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1.0 PRINCIPLE

Benzodiazepines are a class of drugs whose properties include anti-anxiety, hypnotic and sedation. Benzodiazepines are GABA-agonists, acting via specific allosteric-activating receptors on the GABA-A receptor, beta subunit. While benzodiazepines are considered safer than the barbiturate class they have mostly replaced, there is still significant potential for abuse and dependence with continued use. Benzodiazepines are subject to both phase I and phase II metabolism in the liver, and as such, urine samples are subjected to hydrolysis to liberate "parent" benzodiazepine molecules from their glucuronide conjugate analogue.

Benzodiazepines that may be present in blood and/or urine samples that require confirmation by GC/MS are extracted from a buffered, diluted sample aliquot by adsorption onto a solid phase extraction column. Benzodiazepines that may be present are then eluted from the column into Ethyl Acetate with Hexane. After evaporation of the solvent and derivitazation with BSTFA/TMCS (N,O, -bis trimethylsilyl trifluoroacetamide + 1% Trimethylchlorosilane), the extracted drug is analyzed by GC/MS.

Qualitative identification of the Benzodiazepines are based on retention time and ion ratios for 3 ions compared to the corresponding ion ratios from a calibrator extracted and run in the same batch. The concentration of each specific benzodiazepine is determined by single point calibration, using Prazepam as the internal standard. Each GC/MS run is separately evaluated using control and blank samples, and is processed by a custom spreadsheet program, on which calibrator, blank and control results are summarized and tabulated, and the batch review process by both the analyst and technical reviewer is documented. Matrix-specific (blood and/or urine) positive and negative controls are extracted and analyzed in each analytical batch. The presence of Benzodiazepines may be confirmed in urine, blood or other aqueous fluids. Quantitative evaluation is made by single point calibration, using internal standard, and two control samples within the quantitative range.

2.0 SPECIMEN

2.1 Specimens requiring confirmation for Benzodiazepines are listed by lab case number on the clip board marked "Special Benzodiazepines list" which is maintained in the Toxicology Instrument room. Analysts preparing a batch for analysis should derive their case list from this document.

2.2 All evidence transfers, either between individuals or between an individual and a storage location must be documented on the Chain of Custody for the case, either in the LIMS, or on hard-copy COC document maintained in the Case

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2.3 When not in the sampling or aliquot process, samples in the toxicology section must be stored in a secure and locked area.

2.4 Samples must be maintained in such a manner so that they are protected from contamination or deleterious change. Depending on the nature of the sample, this may mean refrigeration or freezing when not in the analytical process.

2.5 When analysis of samples is complete, samples in the toxicology section must be maintained "Under Proper Seal." This is interpreted to mean that the sample, or a container in which the sample is kept is sealed with tamper-evident tape, with the initials and date of person placing the seal on the seal as well.

2.6 Samples are maintained in the Toxicology Section, for 8 weeks after case is completed, in the absence of notification of any legal action, or reason to maintain the samples. After this period, samples are discarded in the appropriate medical waste container. Sample from fatalities, or cases with requests for retention are maintained by the laboratory

3.0 EQUIPMENT:

GC/MS and associated data station/computer (HP6890/5973 or equivalent)
General laboratory glassware and equipment
Solid phase extraction manifold and associated vacuum equipment.
Analytical evaporator Zymark Turbovap or equivalent.
Precision water bath.
UCT; ZSDAU020 "Clean Screen" extraction columns.

4.0 REAGENTS:

4.1 Reagents available as stock items:
Methanol (Baker Ultra-Resi Analyzed or equivalent)
Ethyl acetate (Baker Ultra-Resi Analyzed or equivalent)
Acetonitrile (Baker reagent grade or equivalent)
Blank Blood (Received from Hartford Hospital – Outdated supply)
Synthetic urine No. 13015 Alltech or equivalent
Alltech, Cerilliant or Equivailent drug Stocks: 1 mL vial 1.0 mg/mL
Prazepam internal Standard 1mg/mL

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Deionized water (DIW; Millipore or equivalent In-House supply)
β-Glucuronidase
Glacial Acetic acid (Baker reagent grade or equivalent)
Sodium Acetate Trihydrate (Baker reagent grade or equivalent)
BSTFA with 1% TMCS
Sodium Phosphate Dibasic (Na_2HPO_4).
Sodium Phosphate Monobasic (NaH_2PO_4).

4.2 Reagents prepared in the Toxicology Laboratory:

4.2.1 1 M Acetate buffer pH 5.0; Dissolve 42.9 g sodium acetate trihydrate in 400 mL DIW; add 10.4 mL glacial acetic acid. Dilute to 500 mL with DIW.

4.2.2 0.1M Acetate buffer pH 5.0; Dilute 40 mL 1.0 M acetate buffer to 400 mL with DIW.

4.2.3 β-Glucuronidase solution; Dissolve 100,000 Fishman units lyophilized powder with 20 mL 100 mM Acetate buffer pH 5.0.

4.2.4 0.1M phosphate buffer pH 6.0; Combine 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 and dilute to 1000 mL using DIW.

4.2.5 20% Acetonitrile in 0.1 M Phosphate buffer pH 6.0; Mix in proportion as needed.

Note: Reagent Preparation and Validation is documented in the Toxicology "Reagent Preparation/Validation Logbook" maintained in the Toxicology section. Validation is addressed in section 4.5, below.

4.3 Calibrators and Internal Standard:

Note: Preparation of all calibrator and control solutions is documented in the "Calibrator and Control Preparation Log" (maintained in the Toxicology Wet Laboratory)

Note: All Glass pipettes and Flasks used in this SOP shall be Grade A.

4.3.1 Prazepam internal standard stock solution; Dilute 100 uL of prazepam (1mg/mL) to 10.0 mL with Methanol in a volumetric flask

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4.3.2 Stock Calibrator solutions: Various benzodiazepine drugs as needed (Alltech or Cerilliant; 1 mg/mL); e.g. Alprazolam, Lorazepam; Temazepam; Oxazepam; Nordiazepam; etc. Add 100 uL of each standard solution and dilute up to 10 mL in a volumetric flask with methanol. Standards may be combined in calibrator stock solution.

4.4 Controls:

4.4.1 Benzodiazepine Control stock solution: Prepared as per 4.3.2, above.

Note: Each benzodiazepine batch must incorporate a high and low control for each analyte. Blood matrix controls may be used to validate urine results, but not the reverse. Controls are prepared by addition of benzodiazepines from validated stock solutions to blank sample matrix aliquots, prior to extraction, (as detailed below). Acceptable control performance is target value +/- 20%.

4.4.2 Glucuronide Control stock solution: Add 100 uL of 1.0 mg/mL Oxazepam glucuronide standard solution and dilute up to 10 mL in a volumetric flask with methanol.

4.5. Validation of Reagents: Acceptable performance of all batch control materials and overall batch acceptability (although individual samples may fail) is considered as validation of reagents. Validated reagents are marked with a green dot, detailing the specific procedure for which the reagent was validated, and the batch on which that process was documented. Newly prepared reagents may be evaluated for validity on an analytical batch, prior to any consideration of sample results. Reagents so validated are marked with a green sticker as noted above. Preparation of reagents, and their validation is documented in the Toxicology Section Reagent Preparation Validation Logbook, maintained in the Toxicology laboratory. See SOP #11.

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5.0 **PROCEDURE**

Note: Departure from procedures as specified in this SOP is not anticipated. Should an issue arise that may require such a departure, the issue must be raised with the Section Supervisor, Quality Manager and/or the Director. If the proposed change will not present a change of such a magnitude so as to require validation,

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the change may be approved, and the Director will modify and re-issue the SOP accordingly.

Any such procedural changes would be subject to the review process afforded by the quality control measures of the analytical scheme described herein. Hence, any modification or change that produces an unexpected deleterious effect on the analytical procedure would be expected to trigger analysis or batch failure in the QC review stages.

5.1 Batch Format: Analytical batches for Benzodiazepine confirmations should follow the format indicated below: Note that samples are analyzed in duplicate.

Calibrator
Matrix blank
Control High
Control Low
Glucuronide Control
Sample 1 rep 1
Sample 1 rep 2
Samples 2-10 Rep 1 and 2
Control High (Final control, in the absence of additional samples)
Additional Samples
Final control (may be high or low)

5.2 Label a 16x100 screw top culture tube for each sample replicate, blank, calibrator and control (including “glucuronide control”).

5.3. Using a validated dispensing pipette, place 1.0 mLs aliquot of each sample replicate into the appropriately labeled tube

Note: Samples requiring dilution as a function of concentration greater than the high control, should be diluted with 0.1M pH 6.0 Phosphate buffer as appropriate. The initial quantitative values may be used as a guide for the dilution process. The final dilution volume should be greated than the 1.0 ml aliquot that will be taken. The dilution process shall be documented on the batch worksheet, including the pipette(s) used in the process:

Example: Blood sample; 1st run result, ~ 1250 ng/mL Diazepam. Since a 1:1 dilution would be adequate (final result would be expected to be ~ 600 ng/mL, a 1:1 dilution is selected. Therefore, 600 uL sample is

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added to a 13 x 100 test tube using a 0 – 1 mL adjustable volume pipette, along with 600 uL 0.1 M pH 6.0 Phosphate buffer. (Note; because equal volumes of sample were utilized, precision alone determines the accuracy of the dilution). The solution is mixed, and aliquotted as per step 5.3. The dilution details, including the dilution factor (Volume sample:Volume Diluent + Volume Sample) are documented on the batch worksheet.

5.4 Add 1 mL of blank urine or blood, as appropriate, to each labeled control, calibrator tube .

5.5 Using a validated dispensing pipette, add 100 uL of prazepam internal standard stock solution to each replicate, blank, calibrator and control tube.

5.6 Using a validated dispensing pipette, add 50 uL of Benzodiazepine drug calibrator standard stock solution to the tube labeled Calibrator.

5.7 Using a validated dispensing pipette, add 100 uL of Benzodiazepine control standard stock solution to the tube labeled High Control.

5.8 Using a validated dispensing pipette, add 20 uL of Benzodiazepine control standard stock solution to the tube labeled Low Control.

5.9. Using a validated dispensing pipette, add 20 uL of Oxazepam Glucuronide control standard stock solution to the tube labeled Low Control.

5.10 To urine samples, blanks, calibrators and controls only, add 0.5 mL of β -Glucuronidase solution. Hydrolyze for 3 hours at 60°C in water bath. For blood samples, add 3 mL of Deionized water and vortex for 30 seconds. Let sit for 5 minutes and decant supernate.

5.11 Label SPE columns to correspond with each culture tube, place a clean thru tip on each end and load in the manifold. Add 3 mL pH6 buffer, and allow to drain through the column.

5.12 Condition the columns by passing through as follows:
1 x 3 mL methanol; aspirate
1 x 3 mL DIW; aspirate
1 x 1 mL 0.1 M Phosphate buffer 6.0; aspirate

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DO NOT LET COLUMN GO DRY!

5.13 Transfer contents of each tube to the appropriately labeled SPE column, and allow gentle drop wise flow until the level reaches the top if the column bed.

5.14 Wash column as follows:
1 x 2 mL DIW; drain (vacuum assist)
1 x 2 mL 20% acetonitrile/0.1 M Phosphate acid pH 6.0; drain (vacuum assist)
1 x 2 mL Hexane
Dry column (15 minutes at > 10 inches Hg)

5.15 Position appropriately labeled 13x100 test tubes under each SPE column, with clean thru tip inside collection test tube.

5.16 Elute Benzodiazepine analytes as follows:
1 x 5 mL Ethyl acetate
Collect Elute at 1 to 2 mL/minute.

5.17 Remove receiver tubes and evaporate to dryness at < 40 C using a gentle flow of Nitrogen (Turbovap).

5.18 Reconstitute eluent with 150 uL of ethyl acetate and transfer to gc/ms vial with limited volume insert. Add 25 uL BSTFA (with 1% TMCS) and cap. React 20 minutes at 70 degrees C. Remove from heat source to cool. NOTE: Do not evaporate BSTFA solution

5.19 Reserve for GCMS analysis, below:

6.0 CHROMATOGRAPHY AND MASS SPECTROSCOPY

6.1 Instrument and Setup:

GCMS/Autosampler: (Hewlett-Packard 6890/5973, or equivalent)
Column: 30M RTX-5MS (0.25 mm ID; 0.25 micron film)
Injection Temp. 250 ^{o}
Det. Temp. 80 ^{o}
Oven (init.): 80^{o}, 25 ^{o} /min to 300 ^{o}, (11 min hold).
19.8 min total run time

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1 uL injection

6.2 Injection sequence: For each fraction of samples: injections on the GC/MS in the following sequence:

Calibrator
Matrix blank
Control High
Control Low
Sample 1 rep 1
Sample 1 rep 2
Samples 2-? Rep 1 and 2
Control High
Additional Samples
Final Control

6.3 DETECTION AND IDENTIFICATION: Determination of the presence of Benzodiazepines in the sample extract are identified by appearance and ratio of the 3 ions characteristic of each species at the appropriate retention time, hence both retention time (a GC characteristic) and fragmentation pattern and ratio (MS characteristics) are used as the basis of qualitative identification. For a positive Identification of Benzodiazepines, the retention time must be within 5% of the corresponding analyte in the calibrator injection, and the ion ratios for both qualitative ions must be within 20% of the corresponding ratio calibrator sample. Qualitative identification of each analyte is independent.

6.4 CALIBRATION AND QUANTITATION

6.4.1 Calibration: Calibration for each batch and fraction is done Independently. Hence, no sample analysis conducted under DESPP guidelines is quantitated based on an historical calibration curve. Calibration is accomplished by the incorporation into the sample procedures of a blank sample of the matrix being analyzed that has known quantities (1 mg/L for Benzodiazepines) in addition to the internal standard. The response of the system to this calibrator, and the assumption of a 0 response to a 0 concentration, defines a run-specific standard curve that is used as the basis for the quantitative calculation in all controls and samples. The system is therefore “single-point calibration,

multi-point control".

6.4.2 Quantitation: Quantitation is accomplished by the comparison of the response ratio of the analyte in a specific sample, to the response ratios of the calibrators as expressed as a standard curve. The concentration of the analyte in the sample is then extrapolated from the standard curve, and corrected for any dilution that may have been performed to acilitate the analysis of relatively concentrated samples.

7.0 QUALITY CONTROL AND RUN EVALUATION

7.1 Verification of Vial Sequence; The vial sequence is checked both prior to and after the injection of samples when the auto injector is used. The check after the samples are injected is documented on the run summary sheet.

7.2 Chromatography - Evaluation and Acceptance Criteria: Chromatographic quality is evaluated for each peak. While general guidelines are that a peak should be symmetrical, and be resolved to baseline on at least one side, with 90% resolution on the other side, significant departures from those guidelines may be experienced with forensic samples. In many cases, chromatographic quality will warrant rejection of the chromatographic run, or specific samples, by the operator. Any such action should be clearly documented on the batch summary sheet. Questionable chromatographic peak shape, resolution, or other problems with chromatography can be discussed with the Section Supervisor, Director or the Quality Manager.

7.3 Evaluation of Potential Carryover: Carryover in the chromatographic quality is evaluated by injection of a blank sample immediately following the calibrator. Carryover of greater than 2% (20 ng/mL requires batch rejection, and remedial action for the instrument (e.g. replacement of injector insert, new septum and perhaps column trim or even replacement). Demonstrated carryover If less than 2% will require operator consideration with regard to the potential for effects on specific samples, and may require re-extraction of specific samples. Carryover is further evaluated on a per sample basis by the requirement that quantitative results between replicates agree within 20%. Any significant carrover effect should cause the first of the two replicate samples to exceed the second by an

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amount in excess of the 20% differential. If not, any carryover may be considered inconsequential. In practice, when a question of potential carryover exists, the potentially affected sample replicates may be repeated at the end of the batch.

7.4 Control Results: For the batch to be considered acceptable for any particular analyte, both control results for that analyte must be within 20% of the target value.

7.5 Internal Standard: For any individual injection to be acceptable, the internal standard abundance should be at least 20% of the corresponding abundance in the calibrator.

7.6 LINEARITY: Linearity of the calibration curve is demonstrable in each batch, for each analyte as a function of quantitative results of control materials.

7.7 SENSITIVITY- LIMITS OF DETECTION (LOD) and QUANTITATION (LOQ): For the purposes of this procedure, the LOD and LOQ are defined as equal to the lowest concentration of the lowest control. Qualitative Identification and/or Quantitative analysis below the concentration of the low control may be accepted on a case by case basis with the concurrence of the analyst, technical reviewer, Director and/or Quality Manager.

7.8 ACCURACY AND PRECISION: Precision of the procedure is evaluated on a yearly basis, by repeat analyses of control or PT materials. Accuracy is expressed as a mean (absolute value) percentage differencebetween mean quantitative value of 10 reps of the specific control, and the target value. Precision is expressed as the CV of that value

7.9 SPECIFICITY: Specificity is a function of both the resolution of target analyte during the analytical process, and the mass spectral fragmentation that analyte molecules undergo during the instrumental analysis. There has been no report of any material other than Benzodiazepines shown in this SOP that elute within 5% if the retention time of known standard materials, and produce the same fragmentation ions and ratios.

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8.0 **REPORTING OF RESULTS**: Benzodiazepine runs runs are performed as part of GC/MS batches, containing controls and calibrators. The complete batch packets are in the Toxicology Laboratory. This packet contains all run evaluation documentation. Specific chromatograms for each case are filed in the appropriate case file. Results are documented on the "Batch Summary" sticker on each case file and include a reference number for the batch as a whole. A Batch summary sheet will be produced with each batch. Data on each batch should include fields such as: Sample name, Batch ID (Date of Batch), analysts who generated data, matrix, analyte found (and concentration if applicable), controls run with the batch and results obtained. If controls do not meet the criteria, the batch can be rejected as a whole or by a case by case basis. The supervisor is notified and proper action is taken to correct any problem. Batches and/or cases shall be repeated as needed.

Procedural Uncertainty is reported with all quantitative results, and is calculated and tabulated annually for each analytical method, (See SOP TX-19 section 6.3).

9.0 **QUALITY ASSURANCE**: Quality Assurance is provided by the multiple layers of checks that are performed both during and after analysis. Specifically:

9.1 The GC/MS run is thoroughly checked by the operator, including vial position on the autosampler, both prior to and following the injection of samples.

9.2 The GC/MS run is reviewed and signed off by a reviewer distinct from the operator, with this review including an evaluation of qualitative and quantitative (where applicable) results, including:

a. Control Results
b. Chromatographic Characteristics
c. Transcription errors

9.3 The results, as transcribed in the Case Summary Form are checked against the original run summary sheet during the process of report preparation, and during the administrative review of case results.

9.4 The original run is compared to the Final Report during the final Director's review, prior to case sign-off.

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10.0 **SOURCES OF ERROR**

The utilization of 3-ion SIM methodology, with reference to procedural, controls and calibrators yields qualitative drug identification with essentially no uncertainty. Urine drug analyses are reported only as qualitative results.

11.0 **REFERENCES**

Clarke's Isolation and Identification of Drugs. 2nd Edition

UCT United Chemical Technologies; Solid phase extraction methods

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Benzodiazepine Analysis

GC/MS temperature program specifications

Parameter			
Initial temp	80° C		
Initial Time	0.00min		
Ramps Rate/final temp/final time	rate	temp	time
	25.0	300	11.0 min
Post temp	80°c		
Post time	0.00 min		
Run Time	19.80 min		
Front inlet			
Mode	Pulsed splitless		
Initial temp	250°c		
Pressure	8.56 psi		
Pulse pressure	25 psi		

Pulse time	0.50 min
Total flow	43.4 mL/min
Gas type	Helium
Injection volume	1 microliter
Post injection washes Solvent A / Solvent B	Solvent A -3 Solvent B - 3
Tune file	STUNE
Acquisition mode	SIM
Solvent delay	8.70 minutes
M/Zs group 1 Start time 0.00 M/Zs group 2 Start time 9.20 M/Zs group 3 Start time 9.55 M/Zs group 4 Start time 10.25 M/Zs group 5 Start time 12.3	341, 342, 343 429, 430, 313 429, 430, 347 343, 269, 257, 241, 283, 324 308, 279, 204
Dwell	50
MS Quad	150°c max 200
MS Source	230°c max 250

Note: Times are Approximate.
Example of controlled Benzo batch document TX Benzo-1. Batch documents can vary based on nature of batch.

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CT DPS; Toxicology Laboratory Batch ID: 3-22-11

Benzodiazepine GC/MS Summary Page 1

Vial position	Sample	Specimen	Specimen Volume, (ml)	Int. Std. Volume (ul)	Analyte	I.S. abund. (x 1 E 3)	Theoretical Concentration (mg/L)	Observed Concentration (mg/L)	Percent Recovery (Acceptable: 80 - 120)	Analyst Qualitative	Reviewer Qualitative
3	Calibrator	Blood	1.0	20	Nordiazepam 6.66	348	1.00	1.00			
					Oxazepam 7.01		1.00	1.00			
					Lorazepam 7.37		1.00	1.00			
					Temazepam 7.69		1.00	1.00			
4	Blank	Blood	1.0	20	Nordiazepam	368	0.00	ND			
					Oxazepam		0.00	ND			
					Lorazepam		0.00	ND			
					Temazepam		0.00	ND			
5	Control 0.500	Blood	1.0	20	Nordiazepam	276	0.500	0.297	59.4		
					Oxazepam		0.500	0.398	79.6		
					Lorazepam		0.500	0.442	88.4		
					Temazepam		0.500	0.495	99.0		
6	Control 0.200	Blood	1.0	20	Nordiazepam	408	0.200	0.200	100		
					Oxazepam		0.200	0.188	94.0		
					Lorazepam		0.200	0.197	98.5		
					Temazepam		0.200	0.226	113		
7	TX-11-354-3	Blood	1.0	20	Oxazepam	361		0.272			
8	TX-11-354-3	Blood	1.0	20	Oxazepam	365		0.205			
6	Control 0.200	Blood	1.0	20	Nordiazepam	414	0.200	0.191	96		
					Oxazepam		0.200	0.198	99		
					Lorazepam		0.200	0.214	107		
					Temazepam		0.200	0.228	114		

Samples Extracted by: _____ Date: _____ GCMS Run Date: _____

Vial position verified prior to sample removal _____

Derivitizing Agent Valid: _____Yes _____ No

*** Acceptability; Peak Shape, Retention Time, IS Area, Fragment Ratio all must be acceptable.

Analyst Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No

Acccepted quantitative results have internal standard abundances of at least 20% of internal standard abundance observed in calibrator.

**: For Prazepam Internal std, this method only collected 2 ions; 324 and 241.

*1: Only report if found on other Batch.

*2: poor correlation between quant results, report as ID only

Technical Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No

Reviewer Comments: