Idaho State Police
Forensic Services
Toxicology Section

Section Two
Urine Toxicology

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

2.3.3 Extraction of Benzodiazepines Employing the Ansys® Diagnostics Spec·Plus™ DAU Column

2.3.3.1 BACKGROUND

Refer to manual section 2.4.3.

2.3.3.2 PRINCIPLE

This procedure outlines the use of the ANSYS® Diagnostics, Inc SPEC·PLUS™ 3ml SPE column for the extraction of benzodiazepines from urine. ANSYS Technologies' SPEC™ Solid Phase Extraction products are manufactured with polypropylene plastic and bonded-silica impregnated on a glass fiber disc. For benzodiazepines, a non-polar phase (reversed phase) retention mechanism is utilized. to interact effectively, with analytes of interest and minimally with interfering substances in the urine sample. The non-polar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[4]

Benzodiazepines form glucuronide conjugates to facilitate their excretion. An enzymatic hydrolysis is required to free them from the glucuronide sugar moiety. For the extraction of benzodiazepines, the urine is adjusted to pH 10.8 with a phosphate buffer, to maximize the hydrophobic/non-polar character of the analyte and the sorbent, and applied to a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The column is subsequently washed with an aqueous solvent, to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of solvent. When the column is dry, the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.3.3 EQUIPMENT AND SUPPLIES

2.3.3.3.1 SPEC·PLUS™ - 3mL DAU SPE column (Ansys 532-DAU or equivalent)
2.3.3.3.2 Drybath (Fisher or equivalent)
2.3.3.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.
2.3.3.3.4 Vacuum Manifold/pump

2.3.3.3.5 Glassware
16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)
pH paper (Fisher 09-876-17 or equivalent)

2.3.3.3.6 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs, compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.3.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.3.4.1 Isooctane (2,2,4 – trimethylpentane) (Fisher O-299-1 or equivalent)

2.3.3.4.2 1.0M Acetate Buffer, pH 3.8

2.3.3.4.3 1.5 M Phosphate Buffer, pH 10.8

2.3.1.4.4 Ethyl Acetate (Fisher E145-4 or equivalent)

2.3.1.4.5 Ammonium Hydroxide (Fisher A669-500 or equivalent)

2.3.3.4.6 Elution Solvent
To 98mL ethyl acetate add 2ml NH_4OH, mix.
Make Fresh.

2.3.3.4.7 Deionized/distilled water

2.3.3.4.8 β-Glucuronidase (Patella vulgata) Options
- Prepare from Patella vulgata Type L-II powder (Sigma G8132 or equivalent)
- Prepared Helix pomatia Type H-2 Solution (Sigma G0876 or equivalent)

2.3.3.4.9 Silylation Reagent Options
- MTBSTFA / 1% t-BDMCS (Pierce 48925 or equivalent)
- MSFTA (Pierce 48910 or equivalent)
- BSTFA (Pierce 38830 or equivalent)

2.3.3.5 CONTROL

2.3.3.5.1 Liquid Urine Control containing a minimum of Oxazepam or Nordiazepam (BioRad 443, Utak 88121 or equivalent)

2.3.3.5.2 Drug Mix (Alltech 601826 {Medazepam, Oxazepam, Lorazepam, Diazepam, Temazepam, and Bromazepam}or similar)

2.3.3.5.3 Oxazepam Glucuronide (Alltech 01541 or equivalent.

2.3.3.6 STANDARDS

2.3.3.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards in Solution	*Potential Vendors*
Alprazolam	Cerilliant A-903, Alltech 01427
α-Hydroxyalprazolam	Cerilliant A-907, Alltech 01545
Bromazepam	Cerilliant B-903, Alltech 6013563
Chlordiazepoxide	Cerilliant C-022
Norchlordiazepoxide	Alltech 6013433
Clonazepam	Cerilliant C-907, Alltech 017943
7-Aminoclonazepam	Cerilliant A-916
Diazepam	Cerilliant D-907, Alltech 017213
Estazolam	Cerilliant B-901, Alltech 601560
Flurazepam	Cerilliant F-003, Alltech 017953
Flunitrazepam	Cerilliant F-907, Alltech 6015123
7-aminoflunitrazepam	Cerilliant A-911
Lorazepam	Cerilliant L-901, Alltech 013583
Medazepam	Alltech 013573
Midazolam	Cerilliant M-908
4-hydroxymidazolam	Cerilliant H-902
Nitrazepam	Cerilliant N-906, Alltech 017933
Nordiazepam	Cerilliant N-905, Alltech 013453
Oxazepam	Cerilliant O-902, Alltech 013703
Temazepam	Cerilliant T-907, Alltech 013833
Triazolam	Cerilliant T-910, Alltech 014283
α-Hydroxytriazolam	Cerilliant T-911

2.3.3.7 PROCEDURE

2.3.3.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Oxazepam, temazepam, nordiazepam and lorazepam. Add 10µL of stock solution to labeled tapered bottom centrifuge tube.

2.3.3.7.2 Initial set-up

Label SPE·PLUS™·3ml·DAU extraction column, test tubes, tapered-bottom derivatization tubes and GC/MS vials with microinserts for the negative control (NC), positive control (PC), Oxazepam Glucuronide control, Standards, and appropriate laboratory numbers.

2.3.3.7.3 Manual Extraction Procedure Utilizing the SPEC·PLUS™ •3ml DAU column

2.3.3.7.3.1 Sample Preparation

Transfer 1.0mL of urine specimen, negative urine or positive control to labeled extraction test tube.

2.3.3.7.3.2 Sample Hydrolysis

For each extraction tube:

- Add 200µL 1.0M acetate buffer, pH 3.8
  - Vortex.
  - Verify that the resulting pH is approximately 4.
  - If necessary adjust pH.
- Add 200µL β-Glucuronidase.
  - Cap and vortex *gently* to mix.
- Place in 37°C laboratory oven or waterbath for three hours.
- Allow samples to cool before proceeding with solvent extraction.

2.3.3.7.3.3 Extraction

For each extraction tube:

- Add 1.5mL of phosphate buffer (pH 10.8)
  - Vortex.
  - Resulting pH should be approximately 10.
  - If necessary, adjust pH.
- Centrifuge at 3500 rpm for ≥5 minutes.
- Insert labeled SPE·PLUS™·3ml·DAU column in the vacuum manifold.
- Add 200µL of methanol to the column. Wait for 1 minute.
- Decant sample into column and aspirate at approximately 3-5 in. Hg (10-17kPa)
- Add 1mL of deionized water to column and aspirate at approximately 3-5 in. Hg (10-17kPa)
- Increase vacuum to 10-20 in. Hg (34-68kPa) and dry extraction disc for approximately 5 minutes.
- Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.

- Add 800µL of elution solvent and apply ***gentle*** vacuum of <3 in. Hg (10 kPa) to aspirate the sample into the collection tube
- Increase vacuum to approximately 5 in. Hg (17 kPa) to assist the final amount of elution solvent through the disc.
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at 60°C.

2.3.3.7.3.4 Derivatization

- In hood, add 100µL silylating agent.
  - Cap tubes with snap caps.
  - Vortex.
- Heat tube in 90°C dry bath for 30 minutes.
- Remove from dry bath and allow to cool.
- Add 100µL of isooctane.
  - Vortex.
- Transfer derivative to labeled GC/MSD ALS vial with microinsert.

2.3.3.7.4 Automated Extraction Procedure Utilizing SPEC·PLUS™ - 3ml DAU column.

2.3.1.7.6.1 Refer to the following attached methods/printouts.

2.3.3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.3.3.8.1 Analysis Parameters

2.3.3.8.1.1 Inject 1 µL into GC/MSD using the ALS.

2.3.3.8.1.2 Analyze sample extract in full scan acquisition.

2.3.3.8.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.3.3.8.2 Detection and Identification Criteria

2.3.3.8.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.3.3.9 REFERENCES

2.3.1.9.1 Automated SPEC® · Solid Phase Extraction Protocols for Drugs of Abuse Using the RapidTrace™ SPE Workstation, ANSYS Diagnostics, 1997.

2.3.1.9.2 SPEC·PLUS™·3ML·DAU Drugs of Abuse in Urine Extraction Applications, ANSYS Diagnostics, 1999.

2.3.1.10.3 Instructions for Urine of SPEC·Solid Phase Extraction Columns, SPEC·PLUS™ Solid Phase Extraction Columns with Filter, ANSYS Diagnostics, 1997.

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