Name of Procedure:

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

Suggested Uses:

This is an extraction procedure for acidic drugs using United Chemical Technologies Clean Screen Extraction Columns® and the Zymark RapidTrace SPE Workstation. This procedure is designed to extract acidic drugs for confirmation by mass spectrometry. The extraction procedure targets butalbital, pentobarbital, and secobarbital at a concentration of 1000 ng/mL in a 2.0 mL blood sample or a 5.0 mL urine sample and phenobarbital at 5000 ng/mL in a 2.0 mL blood sample or a 5.0 mL urine sample. Calibration standards may be utilized for quantitative analysis. After collection of the acidic fraction from the sample, a base fraction may be collected from the same sample (see procedure J-5 for details).

Apparatus Needed 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® CSDAU203
Zymark TurboVap LV
Nitrogen

Reagents Needed:

Analytical Grade Drug Standards
- Butalbital
- Phenobarbital
- Pentobarbital
- Secobarbital

Hexobarbital
Methylene chloride
Deionized water
Methanol
Hexane
Ethyl Acetate

Acetic Acid, 1.0 M

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.

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. Acid Drug Internal Standard: Prepare an internal standard solution that contains 40 micrograms/mL (40,000 ng/mL) of hexobarbital. Label the flask "Acid Drug Internal Standard". Include on the label initials, date prepared, and the expiration date which is determined from the date listed on the acquired drug standard.
   Example: From a drug standard containing 1.0 mg/mL of hexobarbital dilute 1.0 mL with methanol to 25 mL in a volumetric flask.

Calibration Solutions

a. Acid Drug Calibrator: Prepare an Acid Drug Calibrator solution that contains 40 micrograms/mL (40,000 ng/mL) of each of the following drugs:
   Butalbital
   Phenobarbital

Pentobarbital
Secobarbital

Label this solution "Acid Drug Calibrator" and include initials, the date prepared, and the expiration date which is determined from the earliest expiration date of the acquired drug standards in solution.

Example: For the acquired drug standards that contain a drug concentration of 1.0 mg/ml dilute 1.0 ml to 25 ml with methanol in a volumetric flask.

An acid drug verification solution must be prepared for each class of drugs using the same procedure to prepare the stock calibrators. Label this solution appropriately.

b. 1000 ng/mL acid calibration/verification standard: Add 50 microliters of acid drug standard to 2.0 mL of drug free blood. Add 100 microliters of the acid drug internal standard solution. Vortex the mixture.
c. 2000 ng/mL acid calibration/verification standard: Add 100 microliters of acid drug standard to 2.0 mL of drug free blood. Add 100 microliters of the acid drug internal standard solution. Vortex the mixture.
d. 5000 ng/mL acid calibration/verification standard: Add 250 microliters of acid drug standard to 2.0 mL of drug free blood. Add 100 microliters of the acid drug internal standard solution. Vortex the mixture.

Procedure:

1. Blood Sample Preparation:
   a. To 2 mL of blood add 4.5 mL of DI H_2O and 100 microliters of the acid drug internal standard (add an appropriate base drug internal standard if a basic fraction is to be collected).
   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 approximately 5 mL of urine with 500 microliters of the acid drug internal standard added. Before extraction check the pH to see it if it is 6.0 +/- 0.5. If it is not then adjust pH

accordingly with monobasic or dibasic sodium phosphate.

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 1 mL acetic acid, 1.0M
g. Dry column for 5 minutes with nitrogen.
h. Rinse the column with 2 mL of hexane
i. Collect acidic drugs with 6 mL methylene chloride

Add the following three steps to collect a basic fraction from the same sample.

j. Rinse the column with 3 mL of methanol
k. Dry column for 2 minutes with nitrogen
l. Collect basic drugs with 3 mL of MeCL:IPA:NH_4OH

Reagent flow must be between 1 to 15 mL per minute, except for elution reagent and samples which cannot be no more than 5 mL per minute.

Quality Control:

Quality control is verified for each extraction by utilizing the 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. If the anticipated drug to be extracted is butalbital, pentobarbital, or secobarbital then it is recommended that the 1000 ng/mL verification standard be extracted. If the anticipated drug to be extracted is phenobarbital then it is recommended that the 5000 ng/mL verification standard be extracted. If multiple barbiturates are anticipated or if the anticipated drug is unknown then it is recommended that the 1000 ng/mL and 5000ng/mL verification standards both be extracted. Extractions confirmed using mass spectrometry in full scan mode do not require a positive control, but do require that the acid 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.

Post Extraction Procedures:

Evaporation of the solvent from the collection test tube and reconstitution with an appropriate solvent is all that is required for analysis by full scan mass spectrometry. Examine the collected fraction utilizing an appropriate method on the gas chromatograph or liquid chromatograph/mass spectrometer. If selected ion monitoring is to be employed then follow the steps listed below. Follow the post extraction procedures in J-5 if a basic fraction was collected.

1. Evaporate the solvent from the collection test tube.

2. Add 100 microliters of ethyl acetate to the collection test tube, cap and mix.

3. Examine the collected fraction utilizing gas chromatography or liquid chromatography/mass spectrometry.

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. Ethyl acetate should be handled in a fume hood with 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.