Modification of J-5
Prepared By: R. W. Waggoner, Jr.
Approved By: D. J. Koontz
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Name of Procedure:

Toxicology
Extraction Procedure for Base Drugs Using United Chemical Technologies Clean Screen Extraction Columns®

Suggested Uses:

This is an extraction procedure for base drugs. The procedure is designed to extract base drugs from blood or urine for confirmation by mass spectrometry. It utilizes United Chemical Technologies Clean Screen Extraction Columns® and the Zymark RapidTrace SPE Workstation. The extraction procedure targets cocaine, benzodiazepines, opiates, amphetamine, methamphetamine and their respective metabolites at a concentration of 50 ng/mL. Calibration standards may be utilized for quantitative analysis.

Apparatus Used to Perform Procedure:

Test tubes, 16 x 125, 13 x 100, 12 x 75
Test tube caps or stoppers
Vortexer
Centrifuge
pH meter
Eppendorf Pipettes
Pipet tips
Volumetric flasks
Zymark RapidTrace SPE Workstation
World Wide Monitoring Clean Screen Extraction Columns®
Zymark TurboVap LV
Nitrogen

Reagents Used:

Analytical Grade Drug Standards
- Cocaine
- Benzoylecgonine
- Ecgonine Methyl Ester
- Alprazolam
- Diazepam
- Nordiazepam
- Midazolam

- Prazepam
- Codeine
- Hydrocodone
- Morphine
- 6-Monoacetylmorphine
- Oxycodone
- Amphetamine
- Methamphetamine

Deuterated drug standards

- Cocaine-D_3
- Benzoylecgonine-D_3
- Codeine-D_3
- Morphine-D_3
- 6-Monoacetylmorphine-D_3
- Oxazepam-D_5
- Amphetamine-D_5
- Methamphetamine-D_{11}

Methylene chloride
Deionized water
Methanol
Hexane
Dimethylformamide
BSA (N,O-bis(trimethylsilyl)acetamide)
BSTFA with 1% TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane)
Heptafluorobutyric anhydride (HFBA)
Propionic Anhydride, 99%
Pyridine
Ethyl Acetate

Acetic Acid, 1.0M

a. To 400 mL deionized water add 28.6 mL glacial acetic acid.
b. Dilute to 500 mL with deionized water and mix.
c. Stability - 6 months at room temperature.

Methylene chloride (MeCl) : Isopropyl alcohol (4:1) with 2% Ammonium Hydroxide

a. Make a solution of MeCl:IPA (4 parts MeCl to 1 part IPA)
b. Add NH_4OH to the MeCl:IPA to make it 2% of the total volume.
c. Make fresh daily. MeCl:IPA may be made ahead of time and stored for several months.

Phosphate Buffer

a. Dissolve 1.70 g Na_2HPO_4 and 12.14 g NaH_2PO_4-H_2O in 800 mL DI water and dilute to 1L.
b. Adjust pH to 6.0 +/- 0.1 with monobasic sodium phosphate or dibasic sodium phosphate.
c. Stability: 1 month at 0-5 degrees Celsius (refrigerate when not in use). It is permitted to prepare less than one liter as long as the proportions are equivalent to the ones listed above.

Internal Standard Solutions

a. Internal standards:
Prepare Base Drug Internal Standard Solution(s) containing 2000 ng/mL of the following drug(s) benzoylecgonine-D_3, cocaine-D_3, mo

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-
D_3,
co
dei
ne-
D_3,
6-
mo
no
ac
ety
lm
orp
hin
e-
D_3,
pra
ze
pa
m,
ox
az
ep
am
-
D_5,
am
ph
eta
mi
ne-
D_5
an
d
me
tha
mp
het

amine-D_{11}. Label the flask(s).

Include on the label initials, date prepared, internal standards in the solution(s),

and the expiration date which is determined from the earliest date listed on the acquired drug standards.

Example: To obtain a “Base Drug Internal Standard Solution” from ampules containing a drug concentration of 100 ug/ml, add 1.0 mL of each drug standard

to a 50 mL volumetric flask, and dilute the flask to volume with methanol.

Calibration Solutions

a. Base drug stock calibrators

Prepare the following Base Drug Stock Standard Solution(s) to contain 10,000 ng/mL of the listed drug(s).

Cocaine Stock Calibrator Solution
Cocaine
Benzoylecgonine
Ecgonine Methyl Ester

Benzodiazepine Stock Calibrator Solution
Alprazolam
Diazepam
Nordiazepam
Midazolam

Opiate Stock Calibrator Solution
Codeine
Hydrocodone
Morphine
6-Monoacetylmorphine
Oxycodone

Amphetamine Stock Calibrator Solution
Amphetamine
Methamphetamine

Label the solutions. Include on the label initials, the date prepared, the drugs present in solution, and an expiration date determined by the earliest expiration date of the acquired drug standard(s) in solution.

Example: For the ampules that contain a drug concentration of 1 mg/ml, dilute 1.0 ml to 100 ml with methanol in a volumetric flask.

Stock verification solution(s) must be prepared for each class of drugs using the same procedure to prepare the stock calibrators. Label these solutions

appropriately.

b. Base Drug Working Calibrators
Prepare the following Base Drug Working Calibrator Solutions to contain 1000 ng/mL of drugs:

Cocaine Working Calibrator Solution
Cocaine
Benzoylecgonine
Ecgonine Methyl Ester

Benzodiazepine Working Calibrator Solution
Alprazolam
Diazepam
Nordiazepam
Midazolam

Opiate Working Calibrator Solution
Codeine
Hydrocodone
Morphine
6-Monoacetylmorphine
Oxycodone

Amphetamine Working Calibrator Solution
Amphetamine
Methamphetamine

Example: Place 5 mL of a Base Drug Stock Calibrator solution in a 50 ml volumetric flask and dilute to volume with methanol. Label the solutions. Include initials, date prepared, the drugs present in solution, and the earliest expiration date of the base drug stock calibrators on the label.

Working verification solution(s) must be prepared for each class of drugs using the same procedure to prepare the stock calibrators. Label these solutions appropriately.

c. 100 ng/mL base calibration/verification standard: Add 200 microliters of the appropriate Base Drug Working Calibrator(s)/Verifier(s) to 2.0 mL of drug free

blood. Add 100 microliters of the appropriate internal standard solution(s). Vortex the mixture.

d. 500 ng/mL base calibration/verification standard: Add 100 microliters of the appropriate Base Drug Stock Calibrator(s)/Verifier(s) to 2.0 mL of drug free blood. Add 100 microliters of the appropriate internal standard solution(s). Vortex the mixture.
e. 1000 ng/mL base calibration/verification standard: Add 200 microliters of the appropriate Base Drug Stock Calibrator(s)/Verifier(s) to 2.0 mL of drug free blood. Add 100 microliters of the appropriate internal standard solution(s). Vortex the mixture.

Procedure:

1. Blood Sample Preparation:
   a. To 2 mL of blood add 4.5 mL of deionized H_2O and 100 microliters of an appropriate internal standard(s).
   b. Mix/Vortex and let stand for 5 minutes to lyse red blood cells.
   c. Centrifuge for 10 minutes at >2000 RPM
   d. Decant liquid portion of the sample into 2 mL of 100 mM phosphate buffer (pH6). Mix.
   e. If needed, adjust pH to 6.0 ± 0.5 with 100 mM monobasic or dibasic sodium phosphate.

2. Urine and Liquid Sample Preparation:
   a. No sample preparation is usually needed for urine. Extract 5 mL of urine with 250 microliters (1 milliliter if the extraction targets amphetamine or methamphetamine) of an appropriate internal standard added. Before extraction check the pH to see if it is 6.0 +/- 0.5. If it is not then adjust pH accordingly with monobasic or dibasic sodium phosphate. If a majority of the drug(s) of interest has formed a glucuronide complex, the glucuronide can be hydrolyzed utilizing one of the methods described below.
   b. Hydrolysis Method A:
      1. To 5 mL urine add 2 mL B-glucuronidase (5000 F units/mL Patella Vulgata in 1.0M acetate buffer.)
      2. Mix. Hydrolyze for 3 hours at 65 °C. Cool to room temp.
      3. Adjust pH to 6.0 +/- 0.5 with approximately 0.7 mL of 1.0 N NaOH.
      or
   c. Hydrolysis Method B:
      1. To 5 mL urine add 0.5 mL conc. HCl. Mix.

2. Heat for 30 min. at 120 °C. Cool to room temperature.
3. Add 1.0 mL 7.4 M NH_4OH. Mix.
4. Adjust sample pH to 6.0 +/- 0.5 with 1 to 3 mL 500 mM phosphoric acid.

3. Extraction Procedure

a. Condition column with 3 mL MeOH.
b. Condition column with 3 mL water.
c. Condition column with 1 mL phosphate buffer.
d. Load sample onto column.
e. Rinse column with 3 mL water.
f. Rinse column with 2 mL acetic acid, 1.0M
g. Rinse column with 3 mL MeOH.
h. Dry column for 5 minutes with nitrogen.
i. Collect basic drugs with 3 mL MeCl:IPA:NH_4OH

Reagent flow must be between 1 to 15 mL per minute, except for elution reagents and samples which cannot be more than 5 mL per minute.

<u>Post Extraction Procedures:</u>

Evaporation of the solvent from the collection test tube and reconstitution with an appropriate solvent is all that is required for analysis by mass spectrometry. The following derivatizations can be done to improve analysis by gas or liquid chromatography/mass spectrometry:

a. Silylation of benzodiazepines and cocaine and its metabolites

1. Evaporate the solvent.

2. Derivatize the extract in the collection tube or transfer to a vial using methanol and evaporate the methanol.

3. Derivatize by adding 50 microliters of BSA or BSTFA with 1% TMCS to the vial or test tube and capping. Mix and heat the vial or test tube for 30 minutes at 80 ^{o}C. Remove from heat source and allow the vial or test tube to cool before analysis.

4. Examine the extract using gas or liquid chromatography/mass spectrometry.

b. Acylation of opiates with propionic anhydride

1. Add 50 microliters of 99% or greater purity propionic anhydride and 50 microliters of pyridine.

2. Cap the tubes, vortex, and heat at 80 ^{o}C for at least 30 minutes.

3. Remove the tubes from heat and evaporate at 40 ^{o}C until dry.

4. Reconstitute the residue with 50 microliters of a acetonitrile.

5. Examine the extract using gas or liquid chromatography/mass spectrometry.

c. Acylation of amphetamine and methamphetamine with HFBA

1. Add 50 microliters of dimethylformamide to the eluate.

2. Evaporate the solvent from the collection test tube.

3. Add 100 microliters of heptafluorobutyric anhydride to collection test tube and cap.

4. Heat for 30 minutes at 80 ^{o}C.

5. Remove the test tube from heat and evaporate to dryness in a fume hood using a flow of inert gas.

6. Reconstitute with 50 microliters of ethyl acetate.

7. Examine the extract using gas or liquid chromatography/mass spectrometry.

Quality Control:

Quality Control is verified for each extraction by utilizing an appropriate internal standard or standards. For each set of extractions a blood blank must be extracted as a negative control. At least one verifier must be extracted as a positive control for extractions that are going to be confirmed using mass spectrometry in the selected ion monitoring mode. Extractions confirmed using mass spectrometry in full scan mode do not require a positive control, but do require that a base drug internal standard be added. Reported quantitative values must be based on a calibration curve using at

least three calibration standards, and at least one verification standard must be extracted to verify the calibration curve. The verification standard must quantify within +/- 20% of the target for quantitative values to be reported.

Personnel Safety Concerns:

1. When working with biohazardous samples use protective measures, such as gloves, eye protection, and work with the samples in a biosafety hood.

2. BSA, BSTFA with 1% TMCS, heptafluorobutyric anhydride should be handled in a fume hood, with gloves, and eye protection.

Maintenance:

Zymark: RapidTrace SPE Workstation
1. Check reagent levels daily before using.
2. Clean protein build-up when needed by passing about 7.5 mL 2N NaOH and 2N HNO_3 through the cannula to the aqueous waste.

Comments:

For an explanation of the operation of the RapidTrace refer to the operation manual.

Clean Screen® Extraction Columns have been used in the Toxicology Unit to extract neutral, acidic and basic drugs and the metabolites of these drugs from whole blood and urine since 1995. Use of the Clean Screen® Extraction Columns to extract neutral, acidic and basic drugs and the metabolites of these drugs has been validated through proficiency testing provided by College of American Pathologists.

Literature References:

RapidTrace SPE Workstation Installation and Quick Reference Manual, Zymark Co., revision 0, 1995.

Clean Screen® Extraction Column Applications Manual, United Chemical Technologies Inc., Bristol, PA.

Kitchen, Chester J.; Telepchak Michael; and August, Thomas F.; “An Automated Solid Phase Extraction Method for Thebaine, 6-Acetylmorphine and Other Opiates in Urine”, United Chemical Technologies.