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2.3.4 Benzodiazepines Determination Employing the United Chemical Technologies (UCT) 200mg CLEAN SCREEN® DAU Extraction Column

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Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for Qualitative GC/MSD Confirmation

2.3.4 Benzodiazepines Determination Employing the United Chemical Technologies (UCT) 200mg CLEAN SCREEN® DAU Extraction Column

2.3.4.1 BACKGROUND

Refer to manual section 2.4.3.

2.3.4.2 SCOPE

This analytical method outlines the use of the 200mg UCT CLEAN SCREEN® DAU Column for the extraction of Benzodiazepines 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 in the urine sample.

The retention mechanisms for the benzodiazepines are hydrophobic interactions and polar adsorption. The nonpolar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[2] Benzodiazepines form glucuronide conjugates to facilitate their excretion. An enzymatic hydrolysis is therefore required to free them from the glucuronide sugar moiety. For the extraction of benzodiazepines, the hydrolyzed urine is 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. The analyte is retained by hydrophobic interaction of the functional groups present on both the analyte and the sorbent. The column is subsequently washed to selectively remove matrix components and interfering substances from the column. 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. Following the elution from the SPE column the evaporated extract is derivatized for confirmation on the GC/MSD.

2.3.4.3 EQUIPMENT AND SUPPLIES

2.3.4.3.1 200 mg CLEAN SCREEN® Extraction Column
2.3.4.3.2 Laboratory Oven or Dry Bath
2.3.4.3.3 Evaporative concentrator equipped with nitrogen tank.
2.3.4.3.4 Vacuum Manifold/pump
2.3.4.3.5 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

2.3.4.3.6 16X100 Round Bottom Test Tubes
2.3.4.3.7 Screw Cap for 16mm O.D. tubes
2.3.4.3.8 pH Indicator Strips
2.3.4.3.9 (Optional) 16X144mm tapered tip centrifuge tubes
2.3.4.3.10 GC/MS Automated Liquid Sampler (ALS) vials
2.3.4.3.11 GC/MS vial microinsert
2.3.4.3.12 Gas chromatograph equipped with a mass selective detector and a nonpolar capillary column with a phase composition capable of efficiently separating drugs of interest (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.4.4 REAGENTS

Refer to Manual section 5.12 for solution preparation

2.3.4.4.1 Ethanol (ACS Grade)
2.3.4.4.2 Ethyl Acetate (ACS Grade)
2.3.4.4.3 Hexane (ACS Grade)
2.3.4.4.4 Deionized/distilled water
2.3.4.4.5 100 mM Phosphate buffer, pH 6.0
2.3.4.4.6 20 % Acetonitrile in 0.1 M phosphate buffer, pH 6.0
2.3.4.4.7 100mM Acetate Buffer, pH 5
2.3.4.4.8 β-Glucuronidase Solution (Patella vulgata)
2.3.4.4.9 BSTFA + 1% TMCS

2.3.4.5 REFERENCE MATERIAL SOLUTIONS

2.3.4.5.1 Positive Control

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

2.3.4.5.1.1 **Positive Control Stock Solution**

Obtain 1mg/mL single component or 250µg/mL multicomponent stock benzodiazepine class drug reference material solutions through Cerilliant, Alltech, Sigma or other appropriate vendor.

2.3.4.5.1.2 **Positive Control Working Solution – 10ng/µL**

Add 100µL 1mg/mL or 400µL 250µg/mL multicomponent stock solution to adjusted amount of 10mL methanol. A minimum of two benzodiazepine compounds must be included in the control. At least one of the compounds must form a TMS derivative.

2.3.4.5.2 Non-extracted Reference Material

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

2.3.4.5.2.2 Dilute 1.0mg/mL reference material solution to 250ug/mL with methanol.

2.3.4.5.3 Non-extracted Derivatized Reference Material

2.3.4.5.3.1 Derivatize reference material as necessary based on current drug therapy and examination of GC/MSD data.

2.3.4.5.3.2 Add 50µL of working solution to labeled tapered bottom centrifuge tube. Derivatize as described in 2.3.4.6.5.

2.3.4.5.4 Internal Standard

2.3.4.5.4.1 **Stock Solutions**
1 mg/mL Prazepam

2.3.4.5.4.2 **Working Internal Standard Solution [10ng/µL]**
Add 100µL Prazepam stock solution to 10mL volumetric ball flask. QS with methanol.
Solution is stable for 1 year when stored at under refrigeration.

2.3.4.5.5 Conjugated Controls

2.3.4.5.5.1 Control is used to verify the β-glucuronidase enzyme's ability to cleave glucuronide conjugated compounds.

2.3.4.5.5.2 Urinary Oxazepam Glucuronide or Morphine Glucuronide can either be spiked into urine with conjugated control working solution or commercially obtained.

2.3.4.5.5.3 Glucuronide conjugated drug must be at a minimum of 375ng/mL.

2.3.4.5.5.4 **Conjugated Stock Solution**
Obtain 1mg/mL stock Oxazepam Glucuronide (or Morphine Glucuronide) drug reference material solution through appropriate vendor.

2.3.4.5.5.5 **Conjugated Working Solution – 10ng/µL**
Add 100µL stock solution to 10mL methanol.

2.3.4.6 PROCEDURE

2.3.4.6.1 Initial set-up
Label SPE column, test tubes, and GC/MS vials with microinserts for the negative control, positive control, Glucuronide controls (with and without glucuronidase) and appropriate laboratory numbers. Label tubes and GC/MS vials with microinserts for non-extracted derivatized reference material.

2.3.4.6.2 Control Samples
Use the same lot of negative urine to prepare both the negative and spiked positive controls.

2.3.4.6.2.1 Negative Control Sample

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

2.3.4.6.2.1.2 Transfer 5mL negative urine to extraction tube.

2.3.4.6.2.2 Positive Control Sample
For a 400ng/mL spiked control, pipette 200µL mixed working control solution to 5mL negative urine or pipette 5mL sample of commercially obtained urine positive control.

2.3.4.6.2.3 Conjugated Reference Material Controls

2.3.4.6.2.3.1 For a 500ng/mL spiked control, pipette 250µL of conjugated working control solution into two 5mL aliquots of negative urine.

2.3.4.6.2.3.2 Prepare one control with and one control without the addition of glucuronidase.

2.3.4.6.3 Casework Samples
Transfer 5mL casework samples to screw top extraction tube.

2.3.4.6.4 Internal Standard Addition

2.3.4.6.4.1 To each prepared sample, add 250µL of internal standard. Vortex to mix.

2.3.4.6.4.2 Allow samples to stand 10 minutes.

2.3.4.6.5 Sample Hydrolysis

2.3.4.6.5.1 **Option One**
Add 2mL 100mM Acetate Buffer, pH 5, to all tubes. Add 100µL β-Glucuronidase Type H-2 crude solution to all tubes except for one conjugated control.

2.3.4.6.5.2 **Option Two**
Add 2mL β-Glucuronidase 100mM Acetate Buffer solution, pH 5, to all tubes except for one conjugated control.

2.3.4.6.5.3 Cap and vortex *gently* to mix.

2.3.4.6.5.4 Place tubes in laboratory oven or waterbath set at 65°C for three hours.

2.3.4.6.5.5 Centrifuge for 10 minutes at 3400 rpm, discard pellet.

2.3.4.6.5.6 Allow samples to cool prior to extraction.

2.3.4.6.6 Pre-conditioning of SPE Column

It is important to aspirate at ≤ 3 inches Hg to prevent sorbent drying. Gravity flow should be employed.

2.3.4.6.6.1 Insert labeled 200mg CLEAN SCREEN® DAU column in the vacuum manifold.

2.3.4.6.6.2 Add 3mL of methanol to the column, aspirate.

2.3.4.6.6.3 Add 3mL of deionized water to the column, aspirate.

2.3.4.6.6.4 1mL of 100mM phosphate buffer (pH 6.0), aspirate.

2.3.4.6.7 Sample Loading

Decant sample into column – flow should be ≤1ml/min. Gravity flow should be employed.

2.3.4.6.8 Column Wash

Aspirate at ≤ 3 inches Hg to prevent sorbent drying. Gravity flow should be adequate.

2.3.4.6.8.1 Add 2mL of deionized water, aspirate.

2.3.4.6.8.2 Add 2mL 20% acetonitrile in 0.1M phosphate buffer (pH 6.0), aspirate.

2.3.4.6.8.3 Increase vacuum to ≥10 in. Hg (≥34 kPa) to dry extraction disc for 5 to 10 minutes.

2.3.4.6.8.4 Add 2mL **Hexane**, aspirate.

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

2.3.4.6.8.6 Add 3mL **Ethyl Acetate** to elute the sample from the column, aspirate.

2.3.4.6.9 Evaporation of Elution Solvent

Evaporate solvent to dryness, under a gentle stream of nitrogen at ≤ 40°C.

2.3.4.6.10 Derivatization

2.3.4.6.10.1 Add 50 µL ethyl acetate, vortex.

2.3.4.6.10.2 Add 50 µL BSTFA-1% TMCS.

2.3.4.6.10.3 Cap tubes. Vortex.

2.3.4.6.10.4 Heat tube for 20 minutes in oven set at 70°C.

2.3.4.6.10.5 Remove from heat and allow to cool.

2.3.4.6.10.6 Transfer derivative to labeled ALS vial with microinsert.

2.3.4.6.11 Preparation for Analysis Run

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

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

2.3.4.6.12 GC-MSD Analysis Parameters

2.3.4.6.12.1 Refer to instrument METHOD printout for current analysis parameters.

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

2.3.4.6.13 Detection and Identification Criteria

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.

2.3.4.7 QUALITY ASSURANCE REQUIREMENTS

2.3.4.7.1 General

2.3.4.7.1.1 Urine samples are to be stored frozen until allowed to thaw prior to analysis.

2.3.4.7.1.2 Urine samples are to be stored under refrigeration after aliquots are removed for analysis.

2.3.4.7.1.3 Post analysis, urine samples are to be stored frozen until appropriate disposal date.

2.3.4.7.1.4 Refer to toxicology analytical methods 5.8 and 5.10 for additional quality assurance and reference material authentication requirements.

2.3.4.8 ANALYSIS DOCUMENTATION

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

2.3.4.8.2 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.

2.3.4.9 REFERENCES

2.3.4.9.1 UCT CLEAN SCREEN® Extraction Columns Application Manual

2.3.4.9.2 Telepchak, M.J., August, T.F. and Chaney, G., Drug Methods for the Toxicology Lab, pp. 190 - 192. *in:* Forensic and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

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

Revision History

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2.3.4 Extraction of Benzodiazepines Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

Revision No.	Issue Date	Revision/Comments
1	02-05-2002	Original Issue in SOP format
2	10-18-2002	Refinements
3	05-07-2007	Addition of internal standard and updated QA measures, reformatting.
4	07-28-2008	Clarified that negative urine used to prepare positive control is the same lot as used for negative control. Clarified that glucuronidase liquid can also be used for hydrolysis.