5.1 Reconstitute the dry extract in 100 µL 1:1 Acetonitrile:Water. (*NOTE: The reagents for this step shall be LC/MS grade.*)

4.1.2.5.2 With a **glass** pipette, transfer most of the reconstituted sample from the evaporation tube into to the corresponding auto-sampler vial with flat-bottom insert, and cap. *Be careful not to transfer any solids. If the reconstituted extract is cloudy or viscous, centrifuge at ~2000 rpm for ~5 minutes, then transfer only the supernatant to the ALS insert.*

4.1.3 Instrument and run set up

4.1.3.1 See Toxicology AM #24 for instrument maintenance and operation.

4.1.3.2 The cycle time for each injection is ~15 minutes.

4.1.3.3 Instrument acquisition method will be printed and stored near the instrument (this may also be done electronically and stored in a central location)

4.1.3.3.1 Acquisition Required Settings

- Column Temperature - 50*C
- Injection volume - 10uL –20uL
- Mobile Phase Flow rate - 0.5 mL/min
- Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0.00	60	40
1.00	60	40
7.00	5	95
10.00	5	95
10.50	60	40

4.1.4 Batch Review

4.1.4.1 The lab criterion for acceptable calibration curve R^2 is $\geq$0.98. Fit for the curve should be linear and weight should be 1/X.

4.1.4.2 The limit of detection (LOD) is the same as the limit of quantitation (LOQ) for blood quantitative analysis results. Once uncertainty of measurement has been established, Δ^9-THC may be reported quantitatively. For all other analytes in blood and all analytes in urine, only qualitative confirmation of results may be made. The established LODs for each compound are as follows:

Analyte	LOD
Δ^9-THC	3 ng/mL
11-nor-Δ^9-THC-9-COOH	10 ng/mL
11-hydroxy-Δ^9-THC	5 ng/mL
Cannabidiol	5 ng/mL

4.1.4.3 The default criterion for Accuracy is that each calibrator result should agree with the target value $\pm$ 20%. For values below 10ng/mL the results should be within $\pm$ 30% of the target value.

4.1.4.4 The default criteria for a positive result are:

4.1.4.4.1 The sample must have a concentration no less than the established LOD for each analyte. Samples with concentrations of qualitatively-reported analytes exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met. For compounds that are quantitatively reported, the concentration shall fall within the range established by the calibrators for each analyte, or reported as >100 ng/mL provided all other reporting criteria are met.

4.2 Quality Assurance Requirements

4.2.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication requirements.

4.3 Analysis Documentation

4.3.1 The printed results for each case sample will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

4.3.2 The reports for the batch, including calibration curves and controls will be stored centrally by the lab in which the analysis was performed. When necessary, a copy of control printouts can be prepared from the centrally stored document.

4.3.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

4.4 Limitations of Method

4.4.1 The hydrolysis process for glucuronides in urine has limited efficiency; based on the validation study, the estimated conversion is about 30-50 percent.

4.4.2 This method has only been evaluated for qualitative identification of the listed compounds in urine and blood. The uncertainty associated with the quantitative values has not been established; therefore, **quantitative values are not to be reported or expressed.**

5.0 Work Instructions

5.1 Reference Material

5.1.1 Calibrators and Controls

Stock Solutions

- 1 mg/mL or 100 µg/mL single component cannabinoid-class reference solutions.

5.1.1.1 Calibrator/Control Working Solutions

NOTE : The calibrator solutions must contain Δ⁹-THC, 11-nor-Δ⁹-THC-9-COOH, and 11-hydroxy-Δ⁹-THC. Cannabidiol is optional.

5.1.1.1.1 - *1.0* µg/mL Target Mix in methanol

- Add 10µL each (1mg/mL) or 100µL (100µg/mL) Stock Solution to ≅ 6mL MeOH (LCMS grade) in 10mL ball flask *EXCEPT carboxy-THC*. QS with MeOH. *Solution is stable for one-year when stored in the freezer.*

5.1.1.1.2 - *0.1/0.5* µg/mL Target Mix in methanol

- Add 1mL 1.0 µg/mL Target Mix + 50 µL (100 µg/mL) carboxy-THC stock solution to ≅ 8mL MeOH in 10mL ball flask. QS with MeOH (LCMS grade). *Solution is stable for one-year when stored in the freezer.*

(NOTE: Alternative calibrator/control working solution preparation options are listed in Appendix 1 of the Analytical Method.)

5.1.2 Calibrator Preparation

5.1.2.1 Add the volume of working calibrator working solution to appropriate tube as indicated below.

Sample Type	1.0/5.0 µg/mL Target Mix (Containing c-THC)	1.0/5.0 µg/mL Target Mix (Containing c-THC)	Or 1.0 µg/mL Target Mix (Without c-THC)	And 100 µg/mL c-THC stock
Blank	-	-	-	-
1/5 ng/mL - Cal 1	10 µL	-	-	-
2/10 ng/mL - Cal 2	20 µL	-	-	-
5/25 ng/mL - Cal 3	50 µL	-	-	-
10/50 ng/mL - Cal 4	100 µL	-	-	-
25/125 ng/mL - Cal 5	250 µL	25 µL	-	-
50/250 ng/mL - Cal 6	-	50 µL	50 µL	2.5 µL
100/500 ng/mL - Cal 7 (Optional for blood)	-	100 µL	100 µL	5 µL

5.1.3 Positive Control

5.1.3.1 Negative urine or blood can be spiked with working solutions, but the compounds in that solution **cannot** be the same lot as was used for the calibrators. At minimum, the control must contain two compounds included in the scope of the method.

5.1.3.2 For urine, a single positive control between the approximate concentrations of 5 ng/mL and 25 ng/mL for all compounds except carboxy-THC shall be included in the run. The response for carboxy-THC should be between 25 ng/mL and 125 ng/mL.

5.1.3.3 For blood, two positive controls are required (one low, and one mid to high). The low concentration control shall fall between 5 ng/mL and 10 ng/mL for all compounds except carboxy-THC, which shall be between 25 ng/mL and 50 ng/mL. The mid- or high-concentration control shall fall between 25 ng/mL and 100 ng/mL for all compounds except carboxy-THC, which shall be between 125 ng/mL and 500 ng/mL. (Note: for the low control, spike with 50-100 µl of the low control working solution and for the mid to high control, spike with 25-100 µl of the high control working solution).

5.1.4 Conjugated Controls (Urine samples only)

Spiked Negative urine (must be the same lot for calibrators and controls)

5.1.4.1 Stock Solution

- 100µg/mL Carboxy-THC Glucuronide

5.1.4.2 Working Glucuronide Solution (10ng/µL)

- Add 10µL 100µg/mL Stock Solution to 990µL MeOH or Acetonitrile. *Solution is stable for one week when stored under refrigeration or in a freezer.*

5.1.5 Internal Standard

5.1.5.1 Stock Solution

- 100 µg/mL Δ^9-THC-D3, 11-nor-Δ^9-THC-9-COOH-D3, 11-hydroxy-Δ^9-THC – D3, Cannabidiol-D3 (optional)

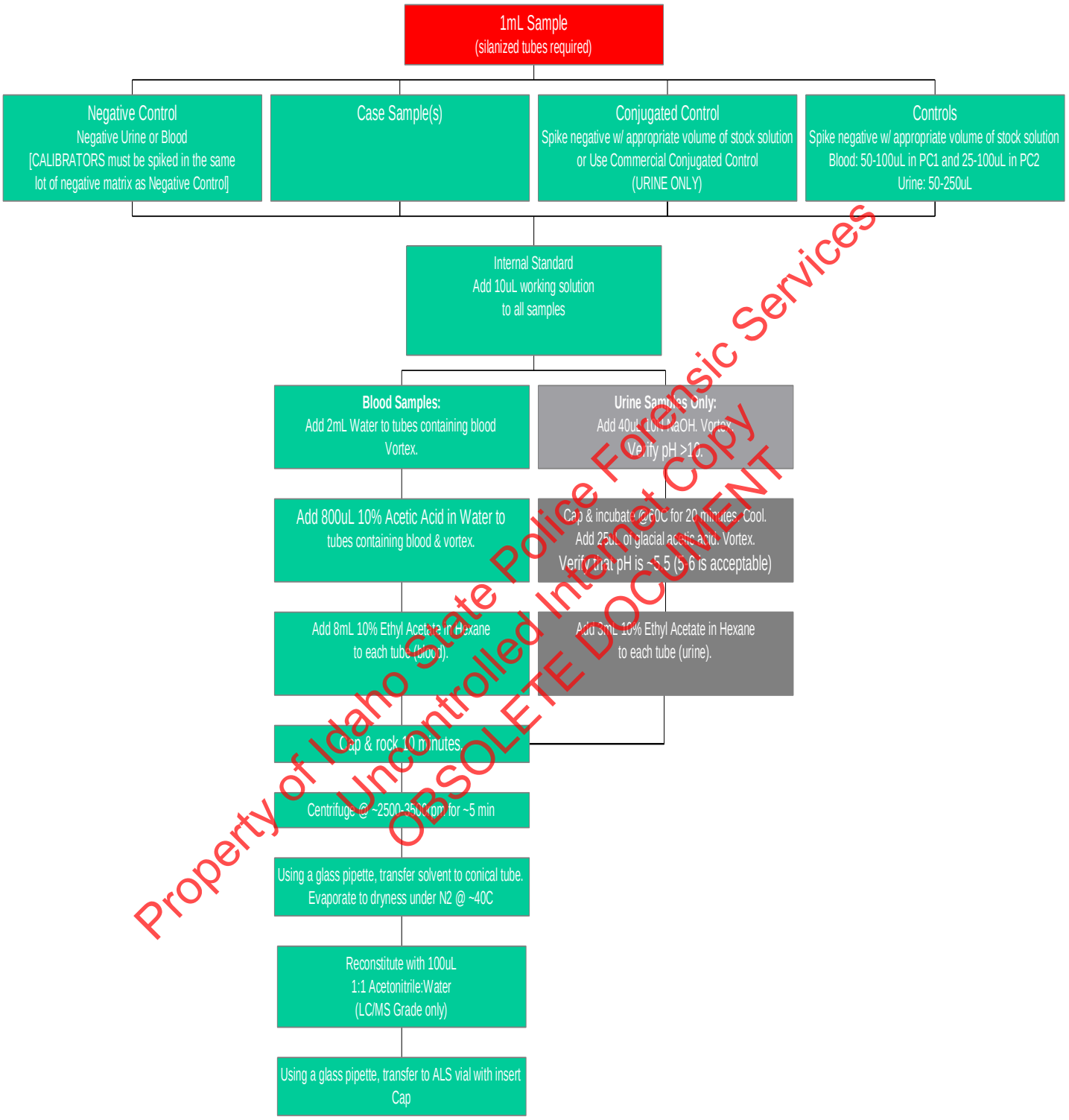
5.1.5.2 Working Internal Standard Solution (1.0 µg/mL ISTD mix in methanol)

- Add 100µL of 100µg/mL stock solution to ≅ 9mL MeOH (LCMS grade) in 10mL ball flask. QS with MeOH (LCMS grade). *Solution is stable for 1 year (or the earliest expiration of the reference materials used for preparation) when stored under refrigeration or in a freezer.*

5.1.6 COMMENTS

5.1.6.1 This method has instructions for the preparation of both urine and blood casework samples.

5.2 Sample Preparation



6.0 Comments

6.1 Appendix 1:

NOTE: All Reagent/Solution preparation will be documented on a prep sheet with an expiration of one year or the date of the earliest Stock Reference material expiration. All working solutions should be stored frozen.

6.1.1 1µg/mL ISTD mix in methanol (LCMS grade)

- Fill a 10 mL volumetric flask about half full with methanol, add 100 µl of 100 µg/mL stock solution of the following compounds. (If the stock solution is a different concentration you will need to adjust addition volumes.)
- D9-THC-D3, 11-nor-D9-THC-9-COOH-D3, and 11-hydroxy-D9-THC -D3. Cannabidiol-D3 is optional.
- QS with methanol and ensure it is thoroughly mixed.

6.1.2 CALIBRATOR/CONTROL Working Solution Preparation Options (High and low working solutions need to be prepared for both calibrators and controls and must be from different vendors or different lot numbers):

6.1.2.1 OPTION 1: High Working Solution (No carboxy-THC): 1 µg/mL Target mix in methanol (LCMS grade)

- In a 10 mL volumetric flask fill the flask about half full with methanol, add 10 µL of 1mg/mL (or 100 µL of 100 µg/mL) stock solution of the following compounds (If the stock solution is a different concentration, you will need to adjust addition volumes):
- D9-THC, and 11-hydroxy-D9-THC. Cannabidiol is optional.
- QS with methanol and ensure it is thoroughly mixed.
- Note: 11-nor-D9-THC-9-COOH is NOT included in this working solution and must be directly spiked into sample.

6.1.2.2 OPTION 1: Low Working Solution: 0.1/0.5 µg/mL Target mix in methanol (LCMS grade)

- NOTE: the 0.1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 0.5 µg/mL.
- In a 10 mL volumetric flask fill the flask about half full with methanol. Add 1mL of 1 µg/mL Target Mix working solution (See OPTION 1 for Target Mix preparation) AND 50 µL (100 µg/mL) carboxy-THC stock solution. QS with methanol and ensure it is thoroughly mixed.

6.1.2.3 OPTION 2: High Working Solution (Containing c-THC): 1/5 µg/mL Target mix in methanol (LCMS grade)

- NOTE: the 1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 5 µg/mL.

- In a 10 mL volumetric flask fill the flask about half full with methanol, add 500 µL of 11-nor-d9-THC-9-COOH stock solution (100 µg/mL) AND 10 µL of 1mg/mL (or 100 µL of 100 µg/mL) stock solution of the following compounds (or adjusted volume based on stock concentration):
- d9-THC, and 11-hydroxy-d9-THC. Cannabidiol is optional.
- If the analyst makes this working solution with carboxy-THC, no additional carboxy-THC stock should be spiked into the calibrators as described in Section 4.1.1.1. or in controls in Section 5.1.2.1
- QS with methanol and ensure it is thoroughly mixed.

6.1.2.4 OPTION 2: Low Working Solution (Containing c-THC): 0.1/0.5 µg/mL Target mix in methanol (LCMS grade)

- *NOTE: the 0.1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 0.5 µg/mL.*
- In a 10 mL volumetric flask fill the flask about half full with methanol. Add 1mL of 1/5 µg/mL working solution (See OPTION 2 for High Working Solution preparation). QS with methanol and ensure it is thoroughly mixed.
- If the analyst makes this working solution with carboxy-THC, no additional carboxy-THC stock should be spiked into the calibrators as described in Section 4.1.1.1. or in controls in Section 5.1.2.1

Toxicology AM #15: Verification of POVA Calibration

1.0Background/References

1.1 Background

Colorimetry measures the intensity of a color and relates it to the concentration of the solution. The relationship between concentration and intensity is obtained through determining the degree of light absorbance by a solution at a particular wavelength. The fraction of the incident light that is absorbed by a solution depends on the thickness of the sample, the concentration of the absorbing compounds in the solution, and the chemical nature of the absorbing compound. This relationship is defined by the Beer-Lambert law:

$A = \epsilon bc$ $A = Absorbance$

b = Internal path length (cm) of solution vial

c = Concentration of sample solution

ϵ = Molar absorptivity of sample solution

The ARTEL PCS 2™ pipette calibration system is a colorimetric method for an intermediate check of pipette dispensing accuracy and precision. The system utilizes a photometer coupled with NIST-traceable reagents to measure liquid delivery. The system is set up so that as additional solution (V_{P1}) is added to pre-mixed volume of blank solution (V_B), the absorbance change is proportional to the volume delivered by the pipette. The volume of solution pipetted (V_{P1}) is calculated as follows:

$$V_{P1} = VB\ [A1\ /\epsilon bc - A1]$$

The volume of repetitions (V P2) is determined by the following relationship:

$$V_{P2} = VB + V\ P1\ [A2 - A1\ /\epsilon bc - A2]$$

1.2 References

1.2.1 Standard Operating Procedure for the PCS 2™ Pipette Calibration System, Artel Document #310A2715A, April 1997.

1.2.2 PCS 2™ Pipette Calibration System Procedure Guide, Artel Document # 15A2135, Version 5.1, 03-28-1997.

1.2.3 ASTM Method E 1154-89 (reapproved 2003), **Standard Specification for Piston or Plunger Operated Volumetric Apparatus.**

1.2.4 Segel, I.H., Spectrophotometry and Other Optical Methods. pp. 324-329. In: "Biochemical Calculations", Second ed., John Wiley & Sons, New York, 1976.

1.2.5 Kolthoff, I.M., Sandell, E.B., Meehan, E.J. and Bruckenstein, S., Absorption Spectrophotometry. pp. 967-970, In: "Quantitative Chemical Analysis", Fourth ed., Macmillan, New York, 1969.

1.2.6 Setting Tolerances for Pipette Performance, Artel lab report, Issue 5, March 1999.

1.2.7 Curtis, R.H., *Performance Verification of Manual Action Pipets: Part I*, Am. Clin. Lab. 12(7):8-9; 1994.

1.2.8 Curtis, R.H., *Performance Verification of Manual Action Pipets: Part II*, Am. Clin. Lab. 12(9):16-17; 1994.

1.2.9 Eppendorf Series 2000 Reference Fixed-Volume Pipettes Instruction Manual

1.2.10 Eppendorf Series 2000 Reference Adjustable-Volume Pipettes Instruction Manual

1.2.11 Eppendorf Repeater™ Plus Pipette Instruction Manual

1.2.12 Eppendorf Repeater™ Pipette Instruction Manual

1.2.13 MLA Macro and Macro Selectable Pipette Operator's Manual

2.0 Scope

2.1 The reliability of the volume delivered by piston or plunger operated volumetric apparatus is dependent upon verification of calibration. This method sets forth the requirements for both intermediate checks and calibration. The intermediate check is performed to maintain confidence in calibration. The ARTEL instrument utilizes a system which optimizes the application of the Beer-Lambert Law to provide a reliable, time efficient, pipette intermediate check that is traceable to NIST standards. An approved external service provider performs actual POVA calibration. This analytical method applies to air displacement pipettes as well as syringes attached to dilutors and dispensers.

3.0 Equipment/Reagents

3.1 PCS 2™ Instrument

3.2 Printer

3.3 Printer Paper

3.4 ARTEL Instrument Calibration Kit

3.5 Appropriate ARTEL Reagent Kit

4.0 Procedure

4.1 Refer to manufacturer's *Standard Operating Procedure for the PCS 2 ™ Pipette Calibration System and PCS 2 ™ Pipette Calibration System Procedure Guide.*

4.2 PCS 2™ Instrument Calibration Check

4.2.1 The calibration check of PCS 2™ instrument is valid for one-month providing the instrument stays in proper working order.

4.2.2 PCS 2 calibration check printouts, and/or a copy thereof, are to be initialed and placed in PCS 2™ logbook. A copy is permissible due to the nature of the thermal paper printout.

4.2.3 The calibration kit lot number, imprecision and inaccuracy results of the instrument calibration check should be recorded on the PCS 2™ instrument calibration log sheet.

4.2.4 The results of the calibration check should be evaluated and a pass or fail indicated on the PCS 2™ instrument calibration log sheet.

4.3 PCS 2™ Intermediate Checks

4.3.1 The requirement for a particular pipette or syringe to have periodic intermediate checks will be indicated in the applicable analytical method. As a rule, all methods involving quantitative analysis require periodic checks.

4.3.2 Refer to AM #16 for calibration/intermediate check requirements.

4.3.3 PCS 2™ instrument printouts, or a copy thereof, are to be initialed and placed in PCS 2™ logbook. A copy is permissible due to the nature of the thermal paper printout.

4.3.4 A PCS 2™ intermediate check log sheet must be maintained for each pipette or syringe by serial number or other unique identifier.

4.3.5 The imprecision and inaccuracy results of the intermediate check must be recorded on the appropriate PCS 2™ calibration check log sheet.

4.3.6 The results of the calibration check should be evaluated as described in section 4.4 and 4.5 of this method and a pass or fail indicated on the appropriate PCS 2™ calibration check log sheet.

4.3.7 A minimum of 10-data points is to be collected for each check of the pipette/syringe calibration.

4.3.8 A pipette/syringe not in-use need not be calibrated, however, it must have its calibration verified prior to use.

4.3.9 An intermediate calibration check must be performed after any pipette repair/maintenance.

4.4 Manufacturer Data Acceptance Criteria

4.4.1 Artel recommendations for Piston-stroke Pipette Tolerance Limits

Pipette Volume	Inaccuracy	Imprecision
2µL	5.0%	2.0%
10µL	5.0%	2.0%
20µL	5.0%	2.0%
100µL	5.0%	2.0%
200µL	5.0%	2.0%
1000µL	5.0%	2.0%

4.4.2 Recommendations for Eppendorf Piston-stroke Fixed Volume Pipette Tolerance Limits

Pipette Volume	Inaccuracy	Imprecision
1µL	±2.5%	≤1.8%
2µL	±2.0%	≤1.2%
10µL	±1.5%	≤0.8%
20µL	±1.0%	≤0.5%
100µL	±0.8%	≤0.3%
200µL	±0.7%	≤0.3%
1000µL	±0.6%	≤0.2%

4.4.3 Recommendations for Eppendorf Repeater Plus Pipette Tolerance Limits

	Inaccuracy	Imprecision
Combitip Plus 0.1mL (beige piston)		
2µL	±1.6%	≤3.0%
20µL	±1.0%	≤2.0%
Combitip Plus 0.2mL (blue piston)		
4µL	±1.3%	≤2.0%
40µL	±0.8%	≤1.5%
Combitip Plus 0.5mL		
10µL	±0.9%	≤1.5%
100µL	±0.8%	≤0.6%
Combitip Plus 1mL		
20µL	±0.9%	≤0.9%
200µL	±0.6%	≤0.4%
Combitip Plus 2.5mL		
50µL	±0.8%	≤0.8%
500µL	±0.5%	≤0.3%
Combitip Plus 5mL		
100µL	±0.6%	≤0.6%
1000µL	±0.5%	≤0.25%
Combitip Plus 10mL		
200µL	±0.5%	≤0.6%
2000µL	±0.5%	≤0.25%
Combitip Plus 25mL		
500µL	±0.4%	≤0.6%
5000µL	±0.3%	≤0.25%
Combitip Plus 50mL		
1000µL	±0.3%	≤0.5%
10000µL	±0.3%	≤0.25%

4.5 Intermediate Check Acceptance Criteria

4.5.1 Initially the tolerance limits recommended by ARTEL {Section 4.4 of this method} should be applied.

4.5.2 When a history for an individual pipette or syringe is established, the tolerance limits should be fine-tuned and tightened accordingly.

4.5.3 Refer to ARTEL publication issue 5 (March 1999) for information regarding tolerance setting.

4.5.4 Refer to package insert for tolerance limits observed by manufacturer for each individual pipette.

4.6 Calibration Checks

4.6.1 All pipettes and syringes crucial for the quality of quantitative analysis will be calibrated annually by a qualified external vendor/service provider.

4.6.2 The requirement that a calibrated pipette/syringe is to be used is indicated in the relevant analytical method.

Toxicology AM #16: Pipette Calibration and Intermediate Checks

1.0 Background/References

1.1 Background

The initial calibration of piston or plunger operated volumetric apparatus (POVA) is performed by the manufacturer. Upon receipt of a newly obtained pipette or syringe and thereafter periodically, the calibration must be verified to substantiate that the volume delivered is both accurate and precise. This is accomplished by determining the mass of a volume of liquid of known density that has been delivered into a closed vessel.

1.2 References

1.2.1 ASTM Method E-1154-89 (reapproved 2003), Standard Specification for Piston or Plunger Operated Volumetric Apparatus.

1.2.2 Curtis, R.H., Performance Verification of Manual Action Pipets: Part I, Am. Clin. Lab. 12(7):8-9; 1994.

1.2.3 Curtis, R.H., Performance Verification of Manual Action Pipets: Part II, Am. Clin. Lab. 12(9):16-17; 1994.

1.2.4 Byer, B.J., How to Use and Check Pipetting Equipment, Scientific Newsletters, Inc., 1977.

1.2.5 ISO 8655-6:2002, Piston-operated volumetric apparatus - Part 6: Gravimetric method for the determination of measurement error.

2.0 Scope

2.1 Scope

The reliability of the volume delivered by a pipette is dependent upon verification of calibration. This method sets forth the requirements for both intermediate checks and calibration. The intermediate check is performed to maintain confidence in calibration. Unless specified in the method that a calibrated pipette must be used, the use of a non-calibrated pipettes is permitted as volumes are approximate.

3.0 Equipment/Reagents

3.1 Analytical Balance

- Capable of accurately weighing volumes of interest.

3.2 Thermometer

- Subdivisions of ≤0.5 degree
- Capable of reading 20° - 28°C

3.3 Weighing Vessel

3.4 Appropriate disposable pipette tips

3.5 Water

4.0 Procedure

4.1 Calibration

4.1.1 All pipettes that require calibration (this is designated in the analytical method) will be calibrated within 365 days prior to use by a qualified vendor, or after repair or service. The calibration should take place in-house, if possible. If the pipette is a variable volume apparatus at least one level from the table below will be checked for the volumes that apparatus spans.

4.1.2 After calibration is complete an analyst will review the calibration certificate and the calibration report provided by the vendor will be centrally stored. If the calibration is done off-site, an intermediate check must be performed before the pipette is put back into use. No intermediate check will be required immediately after calibration if the calibration is done in-house.

4.1.3 The criteria listed in table 1A must be met.

Table 1A

Volume	Accuracy	Precision
2-15 uL	± %5	± 2%
16-100 uL	± 2%	± 2%
101-1000 uL	± 2%	± 2%
1000-5000 uL	± 2%	± 2%

4.1.4 The analyst will document the review and approval by initialing and dating the certificate. If the tolerances are not met the analyst will make a notation on the certificate and will take that pipette out service for use in methods that require calibrated pipettes.

4.2 Intermediate Checks

4.2.1 All pipettes that require calibration will have an intermediate check performed within 45 days prior to use. The results will be recorded. The pipette will be clearly marked with the date the next intermediate check is due.

4.2.2 Allow water to equilibrate to room temperature.

4.2.3 Determine and record the water temperature on the logsheet.

4.2.4 Place a volume of water in the weighing vessel, which completely covers the bottom of the container.

4.2.5 Place the weighing vessel on the balance and tare.

4.2.6 Use designated pipette, to dispense the appropriate volume of water. If the pipette is a variable volume apparatus at least one level from the table below will be checked for the volumes that apparatus spans.

Table 1B

Volume	**Accuracy**
2-15 uL	+/- 7%
16-100 uL	+/- 5%
101-1000 uL	+/- 5%
1000-5000 uL	+/- 5%

4.2.7 From the Table 2 below, note the conversion factor (Z) for the mean water temperature. The conversion factor is based upon an air pressure of 1013 hPa.

Table 2

Temperature(°C)	Conversion Factor (Z) (µL/mg)
20.0	1.0029
20.5	1.0030
21.0	1.0031
21.5	1.0032
22.0	1.0033
22.5	1.0034
23.0	1.0035
23.5	1.0036
24.0	1.0038
24.5	1.0039
25.0	1.0040
25.5	1.0041
26.0	1.0043
26.5	1.0044
27.0	1.0045
27.5	1.0047
28.0	1.0048

4.2.8 Calculate the Volume Delivered (V) at the recorded temperature.

$$V = (W) \cdot Z$$

4.2.9 Inaccuracy Calculation

4.2.9.1 Determine inaccuracy by calculating the percent error (E_t) between the expected (V_o) and calculated (V) volume.

$$E_t = V - V_o / V_o \text{ x } 100$$

4.2.9.2 Record the inaccuracy and refer to table 1B above to for acceptable tolerances. Note on the log sheet if the check passes or fails. If the check fails the pipette needs to be taken out of service for quantitative use and repaired, the pipette must be calibrated before being returned to service.

Toxicology AM #17: Balance Calibration and Intermediate Checks

1.0 Background/References

1.1 Background: Refer to manufacturer's manual for balance specific procedures.

1.2 References

1.2.1 ASTM Method E-617-97, Standard Specification for Laboratory Weights and Precision Mass Standards.

2.0 Scope

2.1 The procedure is to ensure accuracy of measurement when an analytical or top-loading balance is used to prepare solutions or reference material for application to toxicology methods.

3.0 Equipment/Reagents

3.1 ANSI/ASTM Type I, Class 1 or Class 2 laboratory weights

3.2 Analytical and/or Top-Loading, Direct-Reading Laboratory Balances

4.0 Procedure

4.1 Intermediate Check Procedure

4.1.1 Inspect balance pan, clean if necessary.

4.1.2 Inspect level bubble, level if necessary.

4.1.3 Tare balance with weighing paper or weigh boat.

4.1.4 Place weight on balance.

4.1.5 If appropriate, add or subtract correction factor for weight as determined by yearly weight calibration certificates. *The correction factor must be applied when it changes whether or not the tolerance is in the accepted range.*

4.1.6 Record weight on balance logsheet.

4.1.7 The acceptable range for the balance is based on whether an analytical or top-loading balance is used.

4.1.7.1 Analytical Balance

Weight	Tolerance	Acceptable Range
100mg (0.1g)	±1.0mg (0.001g)	99.0mg - 101.0g
100g	±0.1g	99.9g - 100.1g

4.1.7.2 Top-loading Balance

Weight	Tolerance	Acceptable Range
10.00g	±0.02g	9.98g - 10.02g
100.00g	±0.2g	99.8g - 100.2g
500g	±0.5g	499.5g - 500.5g
1000g	±1.0g	999.0g - 1001.0g

4.1.8 The verification procedure should be repeated if the value does not fall within the acceptable range. If value is still out of range, contact service vendor to set up a service call. A note must be placed on the balance to indicate that it is not in range and must not be used.

4.2 Quality Assurance Requirements

4.2.1 All balances will be calibrated and serviced yearly by a qualified vendor. Within 45 days prior to use, the balance is to have its calibration checked (intermediate check) against an appropriate set of NIST traceable weights. Results are to be recorded for future reference. The allowable deviation from the standard weights is 0.01g or 0.1%, whichever is greater.

4.2.2 Weights used for intermediate checks of calibration will be NIST traceable and will be calibrated yearly by a qualified vendor.

4.2.3 An in-house intermediate check of balances in use will be performed within 45 days prior to use. Results are to be recorded in logbook.

4.2.4 The weights used for the intermediate check should depend on the application of the balance. Three weights must be used to represent the weight range in question. For instance, if the balance is being used to prepare buffer solutions, then perhaps 100g, 500g and 1000g weights would characterize the weight range. If the balance is used to prepare mg/mL reference material then 1mg, 10mg and 100mg weights may be appropriate.

4.2.5 The type of balance employed should be a consideration. For weights less than 100mg, an analytical balance should be used. For the preparation of a solution involving gram quantities, a top-loading balance should be used.

4.2.6 Do not touch weights with bare hands. Weights should be transferred with forceps or gloves. The weights will be stored and transported in their case.

Toxicology AM #18: Toxicology Proficiency Tests

1.0 Background/References

1.1 Background

As set forth in the Idaho State Police Forensic Services *Procedure and Quality Manual*, proficiency testing is an integral part of a quality program.

2.0 Scope

2.1 This method describes the criteria to be applied to the evaluation of results obtained for proficiency testing for the toxicology discipline.

3.0 Equipment/Reagents

3.1 Not Applicable

4.0 Procedure

4.1 Urine and Blood Analysis Proficiency Testing

4.1.1 Only analytes that are routinely tested for with current ISP-FS analytical methods will be evaluated.–If the parent drug or a metabolite of a drug is reported, additional metabolites of that drug are not required to be reported.

4.1.2 When reported, quantitative values must be within the estimated uncertainty of measurement.

4.1.3 If the analyst does not correctly identify all target analytes and/or quantitative values do not fall within range, the analyst's training will be reviewed and additional training may be required as deemed appropriate by the Toxicology Program Discipline Leader. The analyst may be required to complete a competency test prior to resuming casework. The number of samples will be determined by the nature of the discrepancy.

Toxicology AM #19: Quality Assurance Measures

1.0 Background/References

1.1 Background

The quality assurance measures applied towards analysis of toxicological samples promote confidence in results.

1.2 References

1.2.1 Wu Chen, N.B. Cody, J.T., Garriott, J.C., Foltz, R.L., and et al., Report of the Ad hoc Committee on Forensic GC/MS: Recommended guidelines for forensic GC/MS procedures in toxicology laboratory associated with offices of medical examiners and/or coroners, J. Foren. Sci, 236 (35): 236-242, 1990.

1.2.2 Goldberger, B.A., Huestis, M.A., Wilkins, D.G., Commonly practiced quality control and quality assurance procedures for gas chromatograph/mass spectrometry analysis in forensic urine drug-testing laboratories, For Sci Review, 9(2): 60-79, 1997.

1.2.3 SOFT/AAFS Forensic Toxicology Laboratory Guidelines, 2002

2.0 Scope

2.1 This analytical method addresses general acceptance requirements for qualitative and quantitative analysis data obtained through analysis by gas chromatography equipped with a mass selective detective (MSD). Requirements for analysis with other instrumentation are addressed in relevant analytical methods.

3.0 Equipment/Reagents

3.1 Refer to specific analytical method for necessary equipment and supplies.

3.2 Refer to appropriate analytical method for required reagents as well as Toxicology AM #23 for Solution Preparation instructions.

4.0 Procedure

4.1 Instrument Quality Assurance GCMS

4.1.1 Instrument Maintenance

NOTE: Refer to manufacturer's hardcopy or electronic instrument manuals for maintenance indicators and instructions.

4.1.2 MSD Tuning

4.1.2.1 A Tune must be run within a week prior to analysis of casework.

4.1.2.2 A Tune Evaluation should be completed and parameters should be reviewed for acceptance based on the report's predefined criteria, set by the manufacturer. *Note: H_2O and N_2 values should be monitored for possible instrumentation leaks (acceptance parameters are listed in parentheses on the tune evaluation report).*

4.1.3 Instrument Performance Monitoring

4.1.3.1 Instrument performance will be monitored through evaluation of the positive and negative controls run with every extraction batch. The control requirements for an extraction are found in the applicable analytical methods. The instrument will be determined as working properly if the expected responses are obtained for the controls evaluated.

4.1.3.2 Analysts may use the control data to determine if instrument maintenance is needed.

4.1.3.3 Troubleshooting, repairs and maintenance that takes place should be documented.

4.1.4 Data file back up

4.1.4.1 Data files will be retained and backed up to external media at least once every two months.

4.2 Instrument Quality Assurance LC/MS- Refer to Toxicology AM# 24

4.3 Sample Preparation Quality Assurance

4.3.1 Qualitative Analysis

4.3.1.1 Matrix Controls

4.3.1.1.1 Quality controls are to be prepared and analyzed as designated in the appropriate analytical method.

4.3.1.1.2 Positive controls should exhibit proper retention time and mass spectral characteristics for compounds of interest.

4.3.1.1.3 Negative controls should be examined for compound(s) of interest and interfering substances prior to the completion of analysis, the negative control can be included in the batch.

4.3.1.1.4 Commercially-obtained controls may be utilized for qualitative analysis after the manufacturer's expiration date *provided* all method control requirements are met AND an additional in-run control that is not expired be run. This limited permission is not applicable to expired controls of a unique nature (e.g. EMIT level 0 pre-run control).

4.3.1.1.5 It is applicable, for example, to the use of an expired commercially-obtained ĉ-THC control when performing Toxicology AM# 3; this method requires one (non-expired) spiked controls be run in addition to the commercially-obtained control.

4.3.1.2 GC/MS Solvent Blanks

4.3.1.2.1 An appropriate solvent blank will be run before case sample extracts. If the same sample is injected for more than one instrument method, only one blank needs to be run preceding the sample injections.

4.3.1.2.2 If the solvent blank contains a reportable analyte of interest, the corrected area of the analyte peak in the sample data must be a minimum of 10 times stronger than the corresponding peak in the blank preceding it. Ideally, no contamination should be apparent.

4.3.1.2.3 *Reportable* is defined as a complete fragmentation pattern at the appropriate retention time. Analytes of interest include, but are not limited to, analytes routinely reported.

4.3.1.2.4 If significant contamination is present, as discussed in 4.2.1.2.2, rerun the solvent blank that was used in that analysis and the sample extract in question. If the contamination is still apparent, or the original samples are no longer available, troubleshoot the instrument to determine the source of contamination. In addition, the sample in question must be re-extracted prior to reanalysis on the rectified instrument.

4.3.1.3 LC/MS Solvent Blanks

4.3.1.3.1 As designated in the appropriate analytical method, an appropriate solvent blank containing at minimum one internal standard will be run directly after the highest calibrator and at the end of the run. In addition, if the concentration for any case sample directly preceding another case is above the highest calibrator for that method, the sample will be reinjected and a blank will be run prior to the injection.

4.3.1.3.2- Sections 4.3.1.2.2-4.3.1.2.4 are applicable to LCMS runs if contamination is suspected.

4.3.2 Quantitative Analysis

4.3.2.1 Quality measures are optimized for the analytes in question and are addressed in each individual quantitative analytical method.

4.3.3 Distribution of Quality Data

4.3.3.1 Original data for matrix controls will be stored in a designated central location in the laboratory where the analysis was performed, or electronically on a shared network drive.

4.3.3.2 Copies of all quality assurance control data need not be placed in each case file, except those required under 4.2.3.3.

4.3.3.3 Copies of analytical reference material used to substantiate the identification of each drug compound must be included in each case file, if not otherwise indicated in the relevant analytical method.

4.4 Sample Storage

4.4.1 Blood samples should be stored under refrigeration; this includes Combo Collection Kits.

4.4.2 Urine samples should be stored under refrigeration or frozen. If samples are going to be stored longer than two weeks prior to analysis, they should be frozen. Once analysis is complete, samples should be frozen until they are returned to the agency.

Toxicology AM #20: Testing Guidelines and Reporting Criteria

1.0 Background/References

1.1 Background

To best utilize the resources available to support the ISP-FS toxicology discipline, the degree of analysis pursued should be guided by all available information. It may not always be necessary and/or appropriate to confirm all drug compounds present. With urine analysis, when a subject has admitted to use of prescription and/or over-the counter drugs that may impair driving, confirmation of all drugs present may not serve to strengthen pending charges. With drugs-of-abuse, confirming the presence of all drug compounds may not be necessary, depending on the circumstances. For instance, for Probation and Parole cases, prescription pharmaceuticals are most likely not a consideration. This method also covers reporting criteria.

2.0 Scope

2.1 This method addresses the factors to consider when determining the extent of analysis a toxicology case sample requires. It is intended to provide guidance to analysts; however, the decision to pursue testing remains at the discretion of each analyst. The goal of these considerations is for the efficient utilization of resources in order to provide timely analysis results to user agencies. This method covers reporting criteria to ensure consistent reporting in the lab system and to ensure limitations are properly expressed.

3.0 Equipment/Reagents

3.1 Not applicable

4.0 Procedure

4.1 General

4.1.1 When available, the type of case associated with a toxicology sample should be determined.

4.1.2 The extent of analysis should be based on background information and the charges pending.

4.1.3 If no background information is provided, it is at the discretion of the analyst to perform only basic testing.

4.1.4 When a positive EIA screen result indicates the preliminary presence of a drug or drug class, unless current drug therapy is in agreement, confirmation of EIA results should be pursued if the confirmation of the compound(s) has the potential of providing an additional source of impairment for DUID.

4.1.5 Blood and Urine samples submitted for determination of drugs of abuse and other impairing substances should be analyzed utilizing the criteria considered under sections 4.1.1 through 4.1.4, in essence justifying any potential charge in question. The extent of testing is at the discretion of each analyst; however, the following situations and examples should be factored into the evaluation process.

4.1.6 If the drug in question is recovered in the extraction procedure for another compound, it may be confirmed provided quality assurance requirements are met. Method limitations, if any exist, are discussed in the applicable analytical method.

4.2 Testing Guidelines: Post-Blood Alcohol or Breath Testing Analysis

4.2.1 When the ethanol concentration is 0.10g/100cc, or greater, further testing for additional drugs, in either blood or urine, should not be pursued unless justified by case-related circumstances. This is in consideration that the legal limit for ethanol is 0.08 grams per 100 cc blood.

4.2.2 If a breath test result is listed on the toxicology submittal form, no indication of a problem with the test is noted, and no inhalants are suspected, volatiles testing is not required. If an interferent was noted, it is recommended that the case be referred to the Blood Alcohol Testing Section so that volatiles analysis may be pursued. Analysis requirements will be determined by the applicable Blood Alcohol Analytical Method.

4.2.3 Extenuating circumstances may include the following:

- Fatality or injury accidents.
- Death investigations.
- Sexual assaults.

4.2.3.1 In the case of crashes where the subject is the driver and is deceased and further toxicology testing is requested, testing will be performed on samples that have a blood alcohol content of less than 0.20 grams per 100 cc of blood.

4.2.4 The submitting officer or agency is responsible for providing justification for additional testing. Justification could take the form of a note on the submittal information, memo, e-mail or letter outlining the situation, or a case report.

4.2.5 If the ethanol concentration is 0.10 g/100cc or higher future testing for other impairing drugs will not be pursued if additional testing is not requested. Analysts are encouraged to contact the agency if it is believed that further analysis is recommended. Additional analysis may be prudent if impairment described cannot reasonably be explained by ethanol/other volatiles results.

4.3 Testing Guidelines: Proceeding After EIA Screen

4.3.1 When current prescription drug therapy has the ability to trigger a positive enzyme immunoassay (EIA) response, the presence does not have to be confirmed in all situations.

4.3.2 Example One

4.3.2.1 Positive enzyme immunoassay (EIA) screen result for methamphetamine and benzodiazepines is indicated. The sample is collected as the result of a suspected DUID. The submittal form indicates symptoms consistent with stimulant use and lists diazepam as current drug therapy. When the methamphetamine confirmation data is processed, Nordiazepam is present. The qualitative presence of Nordiazepam may be confirmed in this sample. If no benzodiazepine had been present in the extraction to recover methamphetamine, no additional testing has to be pursued for a benzodiazepines class drug.

4.3.3 Example Two

4.3.3.1 A sample indicates a positive enzyme immunoassay (EIA) benzodiazepine screen. The case is a probation violation. The submittal form lists diazepam as current drug therapy. In this situation, no additional testing needs to be pursued for a benzodiazepine class drug.

4.3.4 Qualifying Statements

4.3.4.1 In the above examples, if no analysis for the e.g. benzodiazepines is pursued, a qualifying statement must be placed on the analysis report.

4.3.4.1.1 Preliminary testing indicates the possible presence of a ***Benzodiazepine class compound***. Confirmatory testing was not pursued because the benzodiazepine ***Alprazolam*** is said to be part of current prescription drug therapy.

4.4 Testing Guidelines: Prescription Drugs Not Covered by EIA Screen

4.4.1 When a prescription drug compound is detected in a general extraction procedure, the confirmation of the drug's presence is not required if other drugs present have the potential to justify the pending charge.

4.4.2 Example

4.4.2.1 Positive enzyme immunoassay (EIA) screen results for methamphetamine and opiates. The sample is collected as the result of a suspected DUID. The submission information indicates symptoms consistent with stimulant and narcotic analgesic use. Effexor (venlafaxine) is listed as current drug therapy. When the methamphetamine confirmation data is processed, venlafaxine is present. It is at the discretion of an analyst of whether or not to run a venlafaxine standard and confirm its presence.

4.5 Testing Guidelines: Enzyme Immunoassay Positive for Several Drugs-of-Abuse

4.5.1 When positive EIA screen results are indicated for several drugs of abuse, all involved drug compounds need not be confirmed.

4.5.2 Example

4.5.2.1 EIA screen is positive for amphetamine, methamphetamine, opiates, and cocaine metabolite. Initial confirmatory analysis indicates the presence of amphetamine, methamphetamine, codeine, morphine and 6-monoacetylmorphine. No cocaine or ecgonine methyl ester is detected. After consideration of all available information, it is at the discretion of the analyst whether or not to pursue the qualitative confirmation of benzoylecgonine.

4.6 Testing Guidelines: Confirmation of Metabolites When Parent Drug is Detected

4.6.1 For qualitative analysis, when a parent drug compound is detected, the confirmation of the presence of associated metabolites is not required.

4.6.2 Example

4.6.2.1 General basic extraction indicates the presence of propoxyphene. The confirmation of the presence of norpropoxyphene is at the discretion of the analyst.

4.7 Reporting Criteria- GC/MS Confirmation

4.7.1 When a drug is confirmed and meets the confirmation criteria outlined in the method, the report will list Drugs Confirmed: any drugs confirmed will be listed.

4.7.2 When no drugs are confirmed in a sample, the report will list Drugs Confirmed: None

4.7.3 When EIA screening results are positive but confirmatory testing is not done, the following comment may be added to the report: Preliminary testing indicated the possible presence of a _____-class drug, confirmatory testing not pursued because _____.

4.7.4 ***Preliminary results that are reported but not confirmed must always be clearly identified on a report, and a reason provided for non-confirmation.***

4.7.4.1 For positive opiate screens in blood where these drugs were not seen in the confirmation, the following comment may be added to the report: Preliminary testing indicated the presence of opiate-class compounds; however no opiate-class compounds were confirmed. This may be due to current limitations in the types and concentrations of opiates that can be confirmed by ISP Forensic Services.

4.7.5 When a drug is indicated in a confirmatory test but does not meet the criteria for identification in the analytical method, at the analysts' discretion the following statement may be included.

4.7.5.1 Inconclusive for _____, as it does not meet ISP Forensic Services toxicology criteria for identification. This is due to _____.

4.7.5.1.1 *Example*: Inconclusive for zolpidem, as it does not meet ISP Forensic Services toxicology criteria for identification. This is due to mass spectral differences between sample and reference material.

4.7.6 Reporting listed Rx therapy will be at the analyst's discretion, but is recommended for cases where it could alleviate confusion of where a drug came from. For example if Oxazepam was detected in urine, the comment could read: Prescription drug therapy is said to include Valium (diazepam), oxazepam is an active metabolite of diazepam.

4.8 Reporting Criteria- LC/MS Confirmation

4.8.1 Criteria for reporting LCMS results are located in the appropriate analytical method.

Toxicology AM #21: Authentication of Reference

1.0 Background/References

1.1 Background

The quality assurance measures applied towards analysis promote confidence in results. This analytical method was created so that the shared requirements did not have to be included in every toxicology discipline analytical method.

1.2 References

1.2.1 Wu Chen, N.B. Cody, J.T., Garriott, J.C., Foltz, R.L., et al., Report of the Ad Hoc Committee on Forensic GC/MS: Recommended guidelines for forensic GC/MS procedures in toxicology laboratory associated with offices of medical examiners and/or coroners, J. Foren. Sci, 236 (35): 236-242, 1990.

1.2.2 Goldberger, B.A., Huestis, M.A., Wilkins, D.G., Commonly practiced quality control and quality assurance procedures for gas chromatograph/mass spectrometry analysis in forensic urine drug-testing laboratories, For Sci Review, 9(2): 60-79, 1997.

1.2.3 SOFT/AAFS Forensic Toxicology Laboratory Guidelines, 2002.

2.0 Scope

2.1 This analytical method addresses qualitative and quantitative authentication of reference materials. Reference materials include both standards and controls.

3.0 Equipment/Reagents

3.1 Refer to appropriate analytical method for necessary equipment and reagents.

3.2 Refer to appropriate analytical method for solution preparation instructions.

4.0 Procedure

4.1 General

4.1.1 Appropriate authentication must be documented for reference materials prior to an analyst reporting a conclusion in casework in which that reference material was used. Authentication data should be stored centrally. If more than one laboratory within the ISP Forensic Services system will use the same lot of a commercial control or reference material, authentication at each individual laboratory is not required, provided personnel in each laboratory have ready access to authentication data.

4.1.2 When a reference material or control contains more than one constituent, only the compound(s) of interest need be authenticated. It should be clearly marked what compounds are authenticated.

4.1.3 Whenever possible, the source of reference material used to prepare matrix controls must differ from that used to prepare a quantitative response curve. If different vendors are not available, a different lot number should be used. As a last resort, if different lot numbers are not available, a second qualified analyst may prepare one of the working solutions.

4.1.4 Unauthenticated reference material must be stored in a designated area or clearly marked that authentication is needed.

4.1.5 It is the responsibility of each analyst to verify that each standard or control used has been properly authenticated. If an analyst uses a previously authenticated reference material, the analyst will initial the authentication data cover sheet indicating that that have looked at the authentication paperwork.

4.2 Qualitative Reference Material Authentication

4.2.1 Qualitative standards will be authenticated by an instrument that provides structural information (such as GCMS or FTIR) and has been validated and approved for use in the lab. A standard will be considered authenticated when the match (Q) is at least 85%, as compared to a library search *and* the analyst confirms that the spectra matches with no significant differences. If the spectra does not have a library match of 85% or greater, the spectra may be authenticated by comparing it to a peer reviewed scientific journal, reference standard compendium or a library match that is less than 85%. For these three options, two analysts trained to use the authentication instrumentation must initial the documentation signifying that it is an appropriate match.

4.2.1.1 Reference material used for LCMS analytical methods may be authenticated using the appropriate acquisition method. Documentation of the authentication will be the calibration curve print out for the compound of interest. The calibration curve and COA should be filed.

4.2.2 When comparison to a journal, compendium or other document is not an option, mass spectral interpretation may be used in conjunction with the COA (certificate of analysis). This would apply in cases where instrumental data for a drug metabolite is not yet published, but a structurally similar compound is available to assist with interpretation. A second trained analyst must also review and initial the printout verifying the interpretation.

4.2.3 A coversheet providing the information necessary for authentication will be prepared and placed with the MSD or FTIR data and a copy of the reference spectra. The coversheet must, at a minimum, list the lot number, vendor, date of analysis, analyst name, and mode of authentication.

4.2.4 Reference materials used for qualitative purposes do not have expiration dates; if the compound breaks down and is no longer performing as intended, the reference material will be discarded (or clearly marked invalid for casework since it may be used for training purposes).

4.3 Quantitative Reference Materials Authentication

4.3.1 The qualitative properties of these reference materials will be evaluated using the procedures described in 4.2.

4.3.2 The quantitative values on the COA will be accepted. The COA will be centrally stored for quantitative reference materials. Quantitative reference materials will be marked or stored in a designated location to prevent those that are only authenticated for qualitative use from being inadvertently used in quantitative applications.

4.3.3 The manufacturer of reference materials used for quantitative purposes must either utilize balances calibrated with weights traceable to National Institute of Standards and Technology (NIST) standards *or* be 17025 certified to produce reference materials. The certificate of analysis or manufacturer's accreditation certificate(s) and scope must be consulted to verify compliance with this requirement.

4.3.4 Quantitative reference materials will be discarded or designated for qualitative use only after they expire.

4.4 Qualitative and Quantitative Matrix Control Authentication

4.4.1 Matrix controls are analyzed in parallel with casework samples to demonstrate that a procedure performed as intended.

4.4.2 Quantitative Matrix controls also serve to verify the accuracy of a response curve.

4.4.3 Matrix controls may be prepared with authenticated reference materials or obtained through a vendor. The quantitative and qualitative properties of these controls will be based on the certificate of analysis, or the in-house preparation information. In addition, controls used in qualitative analysis may be authenticated following 4.2, if a certificate of analysis is not available.

4.4.4 The qualitative identity and quantitative values of component(s) in a commercially obtained matrix control will be based on the package insert or certificate of analysis. Certificates of Analysis (COA) and package inserts for commercially obtained matrix controls will be stored centrally in the laboratory in which they are used.

4.4.5 To authenticate the qualitative presence of components when the manufacturer does not provide a certificate of analysis or package insert, the analyst will authenticate each compound in the same way a qualitative reference material is authenticated (see 5.10.5.2). If a previous lot of that control has been authenticated with this process, the analyst may simply compare the new lot to the previously authenticated lot.

4.5 Internal Standard Authentication

4.5.1 Internal standards can be used to demonstrate the efficiency of an extraction, that the injection on the instrument worked properly, and for quantitation.

4.5.2 The qualitative identity and quantitative values of component(s) used as internal standards will be based on the package insert or certificate of analysis. Certificate of Analysis (COA) and package inserts for internal standards will be stored centrally in the laboratory in which they are used.

4.5.3 If the Certificate of Analysis is not available for an internal standard that is only used in qualitative analysis, it may be authenticated the same way a qualitative reference material is authenticated (see 4.2).

4.6 Qualitative and Quantitative ToxBox Internal Standard, Reference Material and Control Authentication.

4.6.1 The qualitative identity and quantitative values of component(s) in the ToxBox kits from Cayman Chemical will be based on the certificate of analysis. The Certificates of Analysis (COA) will be stored electronically on a common drive.

Toxicology AM #22: Key GC/MS Ions for Commonly-Encountered Compounds

1.0 Background/References

1.1 Background

This method was created to aid the analyst in the identification of the wide variety of commonly encountered compounds in blood and urine by GCMS.

2.0 Scope

2.1 This method provides the key ions to be used to establish the presence of compounds of interest in blood and urine. In order to familiarize the analyst with Drug Recognition Examination (DRE), the compounds are arranged according to DRE categories. DRE categories include central nervous system (CNS) depressants, CNS stimulants, narcotic analgesics, PCP, hallucinogens and cannabis. Additional compound information includes drug class, intended use and examples of trade names.

3.0 Equipment/Reagents

3.1 Refer to appropriate analytical method.

4.0 Procedure

4.1 With the assistance of case history and screening results, use the following table to detect drug compounds and their metabolites in urine and blood. When the presence of a compound is supported by the listed ions, the analyst must analyze relevant reference material to establish the compound's retention time in order to complete the identification process.

4.2 DRE CATEGORIES

4.2.1 CNS Depressants

Compound	***Key Ions***							***Suggested Window***	***Class***	***Background Info.***
	Base	Prominent Ions					**MW**			
Anticonvulsants										
Carbamazepine	193	192	236	191	165	44	**236**	250	Ureas – Ethylene bridged 1,1-diphenylurea	Tegretol® (Novartis) Tonic-clonic and partial seizures
Carbamazepine-M/artifact	193	165	96	83	139	177	**193**	210	---	Diphenylurea
Carbamazepine Epoxide	180	193	207	252	152	223	**252**	280	---	Metabolite
Gabapentin	153	81	67	110	96	68		240	Structurally related to GABA	Neurontin® (P-D), Excreted primarily unchanged
Phenytoin	180	252	77	104	223		**252**	280	Hydantoins	Dilantin® Structurally related to barbiturates. All seizure types except absence

Anticonvulsants										
Primidone	146	190	117	161	103	91	**218**	230	5-Ethyldihydro-5-4,6-(1H,5H) pyrimidinedione	Mysoline® (Wyeth-Ayerst) 2-desoxy-phenobarbital, converts to phenobarbital & PEMA. Good for all types of seizures except absence.
Topiramate	43	324	59	110	127	189	**339**	360	Sulfamate-substituted monosaccharide	Topamax® (Ortho-McNeil Has numerous other indications
Valproic Acid	73	102	55	41	57	115	**144**	140	2-Propyl pentanoic acid	Depakote® / Depakene® (Abbott) Multiple sezure types including absence. Also for Mania
Antidepressants/-M										
Amitriptyline	58	202	215	189	178	165	**277**	300	Tricyclic (TCA) Tertiary Amine	Elavil® (Zeneca)
Nortriptyline	44	202	215	220	115	91	**263**	300	TCA Secondary Amine	Pamelor® (Norvartis) Norpramin®, Pertofrane®. Parent **or** metabolite of amitriptyline
Citalopram	58	238	208	42	190	221	**324**	340	SSRI Bicyclic Phthalane Derivative	Celexa® (Racemic) Lexapro® (S-Citalopram)
Desmethylcitalopram	44	238	310	138	208	57		340	---	Citalopram metabolite
Amoxapine	245	257	193	247	228	164	**313**	350	TCA Secondary Amine	Asendin® (Lederle) Depression w/anxiety or agitation.
Loxepine	257	70	83	193	228	259	**327**	350	TCA	Loxitane® (antipsychotic), Parent **or** metabolite of amoxapine
Bupropion	44	100	57	139	111	224	**239**	250	Aminoketone	Wellbutrin® (GlaxoWellcome)
Imipramine	234	235	58	193	195	220	**280**	300	TCA Dimethylamine Tertiary amine	Tofranil® High 5-HT/NE uptake ratio Anticholinergic and sedataive effects tend to be marked
Imipramine-N-Oxide	194	41	42	235	193	192	**296**	310	---	Metabolite
Desipramine	234	195	193	235	208	266	**266**	280	TCA Secondary amine	Norpramin (Hoechst Marion Roussel®) Parent **or** metabolite of Imipramine Less sedative effects than imipramine Higher NE than 5-HT uptake blocking capacity
Clomipramine	58	85	268	228	314	130	**314**	320	TCA Tertiary amine	Anafranil® (Novartis) Obsessive-compulsive disorder (OCD)
Doxepine	58	42	165	152	178	189	**279**	290	TCA Tertiary amine	Sinequan® (Roerig) Also for anxiety.
Nordoxepine	44	178	165	222	204	128	**265**	280	---	Metabolite of Doxepin
Fluoxetine	44	104	91	59	309	148	**309**	320	Unrelated to other Anti-depressants	Prozac® (Dista) Selective 5-HT reuptake inhibitor For OCD also.
Norfluoxetine	134	104	191	162	77	251	**295**	300	---	Metabolite of Fluoxetine
Maprotiline	44	203	202	277	189	59	**277**	300	Tetracyclic	Ludiomil® (Novartis)
Mirtazapine	195	194	208	196	180	167	**265**	280	Tetracyclic	Remeron®
Paroxetine	192	44	138	329	70	109	**177**	200		Paxil® Selective 5-HT reuptake inhibitor

Antidepressants/-M										
Sertraline	274	276	159	262	239	306	**304**	330		Zoloft® Low dose has no CNS depression Selective 5-HT reuptake inhibitor For OCD also.
Trazodone	205	70	176	231	278	56	**371**	390	Chemically unrelated to TCA or other ADs Inhibits 5-HT uptake	Desyrel® High temp GC/MS program
Venlafaxine	58	134	179	91	119	121	**202**	290	Phenethylamine	Effexor® Potent inhibitor of 5-HT and NE reuptake
O-Desmethyl Venlafaxine	58	120	165	107	91	202	---	270	---	Metabolite
Antihistamines										
Brompheniramine	247	249	167	58	72	168	**318**	260	Propylamine Deriviative	Dimetane® Produce Drowsiness
Chlorpheniramine	203	205	202	167	58	139	**274**	290	Propylamine Deriviative	Chlor-Trimeton® Produce Drowsiness
Diphenhydramine	58	73	165	152	42	227	**255**	260	Aminoalkyl ether	Benadryl® Significant Anti-Chol. activity Produce Drowsiness
Doxylamine	71	58	167	180	182	72	**270**	290	Aminoalkyl ether	Unisom®, Decapryn® Produce Drowsiness
Promethazine	72	284	180	198	213	152	**284**	310	Phenothiazine Derivative	Phenergan®
Antiparkinson Agents										
Trihexyphenidyl	98	218	55	77	284	300	**301**	350	Anticholinergics	Artane®
Antipsychotics										
Clozapine	243	256	192	227	326	70	**326**	350	Dibenzapine Derivative	Clozaril® Severely ill schizophrenics
Haloperidol	224	42	237	226	123	206	**375**	380	Fluorobutyophenones	Haldol®
Olanzapine	242	229	213	198	42	169	**312**	340	Dibenzapine Derivative	Zyprexa® Psychotic disorders
Quetiapine	210	144	239	209	251	321	**383**	400	Dibenzothiaze-pines	Seroquel®
Quetapine-M	227	210	239	139	251	183	**295**	310	---	Metabolite
Thioridazine	98	370	70	126	185	244	**370**	390	Phenothiazine Derivative	Mellaril®
Antitussives										
DextroMethorphan	271	59	150	214	270	171	**271**	300	d-isomer of levorphanol	Vicks Formula 44, Robitussin, Street name: DMX
Sedative/Hypnotic										
Amobarbital	156	141	157	142	197	98	**226**	220	Barbiturate Alkyl-substituted barbituric acid	Amytal®
Butalbital	168	167	124	141	153	209	**224**	220	Barbiturate Alkyl-substituted barbituric acid	Fiorinal®, Esgic®
Eszopiclone	143	245	99	217	112					Lunesta®
Pentobarbital	156	141	157	98	197	69	**226**	230	Barbiturate Alkyl-substituted barbituric acid	Nembutal®
Sedative/Hypnotic										
Phenobarbital	204	232	117	161	146	217	**232**	240	Barbiturate Phenyl/alkyl-substituted barbituric acid	Luminal®, Phenalix®, Solfotin® Long-Acting Also – Anti-convulsant

Secobarbital	168	167	195	97	153	124	**238**	220	Barbiturate Alkyl-substituted barbituric acid	Seconal®, Tuinal®
Zaleplon	248	305	263	262	43	249	**305**	380	Pyrazolopyrim-idine	Sonata®
Zolpidem	235	236	92	65	219	307	**307**	320	Imidazopyridine	Ambien®
Miscellaneous Depressants										
γ-Hydroxybutyric Acid (GHB) -TMS	147	117	233	204	133	59	---	260	Lactone	Potential date rape drug Refer to method for detailed information
Propanolol	72	115	144	100	215	259	**259**	270	Aryloxypropanol-amine	Inderal β-Blocker
Fenfluramine	72	159	44	109	216	56	**231**	250	Sympatho-mimetic Phenethylamine	Anorexient, Sedation and Drowsiness
Vortioxetine	256	119	298	240	136	161				Atypical antidepressant
Benzodiazepines Anxiety/Hyponotic/Anticonvulsant										
Alprazolam	279	204	308	273	77	245	**308**	350	Triazolo-benzodiazepine	Xanax®
α-Hydroxyalprazolam-TMS	381	396	383	293	190	173		420	---	Metabolite of alprazolam
Diazepam	256	283	284	257	221	165	**284**	300	1,4- benzodiazepine	Valium® (Roche) Also – Muscle Relaxant
Midazolam	310	312	311	163	325	75	**325**	340	Fluorinated Triazolo benzodiazepine	Versed®
α-Hydroxymidazolam-TMS	310	73	398	413	383	168		460	---	Metabolite of midazolam
Nordiazepam	242	241	269	270	214	151	**270**	290	1,4- benzodiazepine	Calmday®, Madar®, Stilny® Parent or metabolite of diazepam, prazepam , clorazepate, chlordiazepoxide
Nordiazepam-TMS	341	342	343	327	227	269		350	1,4- benzodiazepine	See above
Oxazepam	205	239	267	177	151	104	**286**	280	1,4- benzodiazepine	Serax® (Wyeth-Ayerst)
Oxazepam-TMS	429	430	313	147	401	415		450	1,4- benzodiazepine	See above.
Temazepam	271	255	300	165	193	228	**300**	320	1,4- benzodiazepine	Restoril
Temazepam-TMS	343	257	345	283	357	372		390	1,4- benzodiazepine	See above.
Lorazepam	239	274	75	276	302	111	**320**	340	Dichloro-1,4-benzodiazepine	Ativan®
Lorazepam-TMS	429	431	147	347	177	449		470	Dichloro-1,4-benzodiazepine	Ativan®
Triazolam	313	238	315	342	203	279	**342**	380	Triazolo-benzodiazepine	Halcion®
α-Hydroxytriazolam									Triazolo-benzodiazepine	Metabolite of triazolam
Clonazepam	280	314	286	315	234	288	**315**	350	7-Nitro benzodiazepine	Klonopin® (Roche) Akinetic and myclonic seizures
7-Aminoclonazepam	285	256	257	258	44	287	---	300	7-Nitro benzodiazepine	Metabolite
Etizolam	342	313	266	224						
Benzodiazepines Anxiety/Hyponotic/Anticonvulsant										
Flurazepam	86	99	87	183	58	315	**387**	400	Fluoro-1,4-benzodiazepine	Dalmane®
Flunitrazepam	312	286	285	313	266	238	**313**	360	7-Nitro benzodiazepine	Rohypnol®
Midazolam										

Muscle Relaxants										
Baclofen	138	103	195	77	140	75	**213**	230	3-(ρ-Chloro phenyl)–γ aminobutyric acid	Lioresal®, GABA analog. Spasticity Depresses synaptic transmission
Carisoprodol	55	58	158	97	104	83	**260**	270	Dicarbamate	Soma® Major side effect is drowsiness
Meprobamate	83	55	71	96	114	144	**218**	170	Carbamate derivative	Miltown® (Wallace), Equanil® Parent or metabolite of carisoprodol
Meprobamate artifact (early R_t)	84	55	56	83	41	101		120	Carbamate	
Meprobamate artifact (mid R_t)	84	55	56	83	41	101		120	Carbamate	
Methocarbamol	118	109	124	77	62	81	**241**	250	Carbamate derivative	Robaxin®
Cyclobenzaprine	58	215	202	216	213	189	**275**	300	Very similar to amitriptyline (TCA).	Flexeril® (Merck) Note 202:215 ratio in MS.
Methaqualone	235	233	250	91	143	132				Quaalude

4.2.2 CNS Stimulants

Compound	Key Ions							Suggested Window	Class	Background Info.
	base	Prominent Ions					MW			
Amphetamine	44	91	65	120	115	134	**135**	145	Sympathomimetic Phenethylamine	Dexedrine®
Methamphetamine	58	91	65	56	134	115	**149**	160	Sympathomimetic Phenethylamine	Desoxyn®
Phentermine	58	91	134	65	115	117	**149**	160	Sympathomimetic Phenethylamine	Ionamin®, Fastin®
Benzphetamine	148	91	65	149	77	42	**239**	250	Sympathomimetic Phenethylamine	Didrex® Methamphetamine as metabolite ($-CH_2C_6H_5$)
Diethylpropion	100	72	77	44	56		**205**	220	Anorexient Sympathomimetic Phenethylamine	Tenuate®, Tepanil® Anorexient
Methylphenidate	84	91	150	56	115	118	**233**	250	Sympathomimetic Phenethylamine	Ritalin® Attention Deficient Disorder (ADD)
Ephedrine/ Pseudoephedrine	58	77/ 71	51	79	105	131	**165**	180	Sympathomimetic Phenethylamine	Numerous cold and sinus preparations.
Mazindol	266	268	231	204	176	115	**284**	300	Sympathomimetic Phenethylamine	Sanorex® Anorexient
Pemoline	176	107	77	89	147	248	**176**	370	Sympathomimetic Phenethylamine	Cylert® ADD, Narcolepsy
Phenmetrazine	71	56	42	77	177	105	**177**	190	Sympathomimetic Phenethylamine	Preludin®
Phendimetrazine	85	57	42	56	191	70	**191**	200	Sympathomimetic Phenethylamine	Plegine® Anorexient
Cocaine	82	182	77	94	105	303	**303**	320	Benzoic acid derivative	Alkaloid obtained from Erythroxylon coca
Ecgonine methyl ester Methylecgonine	82	96	83	199	168	182	**199**	220	---	Cocaine-M (-benzoylester)
Benzoylecgonine	124	168	82	77	105	94	**289**	300	---	Cocaine-M (-methylester)_
Benzoylecgonine-TMS	82	240	105	361	256	346	**361**	380	---	Cocaine-M (-methylester)_
Cocaethylene Ethylcocaine	82	196	94	105	317	272	**317**	330	---	Transesterification occurs with concurrent cocaine + EtOH use
Norcocaine	168	136	68	108	77	289	**289**	320	---	Cocaine metabolite, Demethylation of ecgonine

Propylhexedrine	58	140	55	44	155	67	**155**	170	Aliphatic amine	Benzedrex® Used as a decongestant. inhaler, has abuse potential as an amphetamine substitute

4.2.3 Narcotic Analgesics

Compound	**Key Ions**							**Suggested Window**	**Class**	**Background Info.**
	base	Prominent Ions					**MW**			
Buprenorphine	378	55	43	57	410	379	**467**	480	Thebaine derivative	Subutex®, moderate to severe pain, opiate addiction
Codeine-TMS	371	178	196	234	146	313	**371**	390	Alkaloid	Tylenol 3®
Codeine	299	162	229	214	124	115	**299**	320	Alkaloid Methyl-morphine	Mild to moderate pain. Alkaloid occurs naturally in opium
Norcodeine	285	215	81	148	115	164	**285**	300	---	Codeine-M (-CH_3)
Morphine-2TMS	429	236	196	414	146	414	**429**	450	Alkaloid	
Morphine	285	162	215	115	268	174	**285**	320	Alkaloid	Moderate to severe acute and chronic pain. Papaver somniferum poppy
6-Monoacetylmorphine	327	268	43	215	146	284	**327**	350	Alkaloid	Heroin Metabolite
Dihydrocodeine	301	164	244	284	115	128	**301**	320	Reduction of Codeine	
Dihydrocodeine-TMS	373	236	146	282	315				Reduction of Codeine	TMS
Hydrocodone-TMS	371	234	356	313	282	184		390	Synthetic opiate	See below
Hydrocodone	299	242	214	185	115	96	**299**	320	Synthetic opiate. Catalytic rearrangement of codeine	Hycodan®, Vicodin®, Codone®, Lortab® Moderate to moderately severe pain
Levophanol	257	256	59	200	150	157	**257**	270	Morphinan	Dromoran® (Europe) Severe pain
Meperidine (Pethidine)	71	247	172	218	103	232	**247**	260	Phenyl-piperidine	Demerol® (Sanofi), Moderate to severe pain
Meperidine-M	57	42	56	233	158	91	**233**	260	---	Metabolite
Methadone	72	294	165	223	57	91	**309**	320	Diphenyl-alkylketone	Dolophine®, Methadose® Severe pain, detox and temp. maintenance treatment of narcotic addiction
Methadone-M (nor-) -H_2O	277	276	262	220	165	200	**277**	300	---	Metabolite
Oxycodone	315	230	201	258	115	140	**315**	330	Catalytic reduction of hydroxy-codeinone.	Percolone®, Roxicodone®, Oxycontin®, OxyR®, Moderate to moderately severe pain
Pentazocine	217	110	70	202	230	285	**285**	300	Benzomorphan derivative	Talwin®
Propoxyphene	58	91	105	178	250	265	**339**	280		Darvon®, Darvocet® Mild to moderate pain
Norpropoxyphene/-M	44 220	220 44	205 205	100 100	129 129	307 307		320 320	---	Propoxyphene metabolites
Propoxyphene-M	44	100	234	88	105	57	**325**	320	---	Metabolite
Propoxyphene Artifact (2)	115	208	193	130	179	91	**208**	220	---	Metabolite
Tramadol	58	263	135	77	107	218		280		Ultram® Moderate pain with chronic pain
Tramadol-M (NDT) N-Desmethyltramadol	188	135	150	249	77	55			---	Metabolite

N-Desmethyltramadol Artifact	73	189	121	135	261				Carbamate derivative of NDT	Injection port formed.
Tramadol-M (ODT) O-Desmethyltramadol	58	249	121	77					---	Metabolite
Fentanyl	245	146	189	105	207	253	**336**	340	Opioid/ Anilide Derivative	Sublimaze® Duragesic Post-Op pain, Chronic pain (transdermal)
Hydromorphone	285	162	229	214	124	115	**299**	320	Alkaloid Methyl-morphine	Mild to moderate pain. Alkaloid occurs naturally in opium

4.2.4 PCP

Compound	**Key Ions**							**Suggested Window**	**Class**	**Background Info.**
	base	Prominent Ions					MW			
Phencyclidine	200	91	242	243	186	166	**243**	260		Dissociative agent

4.2.5 Hallucinogens

Compound	**Key Ions**							**Suggested Window**	**Class**	**Background Info.**
	base	Prominent Ions					MW			
Mescaline	182	167	181	211	151	148	**211**	230	2-Phenylethylamine	Peyote cactus
Mescaline Formyl Artifact	44	181	182	58	167	223		240	---	Metabolite/ artifact
Psilocyn	58	204	42	77	117	146	**204**	210	Indolethylamine	Psilocybe mexicana mushroom
3,4-MDA	44	136	135	77	51	81	**179**	190		
3,4-MDMA	58	77	135	51	105	89	**193**	210		

4.2.6 Cannabis

Compound	**Key Ions**							**Suggested Window**	**Class**	**Background Info.**
	base	Prominent Ions					MW			
Carboxy-THC-TMS	**371**	**473**	**488**	474	489	297	**488**	FS=500		
Δ9-THC	**371**	**386**	**306**							

4.2.7 Other Compounds of Interest: Miscellaneous Diluents, Ancillary Compounds

	base	Prominent Ions					MW			
Acetaminophen	109	151	43	80	53		151	160	Aniline derivative	Tylenol®
Aspirin	120	138	92	43	63	121	180	190	Acetylsalicyclic Acid	
Caffeine	194	109	67	82	55	193	194	210	Methylxanthine	Nodoze®
Ibuprofen	163	161	91	107	119	117	118	206	Arylacetic acid derivative	Nuprin®, Motrin®, Advil®
Lidocaine	86	58	234	72	120	77	234	250	Anilides	Xylocaine®
Nicotine	84	133	162	161	42	119	162	180	Alkaloid	
Cotinine	98	176	119	118	175	42	176	190	---	Nicotine-M
Guaifenesin	124	109	198	81	95	167	198	210	Methoxyphenoxy-propane diol	Expectorant Robitussin®
Verapamil	303	304	151	58	260	165	454	470	Calcium channel blocker	Calan®, Isoptin®

Toxicology AM #23: Solution Preparation

1.0 Background/References

1.1 Background: Refer to references

1.2 References

1.2.1 Shugar, G.J., Shugar, R.A. and Bauman, L. Grades of Purity of Chemicals pp. 145-154, pH Measurement. pp. 232-234. in: Chemical Technicians' Ready Reference Handbook, McGraw Hill: New York, 1973.

1.2.2 Ansys, Inc. SPEC Extraction Methods

1.2.3 United Chemical Technologies, Inc. Applications Manual.

2.0 Scope

2.1 This section describes the proper preparation of solutions and buffers used in ISP Forensic Services toxicology methods for the extraction of drug compounds from blood and urine specimens.

3.0 Equipment/Reagents

3.1 Equipment

3.1.1 Glassware

- Adequately sized beakers, volumetric flasks, graduated cylinders and volumetric pipettes

3.1.2 Laboratory balance

3.1.3 pH Meter and/or Indicator Strips

3.1.4 Appropriate buffer solutions for pH meter

3.1.5 Stirring hotplate

3.1.6 Magnetic stirrers

3.1.7 Safety Equipment

- Chemical Fume Hood
- Acid Resistant Apron
- Laboratory Coat
- Safety Goggles and/or face Shield
- Laboratory Gloves

3.2 Reagents

All chemicals must be ACS Grade or better.

3.2.1 Acids

- Acetic, Glacial
- Hydrochloric
- Phosphoric
- Sulfuric
- Formic (this may be ACS or LCMS grade)

3.2.2 Salts

- Ammonium Chloride
- Potassium Hydroxide
- Potassium Phosphate Monobasic
- Potassium Phosphate Dibasic
- Sodium Acetate Trihydrate
- Sodium Bicarbonate
- Sodium Hydroxide
- Sodium Phosphate Monobasic
- Sodium Phosphate Dibasic
- Sodium Tetraborate Decahydrate

3.2.3 Solvents

- Methanol

4.0 Procedure

4.1 Preparation of the following solutions must be recorded on corresponding preparation logs. Solutions may be made in different volumes by adjusting reagent ratios.

Note: Appropriate safety equipment must be worn during the preparation of solutions to minimize exposure to caustic/corrosive solutions. The order of the addition of chemicals may be crucial to minimize exothermic reactions. Refer to appropriate MSDS sheets for more information on handling chemicals.

4.1.1 Acetic Acid

4.1.1.1 **1.0M Acetic Acid (500mL)**

- Place approximately 400mL DI water into a 500mL volumetric flask. Add 29mL **glacial acetic acid**, mix. QS to 500mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.1.2 **20% Acetic Acid (500mL)**

- Place approximately 300mL DI water into a 500mL volumetric flask. Add 100mL glacial acetic acid, mix. QS to 500mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.1.3 **10% Acetic Acid (100mL)**

- Place 90mL DI water into a 100mL graduated cylinder. Add 10mL glacial acetic acid, mix.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.2 Ammonium Chloride

4.1.2.1 **Saturated Ammonium Chloride (500mL)**

- Place approximately 300mL DI water in a beaker and heat/stir over low heat. Add *ammonium chloride* until the solution is saturated. QS to 500mL.
- A positive and negative control will be run with each use. Remake as indicated by control data.

4.1.3 Ammonium Formate

4.1.3.1 **5mM Ammonium Formate +0.01% Formic Acid in LCMS Water**

- Add 0.316 grams ammonium formate to 1000mL of LCMS grade water, spike with 100uL Formic Acid. Mix thoroughly.

4.1.3.2 **10mM Ammonium Formate in LCMS Water**

- Add 0.631 grams ammonium formate to 1000mL LCMS grade water, mix thoroughly.

4.1.4 Ammonium Hydroxide

4.1.4.1 **0.5M Ammonium Hydroxide**

- Add 33.6mL Stock (28-30%w/w) ammonium hydroxide to 466.4mL of DI water.

4.1.5 Borate Buffers

4.1.5.1 **Borate Buffer, pH 9.2**

- Place approximately 500mL DI water into a 1000mL beaker. Heat and stir while adding 50g sodium tetraborate (Na2B4O7·10 H2O). Once dissolved, allow to cool. Bring volume up to approximately 950mL with DI water. Verify pH and adjust as necessary to pH 9.2 ±0.2 with 1N KOH or 100mm HCl. Place solution in 1000mL volumetric flask and QS with DI water.
- Solution is stable for at least six months. After six months, analyst is to verify pH prior to each use. If pH is outside preparation tolerance, the buffer should be remade. Further, a positive and negative control will be run with each use. Remake as indicated by control data.

4.1.5.2 **Borate Buffer, pH 12**

- Place approximately 500mL DI water into a 1000mL beaker. Heat and stir while adding 50g sodium tetraborate (Na2B4O7·10 H2O). Once dissolved, allow to cool. Bring volume up to approximately 900mL with DI water. Add 25mL 10N NaOH and stir. Verify pH and adjust as necessary to pH 12 ±0.2 with 10N NaOH or 6N HCl. Place solution in 1000mL volumetric flask and QS with DI water.
- *Solution is stable for at least six months. After six months, analyst is to verify pH prior to each use. If pH is outside preparation tolerance, the buffer should be remade. Further, a positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.6 Formic Acid for LC/MS Mobile Phases

4.1.6.1 **0.1% Formic Acid in LCMS Grade Water (500mL)**

- Place approximately 300mL LCMS Grade Water into instrument solvent bottle. Add 500µL of Formic Acid, mix . QS to approximately 500mL.
- *Make appropriate volume adjustments if needed, or purchase from an approved vendor.*

4.1.6.2 **0.1% Formic Acid in LCMS Acetonitrile (500mL)**

- Place approximately 300mL LCMS Grade Acetonitrile into instrument solvent bottle. Add 500µL of Formic Acid, mix. QS to approximately 500mL.
- *Make appropriate volume adjustments if needed, or purchase from an approved vendor.*

4.1.6.3 **0.01% Formic Acid in LCMS Grade Methanol (1000mL)**

- Spike 100µL formic acid into 1000mL methanol, prepare in an instrument solvent bottle. Make appropriate volume adjustments if needed, or purchase from an approved vendor (if available).

4.1.6.4 **0.1% Formic Acid in LCMS Grade Methanol (1000mL)**

- Spike 1 mL of formic acid into 1000mL LCMS grade methanol, prepare in an instrument solvent bottle. Make appropriate volume adjustments if needed, or purchase from an approved vendor (if available).

4.1.7 Hydrochloric Acid

4.1.7.1 **0.1M/100mM Hydrochloric Acid (500mL)**

- Place approximately 300mL DI water into a 500mL volumetric flask. Add 4.2mL **concentrated hydrochloric acid**, mix. QS to 500mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.7.2 **1% HCl in Methanol (10mL)**

- Add approximately 5mL of methanol to a 10mL volumetric flask. Pipet 100µL of *concentrated HCl*, QS and mix. Store in a brown glass bottle.
- *Solution is stable for six-months. A positive and negative control will be run with each use. Remake as indicated by control data, or every 6 months.*

4.1.8 Potassium Hydroxide (KOH)

Note: As the addition of KOH to water will generate significant heat, exercise due caution.

4.1.8.1 **1M/1N Potassium Hydroxide (100mL)**

- Dissolve 5.6g potassium hydroxide in approximately 80mL DI water in a 100mL volumetric flask. QS to 100mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.8.2 **11.8N Potassium Hydroxide (1000mL)**

- Gradually (!) add 662g potassium hydroxide to approximately 600mL DI water, stir on stir plate to dissolve. Allow to cool (this takes a while) and QS in a 1000mL volumetric flask.

- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.9 Potassium Phosphate Buffers

4.1.9.1 **Saturated Potassium Phosphate Buffer (1000mL)**

- Place approximately 1000mL DI water in a beaker and heat/stir over low heat. Add potassium phosphate monobasic until the solution is saturated. Allow solution to cool. Adjust pH to approximately 1.8 with concentrated phosphoric acid.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.9.2 **0.1M/100mM Potassium Phosphate Buffer (100mL) - Adjusted to pH 6**

- Dissolve 1.36g potassium phosphate monobasic in approximately 90mL DI water in a 150mL beaker. Adjust to pH 6.0 with 1-M potassium hydroxide. QS in a 100mL volumetric flask.
- *Store in colored-glass container (red or brown). Solution is stable for at least six months. After six months, analyst is to verify pH prior to each use. If pH is outside preparation tolerance, the buffer should be remade. Further, a positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.10 Sodium Acetate Buffers

4.1.10.1 **0.1M/100mM Acetate Buffer, pH 4.5 (500mL)**

- Dissolve 2.93g sodium acetate trihydrate in 400mL DI water in a 600mL beaker. Add 1.62mL glacial acetic acid, and mix well. Adjust to pH 4.5±0.1 with glacial acetic acid or 100mM acetic acid. QS to 500mL in a 500mL volumetric flask.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.10.2 **0.1M/100mM Acetate Buffer, pH 5.0 (500mL)**

- Prepare as with pH 4.5 buffer (5.12.5.9.1). Adjust pH to 5.0 ±0.1.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.10.3 **0.2M Acetate Buffer, pH 4.8 (1000mL)**

- Dissolve 14.14g sodium acetate trihydrate in approximately 800mL DI water. Add 55.2mL glacial acetic acid. Adjust to pH 4.8 and QS to 1000mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.10.4 **2.0M Acetate Buffer, pH 4.8 (1000mL)**

- Dissolve 141.4g sodium acetate trihydrate in approximately 800mL DI water. Add 55.2mL glacial acetic acid. Adjust to pH 4.8 and QS to 1000mL.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.11 Sodium Bicarbonate

4.1.11.1 **50mM Sodium Bicarbonate, pH 11 (500mL)**

- Dissolve 2.1g sodium bicarbonate in 500mL DI water. Adjust to pH 11 as needed.
- *A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.12 Sodium Hydroxide (NaOH)

Note: As the addition of NaOH to water will generate heat, exercise due caution.

4.1.12.1 **2N NaOH (1000mL)**

- Place approximately 250mL DI water into a 1000mL beaker. Gradually add 80g NaOH. Transfer to 500mL volumetric flask and QS to 500mL. (Caution: Exothermic)
- A positive and negative control will be run with each use. Remake as indicated by control data.

4.1.12.2 **10N NaOH (500mL)**

- Place approximately 400mL DI water into a 1000mL beaker. Gradually add 200g NaOH. Transfer to 500mL volumetric flask and QS to 500mL. (Caution: Exothermic)
- This reagent is used in the preparation of other reagents; those reagents are checked with each use.

4.1.13 Sodium Phosphate

4.1.13.1 **100mM Sodium Phosphate Dibasic (200mL)**

- Dissolve 2.84g sodium phosphate dibasic in approximately 160mL DI water. QS to 200mL and mix.
- *Store in glass container. A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.13.2 **100mM Sodium Phosphate Monobasic (200mL)**

- Dissolve 2.76g sodium phosphate monobasic in approximately 160mL DI water. QS to 200mL and mix.
- *Store in glass container. A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.14 Sodium Phosphate Buffers

4.1.14.1 **0.1M/100mM Sodium Phosphate Buffer (1000mL) Adjusted to pH 6**

- Dissolve 1.70g sodium phosphate dibasic (Na_2HPO_4) and 12.14 sodium phosphate monobasic ($NaH_2PO_4 \cdot H_20$) in approximately 800mL DI water in a 1000mL volumetric flask. QS to 1000mL. Adjust to pH 6.0 ±0.1 with 100mM monobasic sodium phosphate (to lower pH) or 100mM dibasic sodium phosphate (to raise the pH).
- *Check pH prior to use for blood toxicology casework; if pH outside preparation tolerance, remake buffer. Store in colored-glass container (red or brown). A positive and negative control will be run with each use. Remake as indicated by control data.*

4.1.15 Sulfuric Acid

4.1.15.1 **0.05M/0.1N Sulfuric Acid**

- Place approximately 800mL distilled/deionized (DI) water into a 1L volumetric flask. Add 2.7mL concentrated sulfuric acid, mix. QS to 1L.
- A positive and negative control will be run with each use. Remake as indicated by control data.

4.2 Quality Assurance

4.2.1 Refer to Toxicology AM #17 for balance intermediate check and calibration requirements.

Note: Balances properly monitored by drug discipline analysts fulfill quality assurance requirements. Additional check need not be performed.

Toxicology AM #24: LCMS-QQQ Instrument Maintenance and Operation

1.0 Background/References

1.1 Background

Recent instrument improvements have led to the accepted use of Liquid Chromatography Mass Spectrometry Mass Spectrometry use in the toxicology field. This instrument utilizes theory of separation of analytes using liquid chromatography, coupled with identification of analytes based on specified fragmentation and transitional patterns.

1.2 References

1.2.1 This method was developed in conjunction with Patrick Friel from Agilent during application training July 23-26, 2012. Additional information was added during validations of new methods completed by ISPFS Toxicologists in conjunction with PinPoint Testing, LLC in 2016.

2.0 Scope

2.1 This method is used for maintenance and operational instructions for Liquid Chromatograph Mass Spectrometer Mass Spectrometers (LC-QQQ or LC Triple Quad) used by the Idaho State Police Forensic Services Toxicology Section.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.3 Agilent 6410B or Equivalent LC/MS/MS system and MassHunter software

3.2 Reagents

3.2.1 LC/MS Low Tuning Solution

3.2.2 LC/MS Grade Methanol

3.2.3 LC/MS Needle Rinse (40/40/20: Water/Methanol/Isopropanol or 75/25 MeOH/Water - this preparation does not require LCMS grade solvents)

4.0 Procedure

4.1 Instrument Maintenance

4.1.1 Refer to Manufacturer's Recommendation for Scheduled Preventative Maintenance. *(Note: Preventative Maintenance is not required as long as response and successful tuning is completed)*

4.2 Instrument Operation

4.2.1 Instrument Start-up

4.2.1.1 Clean the Electrospray Ion Source using lint free cloth and LCMS Grade Methanol, the use ultrafine grit cleaning cloth may be necessary (*ensure thorough rinse with methanol after this type of cleaning*).

4.2.1.2 Select the appropriate Mobile Phase set for the desired method being used. Ensure that the aqueous mobile phase is connected to channel A and the organic mobile phase is connected to channel B. Reset the solvent levels in the acquisition software.

4.2.1.3 Turn LC/MS/MS on, open the pump purge valve and set flow to 1ml/min. Let run for approximately 10 minutes, this will remove any bubbles that may have accumulated since last use.

4.2.1.4 Verify the Column to be used for the desired method.

4.2.1.4.1 If a column change is needed, remove the column that is installed on the instrument, and replace with the dead volume connector.

4.2.1.4.2 Run correct mobile phases at a 50/50 ratio with 1mL/min flow for approximately 5 minutes to flush any previous mobile phase from the system.

4.2.1.4.3 Install the desired column, set solvent flow to appropriate method flow rate and solvent ratio, then let run for approximately 5-10 minutes.

4.2.1.5 Select Tune in MassHunter Acquisition and Run Check Tune, review tune report. If successful continue with pre-run instrument start-up. If unsuccessful perform Autotune, then re-run Check Tune.

4.2.1.5.1 NOTE: Successful Check Tune must have been run within one week prior to running case samples.

4.2.1.5.2 If analysis mode for samples to run is Positive mode, only a Checktune in Positive mode is necessary.

4.2.2 Analytical Run Set-up

4.2.2.1 In MassHunter Acquisition, load the appropriate acquisition method based on the analysis to be run on the instrument. Allow column temperature and LC pressure to stabilize. Verify that the binary pump ripple is <1%.

4.2.2.2 Open or start a new worklist. Enter the calibrators, blanks, controls and samples as needed. Designate the appropriate acquisition method for the samples.

4.2.2.3 Select Worklist then Worklist Run Parameters, and create a Data Path for this Batch (e.g. 110808BZ).

4.2.2.4 Also in Worklist Run Parameters, select Acquisition Cleanup/Standby, to put the instrument in Standby after the Worklist, or if a Not Ready Timeout occurs.

4.2.2.5 Save the Worklist. Use Save As to create a new worklist file. Do not overwrite the Master Worklist file.

4.2.2.6 Allow the instrument pressure and temperatures to stabilize for at least 15 minutes from the time it is turned ON.

4.2.2.7 Verify sufficient volume of needle rinse is present, refill if needed.

4.2.2.8 Begin the Worklist by clicking on the Multiple Vial icon on the top center of the MassHunter Acquisition screen.

4.2.3 Data Analysis

4.2.3.1 Open MassHunter Quantitative Analysis.

4.2.3.2 Select File/New Batch.

4.2.3.3 Navigate to the MassHunter/Data directory, and open the folder containing the data files for the current Batch. Assign a name to the Batch (e.g. 110808BZ), and select Open.

4.2.3.4 Select File/Add Samples, Select All, and OK to add all the samples to the Batch. Any column rinse injections will not contain meaningful results, and can be removed from the Add Samples list and or the batch.

4.2.3.5 Select Method/Open/Open and Apply from Existing File.

4.2.3.6 Navigate to the location of the appropriate Quantitative Analysis Data Analysis Method, select it, and select Open.

4.2.3.7 When the method has been opened and applied, the Batch Table appearance will change, but the results will not yet be populated.

4.2.3.8 Select Analyze Batch, or F5, to complete the Batch analysis, and Save the Batch.

4.2.3.9 The Batch Table view will show the Batch Table with results, Compound Information, and the Calibration Curve. Navigation by Compound can be accomplished by using either the arrows or the drop-down menu in the Compound section of the Batch Table.

4.2.3.10 To update the retention times and qualifier ion ratios for the current Batch, go to Method/Edit, or use F10, to enter the Method Editor view of MassHunter Quantitative Analysis. Ratios should be updated using Update/Average Qualifier ratios. Review the retention times and qualifier ion ratios from the calibrators, and make updates as appropriate. Save the updated method by selecting Save As, use the appropriate name format including the date.

4.2.3.11 To return to the Batch Table and apply the updated retention times and qualifier ion ratios, select the Exit button, answer Yes, and in the Batch Table select Analyze Batch, or F5.

4.2.4 Batch Review

4.2.4.1 The lab criterion for acceptable calibration curve R^2 will be defined in the appropriate analytical method.

4.2.4.2 A **minimum of four calibration points** are required for a valid curve. If the confirmation decision point is removed from the curve, the new administrative cutoff will be the lowest calibrator that meets quality assurance requirements.

4.2.4.3 Outliers are highlighted in the Batch Table with the color codes blue and red, for below or above acceptable limits (respectively).

4.2.4.4 The default criterion for Accuracy is that each calibrator result should agree with the target value ± 20%. For values below 10ng/mL the results should be within ± 30% of the target value.

4.2.4.5 The default criteria for a defining a positive result and reporting criteria are defined in the appropriate analytical methods.

4.2.4.6 Manual integration should not be needed frequently. When it is needed, it is enabled with the Start/End Manual Integration Tool in the Compound Information section of the Batch Table.

4.2.4.7 Manual integration is accomplished by left-clicking and dragging on the black boxes at peak start and end. Spurious peaks can be deleted by selecting the Start/End Manual Integration tool, right clicking in Compound Information, and selecting Zero Peak. (*Adjustments to the Retention time windows in the Data Analysis method should be made if excessive manual integration is needed.)*

4.2.4.8 Review the results for each analyte in the Batch. Check for outliers, R^2 values, and check QC values.

4.2.4.9 When Batch review is complete, Save the Batch a second time.

4.2.5 Generating Reports

4.2.5.1 Select Report/Generate and navigate to the report template (Ex – ISP_Summary_07_ LCMS_1Qual), select it, then select OK. Once the report has generated, print it, then select the appropriate ISTD template report (Ex – Quant Report_ISTD_Calibration_B_05_00) and print it. Alternatively, the generated reports may be saved as electronic files (Ex – pdfs) and stored electronically per any requirements in the ISP-FS Quality Manual.

4.2.5.2 The Queue Viewer, which allows you to track the report generation process, will open automatically. Depending on the size of the Batch, report generation may take approximately 5-20 minutes.

4.3 Quality Assurance Requirements

4.3.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication requirements.

4.4 Analysis Documentation

4.4.1 Case results are to be recorded in the iLIMS system.

4.4.2 Reports for the batch and controls, if printed, will be stored centrally in the lab in which the analysis was performed. A copy of data for controls may be stored electronically in a central location and need not be included in individual case files.

4.4.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

Toxicology AM #25: Blood Multi-Drug Screen by LCMS-QQQ

1.0 Background/References

1.1 References

1.1.1 This method was obtained from PinPoint Testing, LLC. PinPoint provided training and consultation on the method validation and implementation from June 2016 to November 2016.

2.0 Scope

2.1 This method is used to achieve rapid and accurate screening of multiple analytes in biological specimens. This method has simple, automated sample preparation that uses isotopically-labeled internal standards.

2.2 The analytes included are as follows:

Compound	Trade name(s) or other names	Administrative Threshold
6-MAM	6-monoacetylmorphine, 6-acetylmorphine	1
7-aminoclonazepam	Metabolite of Clonazepam	10
7-aminoflunitrazepam	Metabolite of Flunitrazepam	1
Acetyl Fentanyl	N/A	1
Acetyl Norfentanyl	Metabolite of Acetyl fentanyl	1
alpha-hydroxyalprazolam	Metabolite of alprazolam	10
alpha-hydroxymidazolam	Metabolite of midazolam	10
alpha-PVP	N/A	10
Alprazolam	Xanax, Niravam	10
Amitriptyline	Elevil, Endep, Levate	10
Amoxapine	Asendin, Asendis, Defanyl, Demolox	10
Amphetamine	Adderall	10
Benzoylecgonine	Metabolite of cocaine	100
Bupropion	Wellbutrin	10
Carbamazepine	Tegretol	10
Carisoprodol	Soma	10
Chlordiazepoxide	Librium	10
Citalopram	Celexa, Lexapro	10
Clomipramine	Anafranil	10

Clonazepam	Klonopin	10
Cocaine	N/A	10
Codeine	3-methylmorphine, contained in Cheracol,	10
Cyclobenzaprine	Flexeril, Amrix, Fexmid	10
Desipramine	Norpramin, metabolite of Imipramine	10
Dextromethorphan	Delsym	10
Dextrorphan	Metabolite of Dextromethorphan	10
Diazepam	Valium, Diastat	10
Dihydrocodeine	Drocode, Paracodeine, Parzone, Paramol	10
Diphenhydramine	Bendadryl, Nytol, Unisom, ingrediant of Benylin and Panadol	10
Doxepin	Adapin, Sinequan	10
Doxylamine	Unisom	10
EDDP	Metabolite of Methadone	100
Estazolam	Prosom	10
Fentanyl	Duragesic, Actiq, Lazanda, Subsys, Fentora, Sublimaze	1
Flunitrazepam	Rohypnol	10
Fluoxetine	Prozac	10
Flurazepam	Dalmane, Dalmadorm	10
Hydrocodone	Component in Lorcet, Loritab, Norco,	10
Hydromorphone	Dilaudid, Exalgo, Palladone	10
Imipramine	Tofranil	10
Ketamine	Ketalar	10
Levamisole	Ergamisol, Levasole	10
Maprotiline	Deprilept, Ludiomil, Psymion	10
MDA	3,4-methylenedioxy amphetamine	10
MDEA	3,4-methylenedioxyethyl amphetamine	10
MDMA	3,4-methylenedioxymeth amphetamine	10
Meperidine	Demerol	10
Meprobamate	Equanil	10
Methadone	Diskets, Methadose	10
Methamphetamine	Desoxyn	10
Methylphenidate	Ritalin	10
Metoprolol	Lopressor	10
Midazolam	Versed, Hypnovel, Dormicum	10
Mirtazapine	Remeron, Avanza, Zispin	10
Mitragynine	Major psychoactive component of Kratom	10
Morphine	Oramorph, MS Contin, Avinza, Kadian, Roxanol, Kapanol	100
Naloxone	Narcan	10
Naltrexol	Metabolite of Naltrexone	10
Naltrexone	Revia, Vivitrol	10

Norbuprenorphine	Metabolite of Buprenorphine	10
Nordiazepam	Metabolite of Diazepam	10
Norfentanyl	Metabolite of Fentanyl	10
Norhydrocodone	Metabolite of Hydrocodone	10
Normeperidine	Metabolite of Meperidine	10
Noroxycodone	Metabolite of Oxycodone	10
Norpropoxyphene	Metabolite of Propoxyphene	10
Nortriptyline	Metabolite of Amitriptyline, Aventyl, Pamelor	10
O-desmethyl-tramadol	Metabolite of Tramadol	10
Ondansetron	Zofran, Zuplenz	10
Oxazepam	Serax, Murelax, Alepam	10
Oxycodone	Roxycodone, Oxycontin, Oxceta	10
Oxymorphone	Opana, Numorphan	10
Pentazocine	Fortral, Sosegon, Talwin NX	10
Phenazepam	N/A	100
Phencyclidine	PCP	10
Phentermine	Adipex-P, Fastin, Ionamin	10
Phenytoin	Dilantin	100
Prazepam	N/A	10
Primidone	Lepsiral, Mysoline, Resimatil, Primaclone	10
Promethazine	Phenegran	10
Propoxyphene	Darvon, Dolene	10
Protriptyline	Vivactil	10
Pseudoephedrine	Sudafed	10
Quetiapine	Seroquel	10
Sertraline	Zoloft	10
Sufentanil	Sufenta	1
Tapentadol	Nucynta, Palexia, Tapal	10
Temazepam	Restoril, Normison	10
Tramadol	Ultram, ConZip, Ryzolt	10
Trazodone	Desyrel	10
Trimipramine	Surmontil	10
Venlafaxine	Effexor	10
Verapamil	Calan, Isoptin, Verelan	10
Zaleplon	Sonata, Starnoc, Andante	10
Zolpidem	Ambien	10
Zopiclone	Imovane, Zimovane, Lunesta (eszopiclone)	10

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B or other approved LC/MS/MS and MassHunter or equivalent software

3.1.2 Shaker/incubator

3.1.3 Positive Pressure Manifold (Biotage)

3.1.4 SPE dry (Biotage)

3.1.5 Phenomenex Phenyl Hexyl (4.6 x 50mm; 2.6um)

3.1.6 Calibrated Single channel pipette capable of dispending 250ul

3.1.7 Single or multichannel pipette capable of dispending 300ul

3.1.8 Single or multichannel pipette capable of dispensing 900ul

3.1.9 Single or multichannel pipette capable of dispensing 100ul

3.1.10 ToxBox kit with 96 well plate containing internal standards, and controls from Cayman Chemical, 96 well SLE+ plate, and 96 well blank sample preparation plate.

3.1.11 Appropriate sized pipette tips

3.1.12 Heat sealing foil covers for 96 well plate

3.2 Reagents

See AM# 23 for Solution Preparation instructions

3.2.1 10mM Ammonium Formate in LCMS Water(mobile phase A)

3.2.2 0.1% Formic Acid in LCMS Methanol (mobile phase B)

3.2.3 0.5M Ammonium Hydroxide

3.2.4 Formic Acid (LCMS grade)

3.2.5 Water (LCMS grade)

3.2.6 Methanol (LCMS grade)

3.2.7 Ammonium Formate (LCMS)

3.2.8 Ammonium Hydroxide (ACS or higher)

3.2.9 Ethyl Acetate (ACS or higher)

3.2.9.10 20% LCMS Methanol in LCMS Water (Reconstitution Solvent)

3.2.9.11 LC/MS Needle Rinse (40/40/20: Water/Methanol/Isopropanol or 75/25 MeOH/Water –this preparation does not require LCMS grade solvents)

3.3 Quality Assurance: Reference Materials and Controls

3.3.1 Internal Controls, and Internal Standards.

3.3.1.1 Internal controls and internal standards are prepared by Cayman Chemical and contained on the 96 well plate. A minimum of one internal positive control that has a concentration near the administrative threshold level for each compound will be run with each extraction.

3.3.2 External Control

3.3.2.1 External control may be prepared in house or purchased commercially (by a source other than Cayman). The control will have a minimum of 3 compounds at a concentration greater than the minimal control response as noted in Table 1.

3.3.3 A negative control will be run with each extraction. (Drug free sheep or human blood will be used to prepare negative controls)

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Allow refrigerated samples and frozen components to reach ambient temperature

4.1.2 Using a calibrated single channel pipette, pipette 250uL of blank (sheep or human) blood into the appropriate wells of the analytical (standards) plate (those wells containing internal positive and negative controls). Pipette the appropriate sample/case blood into the appropriate wells containing only internal standards.

4.1.3 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.4 Using a pipette (single or multi-channel), pipette 250uL of 0.5M ammonium hydroxide buffer into all wells in use on the analytical (standards) plate.

4.1.5 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.6 Transfer 300uL of blood+base mixture to corresponding wells of SLE+ plate.

4.1.7 Apply positive pressure for approximately 4 seconds or until no liquid remains on top of sorbent. Wait 5 minutes. (Positive Pressure manifold setting for this loading step is between 85-100 PSI)

4.1.8 Add 900uL of ethyl acetate and allow to flow through for approximately 5 minutes under gravity.

4.1.9 Apply positive pressure for approximately 15 seconds. (Positive Pressure manifold setting for this step is between 12-15 PSI).

4.1.10 Add 900uL of ethyl acetate and allow to flow through for approximately 5 minutes under gravity.

4.1.11 Apply positive pressure for approximately 15 seconds. (Positive Pressure manifold setting for this step is between 12-15 PSI).

4.1.12 Remove plate containing eluate. Place on SPE Dry and evaporate to dryness at approximately 35 degrees Celsius.

4.1.13 Reconstitute in 100uL LCMS grade methanol and heat seal plate with foil.

4.2 Instrument and Run set up.

4.2.1 See Toxicology AM #24 for instrument maintenance and operation.

4.2.2 Instrument run parameters and the acquisition methods for this Analytical method will be printed out (paper or electronically) and stored centrally in the lab or in a common file folder electronically.

4.2.2.1 Acquisition Required Settings

4.2.2.1.1 Column Temperature - 35*C

4.2.2.1.2 Injection volume - 5uL

4.2.2.1.3 Mobile Phase Flow rate - 0.5 mL/min

4.2.2.1.3: Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0	95	5
4	0	100
5	0	100
5.1	95	5
9	95	5

4.2.3 Worklist Set up should include internal control, external control, negative control and case samples.

4.2.4 All sample in the batch must be run using Screen Panel 1 and Screen Panel 2 Acquisition methods. Injection to injection cycle time for each of the methods is approximately 14 minutes.

4.3 Evaluation of Results

4.3.1 Minimum Criteria:

4.3.1.1 Signal to noise of Primary Transition greater than 5.

4.3.1.2 Signal to noise of secondary transition greater than 3.

4.3.1.3 In cases that do not meet the criteria above the analyst may also evaluate peak Symmetry/Resolution and it is at their discretion to determine if the sample exhibits a positive response for that compound. Correct peak shape (as compared with QCs) and the sufficient resolution of potential interferents from the analyte of interest can be considered.

4.3.1.4 To ensure the response is adequate, each compound in the internal control will be evaluated using the criteria listed above. If a compound does not meet these standards, samples cannot be evaluated for that compound. At the analysts discretion that compound can be removed from the list of compounds that sample was screened for, or the sample can be re-extracted.

4.3.1.5 Retention time criterion for peak identification is a ±2% or +/-.100 min whichever is greater retention time window relative to the internal control and/or internal standards around the analytes retention time. At the analyst's discretion when the analyte peak falls outside the retention time window and the internal standard shifts comparably that sample may be evaluated as positive if the other criteria are met.

4.3.1.6 Case Samples, external controls and negative controls will generally be considered negative if the primary transition response is less than 5 times less that of the internal control. Samples with a response between 2 and 5 times less than the internal control may be evaluated as negative at the analyst's discretion. If the primary transition response for methamphetamine is less than the internal control it may be evaluated as negative. Internal standard response should be evaluated if this occurs.

4.3.1.7 Samples should have a significant internal standard response, if a sample has little to no internal standard response for a compound, an evaluation for that sample cannot be made. The analyst will need to remove that drug from the list of drugs that sample was screened for or re-extract the sample.

4.4 Quality Assurance Requirements

4.4.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication requirements.

4.5 Analysis Documentation

4.5.1 A print out from the batch table showing compound name, and results for signal to noise for transition 1 and 2, retention time, response, and internal standard retention time and response will be printed out for the case sample and the internal control and included in the analysts' notes.

4.5.2 The print out (paper or electronic) for the negative control, external control and internal controls will be stored centrally in the lab in which they were performed or stored on a network drive.

4.5.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

4.6 Limitation of method

4.6.1 This method is a preliminary screen, the screen results are an indication of drugs that may be present in a sample. The weight or confidence in screening results cannot be given the same as a confirmatory test. If these results are reported it must be clear on the report that it is an indicator the drug is present in the sample, not a confirmation of the drug in the sample.

Toxicology AM #26: Blood THC and Metabolites Screen by LCMS-QQQ

1.0 Background/References

1.1 Background

Δ^9-THC (Figure 1) is the chief psychoactive cannabinoid resulting from exposure to marijuana. Δ^9-THC has a peak blood concentration within 5 to 15 minutes following smoking of a marijuana cigarette.[4,5,6] This blood concentration drops rapidly after cessation of smoking.[4,5] The level may fall to less than 5ng within 30 to 60 minutes, although longer detection times have been reported.[4,5] Detection of low dose (1.75%) post-smoking Δ^9-THC has been reported to vary from 3 to 12 hours.[5] This detection window was based on a limit of quantitation of 0.5ng/mL. The number, duration, spacing of puffs, hold time, and inhalation volume all impact the degree of drug exposure and thus bioavailability.[5] Longer detection times have been observed for frequent users. Δ^9-THC is metabolized to an active metabolite, 11-hydroxy-tetrahydrocannabinol (Hydroxy-THC, THC-OH), and an inactive metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (carboxy-THC). The concentration of the Δ^9-THC inactive metabolite, carboxy-THC, gradually increases and may plateau for several hours.[5] There is poor correlation between blood Δ^9-THC and psychoactive effects since the Δ^9-THC concentrations begin to decline prior to the time of peak effects.[4,5,6] The detection window for the active analytes is much shorter than that of carboxy-THC.[7] Cannabinol and cannabidiol are minor cannabinoids also found in marijuana.[8,9] Due to their low abundance, rapid clearance from blood, and similar pharmacokinetic profiles in chronic versus occasional smokers, the presence of cannabidiol and cannabinol in blood has been suggested to be a sufficient but unnecessary marker for recent marijuana use.[8]

Negative behavioral effects reported from exposure to marijuana include altered time perception, lack of concentration, and impaired learning and memory which can lead to impairment of cognitive and performance tasks.[5] Establishing impairment in an individual is based on evaluation of all available information in conjunction with quantitative blood levels.

For additional background, refer to Toxicology AM #3, obsolete analytical method 3.10.1, and the provided references.

1.2 References

1.2.1 This method was obtained from PinPoint Testing, LLC. The method validation and implementation was completed by ISPFS personnel from October 2016 to November 2016.

1.2.2 Standard Operating Procedure for Blood SPE THC and Carboxy-THC GC/MSD Assay, Edmonton, Canada Office of the Chief Medical Examiners, 2003.

1.2.3 Huestis, M.A., *Cannabis (Marijuana) - Effects on Human Behavior and Performance,* Forensic Science Rev. 14(1/2): 16-60, 2002.

1.2.4 Drummer, O.H., *Cannabis*, pp. 178-212. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.5 Huestis, M. *Marijuana*. pp. 229-244. *in:* Principles of Forensic Toxicology, Second Edition. Levine, B. ed., AACC, 2003.

1.2.6 Desrosiers, N.A.; Himes, S.K.; Scheidweiler, K.B.; Concheiro-Guisan, M.; Huestis, M.A. *Phase I and II Cannbinoid Disposition in Blood and Plasma of Occasional and Frequent Smokers Following Controlled Smoked Cannabis.* Clinical Chemistry, 60:4, pp. 631-643, 2014.

1.2.7 Nadulski, T., et al. *Simultaneous and Sensitive Analysis of THC, 11-OH0THC, THC-COOH, CBD, and CBN by GC-MS in Plasma after Oral Application of Small Doses of THC and Cannabis Extract*. Journal of Analytical Toxicology, Vol 29, pp. 782-789, November/December 2005.

2.0 Scope

2.1 This method is used for preliminary screening of Δ^9-THC, 11-nor-Δ^9-THC-9-COOH (Carboxy-THC) and 11-hydroxy-Δ^9-THC (Hydroxy-THC) in blood.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B or Equivalent LC/MS/MS system and MassHunter software

3.1.2 Shaking incubator

3.1.3 ToxBox kit with 48 Well plate containing internal standards and controls from Cayman Chemical, 48 well SLE+ plate, and 48 well blank sample collection plate.

3.1.4 Test tube rocker

3.1.5 Calibrated pipettes for dispensing blood samples

3.1.6 Single or Multi-channel Pipettes for all other transfers and additions

3.1.7 Positive Pressure Manifold

3.1.8 SPE sample evaporator concentrator

3.2 Reagents

Refer to Toxicology AM #23 for Solution Preparation instructions.

3.2.1 0.1% Formic Acid in Water (LCMS grade)

3.2.2 0.1% Formic Acid in Methanol (LCMS grade)

3.2.3 Methyl Tert-Butyl Ether (MTBE) 99.9%

3.2.4 Hexanes (ACS)

3.2.5 Methanol (LCMS grade)

3.2.6 10mM Ammonium Formate

3.3 Quality Assurance: Reference Materials and Controls

3.3.1 Plate controls and internal standards are prepared by Cayman Chemical and are contained on the 48 well plate. A minimum of one positive plate control with a nominal concentration of 10ng/mL for each drug compound will be included in each run. A minimum of three calibrators will be run to establish a calculated response value for the case samples (one must be between the nominal value of (3 and 5)

3.3.2 A negative control will be run with each extraction (drug free sheep or human blood will be used).

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Print off Method Checklist and allow refrigerated specimens and spiked sample plate to reach room temperature.

4.1.2 Add 1.0mL (**calibrated pipette**) of blank blood to sample wells containing dried down standards, controls, and a blank. Add 1mL of the corresponding case samples to wells with internal standard only. For the external control, spike the well with the working solution, then add 1mL blank blood.

4.1.3 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.4 Add 0.5mL 0.1% formic acid in LCMS water to all of the wells.

4.1.5 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.6 Transfer 800µL of blood+acid mixture to the corresponding wells of the SLE+ plate.

4.1.7 Apply positive pressure until solution penetrates the well sorbent approximately 50% (or until no liquid remains on top of the sorbent) – approximately 4 seconds. *(Recommended setting for Positive Pressure manifold for this loading step is between 80-95 PSI)*

4.1.8 Wait 5 minutes for sample to completely absorb.

4.1.9 Add 2.25mL MTBE and allow to flow for 5 minutes under gravity. *This should be completed in increments, as the sample well accommodates a maximum volume of 1mL. (Recommended for this step is: three (3) repetitions of adding 750µL)*

4.1.10 Apply positive pressure to complete elution- approximately 15 seconds. *(Positive Pressure manifold set between 12-15 PSI)*

4.1.11 Add 2.25mL hexane and allow to flow for 5 minutes under gravity. *This should be completed in increments, as the sample well accommodates a maximum volume of 1mL. (Recommended for this step is: three (3) repetitions of adding 750µL)*

4.1.12 Apply positive pressure to complete elution- approximately 15 seconds. *(Positive Pressure manifold set between 12-15 PSI)*

4.1.13 Evaporate to dryness in the evaporator/concentrator at approximately 35°C under a constant flow of nitrogen. Temperatures for this step are not critical and it is appropriate to use the equipment temperature readings. The temperatures do not need to be monitored or verified with a traceable thermometer.

4.1.14 Reconstitute each sample with 100µL LCMS Grade Methanol. Heat seal plate with foil to prevent evaporation.

4.1.15 Analyze samples immediately or freeze (up to 4 days) for future analysis.

4.2 Instrument and Run set up.

4.2.1 ***See Toxicology AM #24 for instrument maintenance and operation.***

4.2.2 Instrument run parameters and the acquisition methods for this Analytical Method will be printed out (paper or electronically) and stored centrally in the lab or in a common file folder electronically.

4.2.2.1 Acquisition Required Settings

4.2.2.1.1 Column Temperature - 35*C

4.2.2.1.2 Injection volume - 10uL

4.2.2.1.3 Mobile Phase Flow rate - 0.6 mL/min

4.2.2.1.4 Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0	55	45
0.5	0	100
2.0	0	100
2.5	25	75
3.5	55	45
5.0	55	45

4.2.3 Worklist Set up should include a positive control, calibrators, negative control, and case samples.

4.3 Evaluation of Results

4.3.1 Minimum Criteria:

4.3.1.1 Calculated sample concentration of 3ng/mL or greater for THC and THC-OH, a calculated sample concentration of 5ng/mL or greater for Carboxy-THC.

4.3.1.2 Retention time within +/- 2% or +/-.100 min whichever is greater of the average retention time of the calibrators.

4.3.1.3 Additionally the response of the secondary transition for THC-OH may be evaluated if the response is less than 80% that of the 3ng/mL calibrator the sample may be evaluated as negative. This criteria is optional and is evaluated at the analyst's discretion.

4.3.1.4 To ensure the response is adequate, each compound in the internal control will be evaluated using the criteria listed above. If a compound does not meet these standards, samples cannot be evaluated for that compound. At the analyst's discretion that compound can be removed from the list of compounds that sample was screened for, or the sample can be re-extracted.

4.3.1.7 Samples should have a significant internal standard response, if a sample has little to no internal standard response for a compound, an evaluation for that sample cannot be made. The analyst will need to re-inject or re-extract that sample.

4.4 Quality Assurance Requirements

4.4.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication requirements.

4.5 Analysis Documentation

4.5.1 The printed results for each case sample will be included with the analyst's notes. Case results are to be recorded in the iLIMS system.

4.5.2 The print out (paper or electronic) for the calibrators and controls will be stored centrally in the lab in which they were performed or stored on a network drive.

4.5.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

4.6 Limitation of method

4.6.1 This method is a preliminary screen, the screen results are an indication of drugs that may be present in a sample. The weight or confidence in screening results cannot be given the same as a confirmatory test. This is not a quantitative method and quantitative results cannot be reported from this method. The quantitative aspects of this method are only for establishing a minimum response threshold. If these results are reported it must be clear on the report that it is an indicator the drug is present in the sample, not a confirmation of the drug in the sample.

Toxicology AM #27: Quantitative Analysis of THC and Metabolites in Blood by LCMS-QQQ

1.0 Background/References

1.1 Background

Δ^9-THC (Figure 1) is the chief psychoactive cannabinoid resulting from exposure to marijuana. Δ^9-THC has a peak blood concentration within 5 to 15 minutes following smoking of a marijuana cigarette.[4,5,6] This blood concentration drops rapidly after cessation of smoking.[4,5] The level may fall to less than 5ng within 30 to 60 minutes, although longer detection times have been reported.[4,5] Detection of low dose (1.75%) post-smoking Δ^9-THC has been reported to vary from 3 to 12 hours.[5] This detection window was based on a limit of quantitation of 0.5ng/mL. The number, duration, spacing of puffs, hold time, and inhalation volume all impact the degree of drug exposure and thus bioavailability.[5] Longer detection times have been observed for frequent users. Δ^9-THC is metabolized to an active metabolite, 11-hydroxy-tetrahydrocannabinol (Hydroxy-THC, THC-OH), and an inactive metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (carboxy-THC). The concentration of the Δ^9-THC inactive metabolite, carboxy-THC, gradually increases and may plateau for several hours.[5] There is poor correlation between blood Δ^9-THC and psychoactive effects since the Δ^9-THC concentrations begin to decline prior to the time of peak effects.[4,5,6] The detection window for the active analytes is much shorter than that of carboxy-THC.[7] Cannabinol and cannabidiol are minor cannabinoids also found in marijuana.[8,9] Due to their low abundance, rapid clearance from blood, and similar pharmacokinetic profiles in chronic versus occasional smokers, the presence of cannabidiol and cannabinol in blood has been suggested to be a sufficient but unnecessary marker for recent marijuana use.[8]

Negative behavioral effects reported from exposure to marijuana include altered time perception, lack of concentration, and impaired learning and memory which can lead to impairment of cognitive and performance tasks.[5] Establishing impairment in an individual is based on evaluation of all available information in conjunction with quantitative blood levels.

For additional background, refer to Toxicology AM #3 and obsolete analytical methods 3.10.1, as well as provided references.

1.2 References

1.2.1 This method was obtained from PinPoint Testing, LLC. PinPoint provided training and consultation on the method validation and implementation from June 2016 to October 2016.

1.2.2 Standard Operating Procedure for Blood SPE THC and Carboxy-THC GC/MSD Assay, Edmonton, Canada Office of the Chief Medical Examiners, 2003.

1.2.3 Huestis, M.A., *Cannabis (Marijuana) - Effects on Human Behavior and Performance,* Forensic Science Rev. 14(1/2): 16-60, 2002.

1.2.4 Drummer, O.H., *Cannabis*, pp. 178-212. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

1.2.5 Huestis, M. *Marijuana*. pp. 229-244. *in:* Principles of Forensic Toxicology, Second Edition. Levine, B. ed., AACC, 2003.

1.2.6 Desrosiers, N.A.; Himes, S.K.; Scheidweiler, K.B.; Concheiro-Guisan, M.; Huestis, M.A. *Phase I and II Cannbinoid Disposition in Blood and Plasma of Occasional and Frequent Smokers Following Controlled Smoked Cannabis.* Clinical Chemistry, 60:4, pp. 631-643, 2014.

1.2.7 Nadulski, T., et al. *Simultaneous and Sensitive Analysis of THC, 11-OHTHC, THC-COOH, CBD, and CBN by GC-MS in Plasma after Oral Application of Small Doses of THC and Cannabis Extract*. Journal of Analytical Toxicology, Vol 29, pp. 782-789, November/December 2005.

2.0 Scope

2.1 This method is used for the confirmation of Δ^9-THC, 11-nor-Δ^9-THC-9-COOH (Carboxy-THC) and 11-hydroxy-Δ^9-THC (Hydroxy-THC) in blood. The words calibrator and calibration are used to coincide with the terminology in instrument software and manufacturer manuals. The manufacturer's term calibrator refers to what is considered by ISP-FS as reference material that has a certified concentration of drug present.

3.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B or Equivalent LC/MS/MS system and MassHunter software

3.1.2 Shaking incubator

3.1.3 ToxBox kit with 48 Well plate containing internal standards and controls from Cayman Chemical, 48 well SLE+ plate, and 48 well blank sample collection plate.

3.1.4 Test tube rocker

3.1.5 Calibrated pipettes for dispensing blood samples.

3.1.6 Pipettes for all other transfers and additions.

3.1.7 Positive Pressure Manifold

3.1.8 SPE sample evaporator concentrator

3.1.9 UCT Selectra DA 100 x 2.1 mm, 3um LC Column

3.2 Reagents

Refer to Toxicology AM #23 for Solution Preparation instructions.

3.2.1 0.1% Formic Acid in Water (LCMS grade)

3.2.2 0.1% Formic Acid in Acetonitrile (LCMS grade)

3.2.3 Methyl Tert-Butyl Ether (MTBE) 99.9%

3.2.4 Hexanes (ACS)

3.2.5 Methanol (LCMS grade)

3.3 Quality Assurance: Reference Materials and Controls

3.3.1 Plate controls and internal standards are prepared by Cayman Chemical and are contained on the 48 well plate. A minimum of one positive plate control with a nominal concentration of 10ng/mL for each drug compound will be included in each run.

3.3.2 In addition to the quality control that is included on the Cayman plate, one external control will be run. This control can be prepared in house or purchased commercially and must, at a minimum, contain THC at a nominal concentration between 3ng and 10ng/mL. A cannabinoid working solution may be prepared at a concentration of 1ug/mL or 0.1ug/mL and stored frozen for up to one year or the date of the earliest stock reference material expiration. The working solution prep must be documented on a prep sheet.

3.3.3 A negative control will be run with each extraction (drug free sheep or human blood will be used).

3.3.4 A non-extracted blank containing at minimum one internal standard will be run directly preceding each case sample to rule out carryover. The area response of the blank preceding a sample must be at least 10 times less than any compound confirmed in the case sample, and must be below the limit of confirmation for any analyte confirmed in the case sample. If confirmation criteria (e.g. ion ratios, RT, S/N) are not met, the analyte is not considered present.

3.3.4.1 To prepare, spike a tapered bottomed tube with the appropriate amount of internal standard (ie 10 µL if reconstituted in 300 µL reconstitution solvent) dry under Nitrogen, reconstitute in 100ul LCMS grade Methanol

4.0 Procedure

4.1 Extraction Procedure

4.1.1 Print off method checklist and allow refrigerated specimens and spiked sample plate to reach room temperature.

4.1.2 Add 1.0mL (**calibrated pipette**) of blank blood to sample wells containing dried down standards, controls and a blank. Add 1mL of the corresponding case samples to wells with internal standard only. For the external control; spike the well with the working solution then add 1mL blank blood.

4.1.3 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.4 Add 0.5mL 0.1% formic acid in water to all of the wells.

4.1.5 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.6 Transfer 800µL of blood+acid mixture to the corresponding wells of the SLE+ plate.

4.1.7 Apply positive pressure until solution penetrates the well sorbent approximately 50% (or until no liquid remains on top of the sorbent) – approximately 4 seconds. *(Recommended setting for Positive Pressure manifold for this loading step is between 80-95 PSI)*

4.1.8 Wait 5 minutes for sample to completely absorb.

4.1.9 Add 2.25mL MTBE and allow to flow for 5 minutes under gravity. This should be completed in increments, as the sample well accommodates a maximum volume of 1mL. *(Recommended for this step is: three (3) repetitions of adding 750uL)*

4.1.10 Apply positive pressure to complete elution- approximately 15 seconds. *(Positive Pressure manifold set between 12-15 PSI)*

4.1.11 Add 2.25mL hexane and allow to flow for 5 minutes under gravity. This should be completed in increments, as the sample well accommodates a maximum volume of 1mL. *(Recommended for this step is: three (3) repetitions of adding 750uL)*

4.1.12 Apply positive pressure to complete elution- approximately 15 seconds. *(Positive Pressure manifold set between 12-15 PSI)*

4.1.13 Evaporate to dryness in the evaporator/concentrator at approximately 35°C under a constant flow of nitrogen. Temperatures for this step are not critical and it is appropriate to use the equipment temperature readings. The temperatures do not need to be monitored or verified with a traceable thermometer.

4.1.14 Reconstitute each sample with 100µL LC-MS grade Methanol. Heat-seal plate with foil to prevent evaporation.

4.1.15 Analyze samples immediately or freeze for future analysis.

4.2 Instrument and Run set up

4.2.1 See Toxicology AM #24 for LCMS instrument maintenance and operation.

4.2.2 Set up the worklist in MassHunter.

4.2.3 A solvent blank is generally run before the calibration curve, then the calibration curve is run, then a negative control. At minimum, two positive controls will be run with each batch, one will be the external control and the other the plate control that has been aliquoted on the plate.

4.2.4 Non-Extracted solvent blank will be run directly proceeding a case sample.

4.2.5 Instrument run parameters and the acquisition methods for this Analytical Method will be printed out (paper or electronically) and stored centrally in the lab or in a common file folder electronically.

4.2.5.1 Acquisition Required Settings

4.2.5.1.1 Column Temperature - 50*C

4.2.5.1.2 Injection volume - 10uL

4.2.5.1.3 Mobile Phase Flow rate - 0.6 mL/min

4.2.5.1.3: Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0	45	55
5	45	55
5.1	20	80
7	20	80

4.3 Evaluation of Results

4.3.1 Batch Review

4.3.1.1 The lab criterion for acceptable calibration curve R^2 is >0.98

4.3.1.2 Calibration curves for compounds reported quantitatively (Δ^9-THC and 11-hydroxy-Δ^9-THC (Hydroxy-THC)) shall be set to linear with a 1/x weighting. Calibration Curves for qualitatively reported compounds 11-nor-Δ^9-THC-9-COOH (Carboxy-THC) shall be at the analysts discretion.

4.3.1.3 The limit of detection (LOD) is the same as the limit of quantitation (LOQ) for blood quantitative analysis results. The uncertainty of measurement budgets for each compound have been established and will be updated when new instrumentation is approved for use and will be evaluated annually by the discipline lead. The LODs are as follows:

Analyte	**LOD**
THC	3ng/mL
Carboxy-THC	10ng/mL
THC-OH	3ng/mL

4.3.2 The default criteria for a positive result are:

4.3.2.1 The sample must have a concentration no less than the established LOD for that analyte.

4.3.2.2 Samples with a higher response than the 50ng/mL will be reported out as greater than 50ng/mL. The uncertainty of measurement has currently only been evaluated between 3 and 50ng/mL for each compound. The estimated expanded uncertainty will only be reported when it impacts evaluation of a statute, legal requirement, or upon customer request. When the measurement uncertainty is reported it will be on the report in the same units as the measurement and a statement regarding the coverage probability of 99.73% will also be on the report. The current expanded uncertainty is published as a protected document on an ISPFS shared drive and is available to all analysts.

4.3.2.3 The analyst retention time must be within +/- 3% or +/- .100 min whichever is greater of the average retention time of the calibrators for that analyte.

4.3.2.4 For calibrators and controls 10ng and below the accuracy must be within 30%, for calibrators and controls greater than 10ng/mL the accuracy must be within 20%. If the control over 10ng falls outside the accuracy range at the analyst's discretion the compound may be reported qualitatively.

4.3.2.5 The ion ratios for selected transitions must fall within 20% of the averaged calibrators for each run.

4.3.2.6 S/N for each transition of detected analyte must be greater than 10.

4.3.2.7 The negative control will be evaluated as passing if the compound of interest does not meet all of the above listed criteria and the internal standard gives an anticipated response to demonstrate the sample extracted and injected correctly.

4.4 Quality Assurance Requirements

4.4.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication.

4.5 Analysis Documentation

4.5.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results will be recorded in the LIMS system.

4.5.2 The reports for the batch, including calibration curves and controls will be stored centrally in the lab or electronically on a common drive.

4.5.3 The raw data from the run will be stored electronically and will be backed up at least every two months.

4.5.4 Results of the controls will be entered onto the Control Monitoring Chart.

Toxicology AM #28: Blood Multi-Drug Confirmatory Analysis by LCMS-QQQ

1.0Background/References

1.1 Background

1.1.1 This method was obtained from PinPoint Testing, LLC. PinPoint provided training and consultation on the method validation and implementation from June 2016 to November 2016.

2.0Scope

2.1 This method is used to achieve rapid and accurate confirmation of multiple analytes in biological specimens. This method has simple, automated sample preparation that uses isotopically-labeled internal standards.

2.2 The analytes are as follows:

Panel	Analyte	Trade or Other Names	Therapeutic Plasma Range (ng/mL)	Confirmation LOD (ng/mL)	Range
1	6-MAM	6-acetylmorphine	n/a	0.5	0.5-100
1	7-aminoclonazepam	metabolite of Clonazepam	n/a	5	5-1000
1	Acetyl Fentanyl	n/a	n/a	0.5	0.5-50
1	Acetyl Norfentanyl	metabolite of Acetyl fentanyl	n/a	0.5	0.5-100
1	α-hydroxyalprazolam	metabolite of Alprazolam	50	5	5-1000
1	alpha-PVP	Flakka, Gravel	n/a	5	5-1000
1	Alprazolam	Xanax, Niravam	5–50	5	5-1000
1	Amphetamine	Adderall, metabolite of Methamphetamine	20–100	5	5-500
1	Buprenorphine	Buprenex, Butrans	0.5–5	2.5	2.5-100
1	Bupropion	Wellbutrin	10-20; 50-100	5	5-1000
1	Carisoprodol	Soma	10000–30000	5	5-1000
1	Citalopram	Celexa, Lexapro	50–110	5	5-1000
1	Clonazepam	Klonopin	20–80	5	5-1000
1	Codeine	3-methylmorphine, contained in Cheracol, Robitussin A-C,	30–250	5	5-1000
1	Cyclobenzaprine	Flexeril, Amrix, Fexmid	3–40	5	5-500
1	Dextromethorphan	Delsym	10–40	5	5-1000
1	Dextrorphan	metabolite of Dextromethorphan	n/a	5	5-1000
1	Diazepam	Valium, Diastat	100–2000	5	5-500
1	Dihydrocodeine	Drocode, Paracodeine, Parzone, Paramol	30–250	5	5-500

1	Diphenhydramine	Bendadryl, Nytol, Unisom, ingredient of Benylin and Panadol	50-100	5	5-1000
1	Doxylamine	Unisom	50-200	5	5-1000
1	EDDP	metabolite of Methadone	n/a	5	5-500
1	Fentanyl	Duragesic, Actiq, Lazanda, Subsys, Fentora, Sublimaze	3–300	0.5	0.5-100
1	Fluoxetine	Prozac	120–500	25	25-500
1	Hydrocodone	contained in Lorcet, Loritab, Norco, Vicodin, Hycodan, Tussionex	10–50	5	5-1000
1	Hydromorphone	Dilaudid, Exalgo, Palladone	5–50	5	5-1000
1	Ketamine	Ketalar	1000–6000	5	5-1000
1	Lorazepam	Ativan	n/a	5	5-100
1	Meprobamate	Equanil	5000–10000	5	5-1000
1	Methadone	Diskets, Methadose	10–1000	5	5-1000
1	Methamphetamine	Desoxyn	0–100	5	5-1000
1	Morphine	Oramorph, MS Contin, Avinza, Kadian, Roxanol, Kapanol	10–300	5	5-1000
1	Naloxone	Narcan	10–30	5	5-500
1	Naltrexol	metabolite of Naltrexone	n/a	5	5-500
1	Naltrexone	Revia, Vivitrol	3–50	5	5-500
1	Norbuprenorphine	metabolite of Buprenorphine	n/a	2.5	2.5-50
1	Nordiazepam	metabolite of Diazepam	200–800	25	25-1000
1	Norfentanyl	metabolite of Fentanyl	n/a	0.5	0.5-100
1	Norhydrocodone	metabolite of Hydrocodone	n/a	5	5-1000
1	Noroxycodone	metabolite of Oxycodone	n/a	5	5-500
1	O-desmethyl-tramadol	metabolite of Tramadol	n/a	5	5-500
1	Oxazepam	Serax, Murelax, Alepam	200–1500	5	5-500
1	Oxycodone	Roxycodone, Oxycontin, Oxceta	5–100	5	5-1000
1	Oxymorphone	Opana, Numorphan	n/a	5	5-500
1	Phentermine	Adipex-P, Fastin, Ionamin	30–100	5	5-1000
1	Promethazine	Phenegran	50-200	5	5-1000
1	Quetiapine	Seroquel	100–500	5	5-500
1	Sertraline	Zoloft	50-250	5	5-500
1	Sufentanil	Sufenta	0.5–10	2.5	2.5-100
1	Temazepam	Restoril, Normison	0.2–150	25	25-500
1	Tramadol	Ultram, ConZip, Ryzolt	100–1000	5	5-1000
1	Trazodone	Desyrel	700–1000	5	5-1000
1	Venlafaxine	Effexor	35-597	5	5-1000

1	Zolpidem	Ambien	80–200	5	5-1000

5.0 Equipment/Reagents

3.1 Equipment and Supplies

3.1.1 Agilent 6410B LC/MS/MS system or other approved LC/MS/MS and MassHunter or equivalent software

3.1.2 Shaker/incubator

3.1.3 Positive Pressure Manifold

3.1.4 SPE dry

3.1.5 Agilent 120 EC-C18 (2.1 x 100-4 um) or equivalent

3.1.6 Single channel pipette capable of dispending 250 µl

3.1.7 Single or multichannel pipette capable of dispending 300 µl

3.1.8 Single or multichannel pipette capable of dispensing 900 µl

3.1.9 Single or multichannel pipette capable of dispensing 100 µl

3.1.10 ToxBox kit with 96 well plate containing internal standards, and controls from Cayman Chemical, 96 well SLE+ plate and 96 well blank sample preparation plate.

3.1.11 Appropriate sized pipette tips

3.1.12 Heat sealing foil covers

3.2 Reagents

See AM# 23 for Solution Preparation instructions

3.2.1 5mM Ammonium Formate + 0.01% Formic Acid in Water (mobile phase A)

3.2.2 0.01% Formic Acid in Methanol (mobile phase B)

3.2.3 0.5 M Ammonium Hydroxide

3.2.4 Formic Acid (LCMS grade)

3.2.5 Water (LCMS grade)

3.2.6 Methanol (LCMS grade)

3.2.7 Ammonium Formate (LCMS)

3.2.8 Ammonium Hydroxide (ACS or higher)

3.2.9 Ethyl Acetate (ACS or higher)

3.2.9.10 20% LCMS Methanol in LCMS Water (Reconstitution Solvent)

3.2.9.11 LC/MS Needle Rinse (40/40/20: Water/Methanol/Isopropanol or 75/25 MeOH/Water - this preparation does not require LCMS grade solvents)

3.3 Quality Assurance: Reference materials and Controls

3.3.1 Internal Controls, and Internal Standards.

3.3.1.1 Internal controls and internal standards are prepared by Cayman chemical and contained on the 96 well plate. A minimum of one internal positive control that has a concentration near the administrative cut off level for each compound will be run with each extraction.

3.3.2 External Control

3.3.2.1 A minimum of one external control will be run with each batch. The external control may be prepared in house or purchased commercially (by a source other than Cayman). The control will have a minimum of 3 compounds at a concentration greater than the LOD as noted in Table 1, at least one of the compounds will be from panel a and at least one from panel b.

3.3.3 Negative Control

3.3.1.1 A negative control will be run with each extraction. (Drug free sheep or human blood will be used to prepare negative controls)

6.0 Procedure

4.1 Extraction Procedure

4.1.1 Allow refrigerated samples and frozen components to reach ambient temperature.

4.1.2 Add 250 µL (**calibrated pipette**) of blank (sheep or human) blood into the appropriate wells of the analytical (standards) plate (those wells containing internal positive and negative controls). Add 250 µL (**calibrated pipette**) case blood into the appropriate wells containing only internal standards.

4.1.3 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.4 Add 250 µL of 0.5 M ammonium hydroxide buffer into all wells in use on the analytical (standards) plate.

4.1.5 Place plate on shaking incubator at approximately 900 rpm for approximately 15 minutes.

4.1.6 Transfer 300 µL of blood+base mixture to corresponding wells of SLE+ plate.

4.1.7 Apply positive pressure for approximately 4 seconds or until no liquid remains on top of sorbent. Wait 5 minutes. (Positive Pressure manifold setting for this loading step is between 85-100 PSI)

4.1.8 Add 900 µL of ethyl acetate and allow to flow through for approximately 5 minutes under gravity.

4.1.9 Apply positive pressure for approximately 15 seconds. *(Positive Pressure manifold setting for this step is between 12-15 PSI).*

4.1.10 Add 900 µL of ethyl acetate and allow to flow through for approximately 5 minutes under gravity.

4.1.11 Apply positive pressure for approximately 15 seconds. *(Positive Pressure manifold setting for this step is between 12-15 PSI).*

4.1.12 Remove plate containing eluate. Place on SPE Dry and evaporate to dryness at approximately 35 degrees Celsius.

4.1.13 Reconstitute in 100 µL 20% LCMS grade methanol in LCMS Water and heat seal plate with foil.

4.2 Instrument and Run set up.

4.2.1 See Toxicology AM #24 for instrument maintenance and operation.

4.2.2 Set up the worklist in MassHunter.

4.2.3 A solvent blank is generally run before the calibration curve, then the calibration curve is run, then a negative control. At a minimum, two positive controls will be run with each run, one will be the external control and the other the plate control that has been aliquoted on the plate.

4.2.4 Samples do not require a preceding solvent blank if the sample analyzed before does not contain the suspected analyte. IE two samples suspected to contain methamphetamine, would require the worklist sequence to be: case sample, blank, case sample. (it is recommended that a blank follow any samples that exhibit a very strong response in the screen)

4.2.5 Instrument run parameters and the acquisition methods for this Analytical Method will be printed out (paper or electronically) and stored centrally in the lab or in a common file folder electronically.

4.2.5.1 Acquisition Required Settings

4.2.5.1.1 Column Temperature - 55*C

4.2.5.1.2 Panel 1 Injection volume – 5-10uL; Panel 2 Injection Volume 10ul

4.2.5.1.3 Mobile Phase Flow rate - 0.5 mL/min

4.2.5.1.3 Binary Pump Gradient Settings (Pump Time Table)

Time (min)	% Mobile A	% Mobile B
0	95	5
2.00	85	15
4.50	50	50
5.50	5	95
7.50	5	95
7.60	95	5
12.00	95	5

4.3 Evaluation of Results

4.3.1 Batch Review

4.3.1.1 The lab criterion for acceptable calibration curve R^2 is >0.98

4.3.1.2 The confirmation limit of detection (LOD) is defined in the scope of this method. Results will only be reported out qualitatively, no reference to a quantitative value will be given until further review of data to establish Uncertainty of Measurement.

4.3.2 The default criteria for a positive result are:

4.3.2.1 The sample must have a concentration no less than the established confirmation LOD for that analyte. Found in this AM section 2.2.

4.3.2.2 The analyte retention time must be within +/- 5% of the average retention time of the calibrators for that analyte.

4.3.2.3 For calibrators 10 ng and below the accuracy must be within 30%, for calibrators greater than 10 ng/mL the accuracy must be within 20%.

4.3.2.4 The ion ratios for selected transitions must fall within 20% of the averaged calibrators for each run.

4.3.2.5 S/N for each transition of detected analyte must be greater than 10.

4.3.2.6 The negative control will be evaluated as passing if the compound of interest does not meet all of the above listed criteria and the internal standard gives an anticipated response to demonstrate the sample extracted and injected correctly.

4.3.2.7 Controls must give a positive response for each compound if the concentration of the control is greater than the LOD for any given compound. It is not necessary for the control to give a positive response if a particular compound is not being evaluated in a run. In those instances a notation will be made on the control that, that compound is not being evaluated.

4.4 Quality Assurance Requirements

4.4.1 Refer to Toxicology AM #19 and AM #21 for additional quality assurance and reference material authentication.

4.5 Analysis Documentation

4.5.1 The printed results for each case sample and (when applicable) accompanying blank will be included with the analysts' notes. Case results will be recorded in the LIMS system.

4.5.2 The reports for the batch, including calibration curves and controls will be stored centrally in the lab or electronically on a common drive. Calibration curves and control data only need to be evaluated and printed for compounds being evaluated for that run.

4.5.3 The raw data from the run will be stored electronically and will be backed up at least every two months.

4.5.4 Results of the controls will be entered onto the Control Monitoring Chart.