

**FORENSIC TOXICOLOGY LABORATORY
OFFICE OF CHIEF MEDICAL EXAMINER
CITY OF NEW YORK**

**BENZODIAZEPINES
by
SOLID PHASE EXTRACTION
and
GAS CHROMATOGRAPHY/MASS SPECTROMETRY
(Selected Ion Monitoring)**

PRINCIPLE

Benzodiazepines are a widely used class of drugs. This assay is applied to the detection and quantification of the most commonly encountered benzodiazepines. The benzodiazepines are extracted from biological specimens by solid phase extraction. Drugs are temporarily bound to a sorbent in the solid phase cartridge as the prepared sample is poured through the column. The column is washed to remove interfering compounds, followed by elution of drugs from the column. The eluate is evaporated and derivatized with MTBSTFA to form the t-butylsilyl derivatives. Quantitative analysis is performed by SIM GC/MS using a six point calibration curve and deuterated internal standards. Qualitative analysis is also performed by SIM GC/MS using a single point calibrator and deuterated internal standards. Urine samples are hydrolyzed using a glucuronidase to release conjugated benzodiazepines.

SAFETY

The handling of all reagents, samples and equipment is performed within the guidelines which are detailed in the safety manual.

SPECIMEN PREPARATION

The procedure is routinely applied to the following biological specimens and their aliquots unless otherwise specified:

Blood	1.0 mL of the undiluted specimen
Urine	1.0 mL for qualitative identification
Bile	1.0 mL of the undiluted specimen
Brain	1.0 mL of a 1:3 homogenate
Gastric Contents	1.0 mL of a 1:10 dilution
Liver	1.0 mL of a 1:5 homogenate
Vitreous Humor	1.0 mL of the undiluted specimen

Dilution of specimens

Specimens are diluted as follows:

Brain 1:3	5.0 g of brain homogenized with 10 mL of distilled water.
Liver 1:5	5.0 g of liver homogenized with 20 mL of distilled water.
Gastric 1:10	2.0 mL of liquid <i>qs</i> to 20 mL of distilled water, or 2.0 g of a solid specimen homogenized with 18 mL of distilled water.

Note: Use a homogenate which was prepared within two weeks. Do not use homogenates older than two weeks unless low sample size requires it. Discuss with supervisor and note in case record. The entire submitted amount of gastric contents needs to be homogenized prior to sampling.

REAGENTS AND MATERIALS

Chemicals should be analytical reagent (AR) grade or higher. The chemical reagents required for the extraction procedure are prepared as indicated. In each case, the prepared reagent is stable for a minimum of six months. All new reagents are validated against the old ones prior to use.

1. **Deionized water** (distilled can be substituted)
2. **Methanol** (Fisher Scientific - ACS Certified, or equivalent)
3. **Ammonium Hydroxide, NH₄OH** (Fisher Scientific, or equivalent)

Note: Ammonium hydroxide will break down to ammonia and water and the ammonia will evaporate if the container is not kept closed. This will cause the pH to decrease, making the reagent unsuitable for solid phase extraction. Use small lots of working solution (500 mL bottles), open the bottle only briefly to remove aliquots and recap immediately. If the solution appears old, discard and use a fresh bottle.

4. **Ethyl Acetate.** Fisher Scientific or equivalent.
5. **Hexane.** Fisher Scientific or equivalent.
6. **Acetonitrile.** Fisher Scientific or equivalent.
7. **KHCO₃.** Certified ACS. Fisher Scientific or equivalent.
8. **K₂CO₃.** Certified ACS. Fisher Scientific or equivalent.
9. **Sodium acetate trihydrate.** Certified ACS. Fisher Scientific or equivalent.
10. **Glacial acetic acid.** Certified ACS. Fisher Scientific or equivalent.
11. **Certified Negative Blood.** Previously found to have no interfering peaks. Aliquot 1ml validated negative blood into 16 X 125 mm disposable culture tube and Freeze. Stable for 6 months. Revalidate if necessary.
12. **Certified Negative Liver.** Previously found to have no interfering peaks. Aliquot 1ml validated negative liver into 16 X 125 mm disposable culture tube and freeze. Stable for 6 months. Revalidate if necessary.

13. **Certified Negative Brain.** Aliquot 1ml validated negative brain into 16 X 125 mm disposable culture tube and freeze. Stable for 6 months. Revalidate if necessary.
14. **Polycrom Clin II Solid Phase Extraction Column.** CEREX.
15. **System 48 Processor** connected to nitrogen source.
16. **Waste Rack, SPE Rack, Collection Tube Rack.**
17. **Turbovap** or Cerex 48 Sample Concentrator connected to a nitrogen source.
18. **Sonicator**
19. **Vortex**
20. **Centrifuge**
21. **pH Meter**
22. **Laboratory glassware as needed**
23. **100 mM Sodium Acetate buffer (pH 4.5).**

Dissolve 11.72 g sodium acetate trihydrate in 1800 mL DI H₂O; add 6.5 mL glacial acetic acid. Dilute to 2000 mL using DI H₂O. Mix. Adjust pH to 4.5 ± 0.1 with sodium acetate or acetic acid.

Storage: room temperature in glass.

Stability: 6 months. Inspect each day of use for contamination.
24. **100 mM Acetate buffer (pH 5.0).**

Dissolve 17.16 g sodium acetate trihydrate in 1800 mL DI H₂O; add 4.16 mL glacial acetic acid. Dilute to 2000 mL using DI H₂O. Mix. Adjust pH to 5.0 ± 0.1 with sodium acetate or acetic acid.

Storage: room temperature in glass.

Stability: 6 months. Inspect each day of use for contamination
25. **pH 9.0 buffer**

Dissolve 40 g KHCO₃ in 1800 mL DI H₂O; add 20 g K₂CO₃. Dilute to 2000 mL using DI H₂O. Mix. Adjust pH to 9.0 ± 0.1 with KHCO₃ or K₂CO₃.

Storage: room temperature in glass.

Stability: 6 months. Inspect each day of use for contamination.
26. **Eluting solvent:** ethyl acetate:NH₄OH (98:2). Prepare fresh each day of use.
27. **MTBSTFA** (N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide) with 1% TMCS, , Aldrich Chemical, or equivalent.

28. **β -glucuronidase** solution type HP2, greater than 100,000 Fishman units/mL, Helix Pomatia. Prepare working solution by diluting to 1:20 with 100 mM sodium acetate buffer, pH 5.0. Prepared fresh each day of use.

29. **Calibrators and controls**

Calibrator and control primary standards in methanol, Cerilliant or equivalent

<i>Benzo Pool</i>	Group 1
Diazepam	1.0 mg/mL in acetonitrile
Lorazepam	1.0 mg/mL in acetonitrile
Alphahydroxyalprazolam	1.0 mg/mL in acetonitrile
Alprazolam	1.0 mg/mL in acetonitrile
Alphahydroxytriazolam	1.0 mg/mL in acetonitrile
Nordiazepam	1.0 mg/mL in acetonitrile
Oxazepam	1.0 mg/mL in acetonitrile
Temazepam	1.0 mg/mL in acetonitrile
Triazolam	1.0 mg/mL in acetonitrile

Benzo Pool	Group 2
Clonazepam	1.0 mg/mL in acetonitrile
7-Aminoclonazepam	1.0 mg/mL in acetonitrile
7-Aminoflunitrazepam	1.0 mg/mL in acetonitrile

Note: *Clonazepam, 7-aminoclonazepam and 7-aminoflunitrazepam are in a group by themselves, separate from group 1 calibrator and control solutions.*

A. 10 mg/L Calibrator and Control

Using a pipet, deliver 1 mL of 1 mg/mL stock) of each benzodiazepine to a 100 mL volumetric flask. Dilute to mark with acetonitrile. Repeat with a different lot of primary stock solutions (if available) to make the control solution. Label with identity, concentration, solvent, lot number, date prepared, and initials of analyst.

B. 1 mg/L Calibrator and Control

Pipet 10mL of 10 mg/L calibrator solution into a 100 mL volumetric flask. Dilute to mark with acetonitrile. Transfer working calibrator solution to properly labeled containers. Pipet 10 mL of 10 mg/L control solution into a second 100 mL volumetric flask. Dilute to mark with acetonitrile. Transfer working control solution to properly labeled containers.

30. Internal Standards:

Reference Standards are obtained in 1.0 mL ampules from Cerilliant or equivalent.

Alphahydroxyalprazolam- d ₅	100 µg/mL in acetonitrile
Oxazepam-d ₅	100 µg/mL in acetonitrile

5 mg/L internal standard pool:

Using a pipet, deliver 5 mL of 100 µg/mL stock solution (5 x 1 mL ampules) of each deuterated internal standard solution to a 100 mL volumetric flask. Dilute to mark with acetonitrile. Transfer solution to properly labeled containers.

31. Hydrolysis control solution

oxazepam glucuronide (10 mg/L)

Pipet 1 mL of 100 µg/mL oxazepam glucuronide into a 10 mL volumetric flask. Q.S. to 10 mL with acetonitrile. Transfer to properly labeled container.

32. Gas Chromatography Column:

Restek RTX-50 crossbond 50% phenyl -50% methyl polysiloxane. 15M x 0.25 mm ID x 0.25 µm DF, max temperature 320 °C. Model # 10520.

EXTRACTION PROCEDURE FOR BLOOD AND TISSUES

1. Obtain a worklist of benzodiazepine cases to be analyzed. Review with supervisor for any updates of rush cases or other special instructions.
2. Find and collect all samples on worklist and place in an empty rack.
3. Obtain enough 16 x 125 mm disposable culture tubes for calibrators, controls, and all requested cases. Label the tubes appropriately. Tubes should bear the entire toxicology number (e.g., YY-1234, not 1234), the specimen type and any dilution.
4. Obtain 1 mL of validated negative specimen or sample into the 16 x 125 mm test tube labeled as to the contents.
5. Fortify the appropriate amounts of working calibrator solutions to negative matrix specimen tubes.

Six positive calibrators and one blank should be run with each batch of samples.

Positive calibrators are prepared in the following concentrations:

10 ng/mL -	add 10	µL of 1.0 mg/L calibrator solution
25 ng/mL -	add 25	µL of 1.0 mg/L calibrator solution
50 ng/mL -	add 50	µL of 1.0 mg/L calibrator solution*
100 ng/mL -	add 100	µL of 1.0 mg/L calibrator solution
500 ng/mL -	add 50	µL of 10.0 mg/L calibrator solution
1000 ng/mL -	add 100	µL of 10.0 mg/L calibrator solution

Note: *Clonazepam and 7-aminoclonazepam are fortified in calibrator level 50 ng/mL only. This is to be only used as a marker for the analytes in qualitative analysis.

Mix by Vortex.

Note: Deionized water is used as the negative matrix for urine and gastric specimens.

Three controls for verification of the calibrators are included in the run.

50 ng/mL -	add 50	µL of 1.0 mg/L control solution*
200 ng/mL -	add 20	µL of 10.0 mg/L control solution
750 ng/mL -	add 75	µL of 10.0 mg/L control solution

Note: *Clonazepam and 7-aminoclonazepam are fortified in control level 50 ng/mL only. This is to be only used as a marker for the analytes in qualitative analysis.

6. Add 50 µL of internal standard pool to all test tubes. The concentration of the internal standard in each sample is 250 ng/mL.
7. Add 3 mL of 100 mM sodium acetate buffer (pH 4.5). Vortex for about 15 secs then sonicate for 15 min.

Condition each column sequentially with:

2mL ethyl acetate
2mL methanol
2mL water

8. Centrifuge samples at \approx 3000 rpm for \approx 10 min.
9. Decant the supernatant into the Polychrom Clin II column and apply nitrogen at a pressure of 2-4 psi.
10. Wash Column (All wash steps are pressurized at 2-4psi).
Add 2 mL of pH 9.0 buffer.
Add 2 mL distilled or de-ionized water.
Dry columns for 15 min at \approx 40 psi.
Add 2 mL hexane.
Dry for 5 min at \approx 40 psi.

11. Elute Drugs

Place labeled 10 mL conical centrifuge under each column to collect eluate. Elute with 3 mL ethyl acetate:NH₄OH (98:2). Collect eluate at 1 to 2 mL/minute. Apply pressure as necessary to achieve the desired flow rate.

Note: Prepare elution solvent daily.

12. Dry under nitrogen at 40 °C to absolute dryness.

13. Derivatization

Add 50 µL ethyl acetate and 25 µL MTBSTFA (with 1% TMCS). Cap tubes, vortex for 15 secs Incubate 20 minutes at 70 °C. Remove from heat source to cool.

Note: Do not evaporate MTBSTFA solution.

14. Label autosampler vials indicating aliquot and toxicology number (ex: 2-YY-xxxx), specimen type, dilution, analyst and date. Immediately transfer sample into screw cap vial and cap vial. Physically check that the screw cap is tight by attempting to rotate the cap. Do not wait until all transfers have been made to seal the vials.

15. Transfer the vials to the GCMS section for analysis. Be sure to enter a copy of the sequence in the Sequence Logbook. Also document into LIMS the usage log. Enter the date completed in the Dataease database, so the samples are not duplicated by another analyst.

IMPORTANT: DO NOT WAIT UNTIL THE FOLLOWING DAY TO DO THIS!

EXTRACTION PROCEDURE - Urine

1. Obtain a worklist of benzodiazepine cases to be analyzed. Review with supervisor for any updates of rush cases or other special instructions.
2. Find and collect all samples on worklist and place in an empty rack.
3. Obtain enough 16 x 150 mm disposable screw-cap culture tubes for calibrators, controls, and all requested cases. Label the tubes appropriately. Tubes should bear the entire toxicology number (e.g. YY-1234, not 1234), the specimen type and any dilution.
4. Pipet 1 mL of validated negative matrix or sample into test tube labeled as to the contents.
5. Spike the appropriate amounts of calibrator solution to negative matrix specimen tubes.

For urine, one positive calibrator at 500 ng/mL, one positive control at 200 ng/mL, one hydrolysis control at 200 ng/mL and one blank should be run with each batch of samples. The positive calibrator is prepared by adding 50 µL of 10.0 mg/L calibrator solution to 1 mL of blank matrix. Mix by Vortex.

Note: Deionized water is used as the negative matrix for urine and gastric specimens.

Controls for verification of the calibration are included in the run.

a. A positive control of 200 ng/mL should be prepared as follows:

Add 20 µl of 10.0 mg/L control solution to the negative matrix.

b. A hydrolysis control of 200 ng/mL oxazepam glucuronide should be prepared as follows:

Add 32 μL of 10.0 mg/L control solution to the negative matrix.

6. Add 50 μL of internal standard pool to all test tubes. Mix by Vortex.
7. Add 1.0 mL of β -glucuronidase working solution. Mix by Vortex.
8. Hydrolyze for 3 hours at 70 $^{\circ}\text{C}$.
9. Centrifuge for \approx 10 minutes at \approx 3000 rpm. Cool before proceeding.
10. Condition each column sequentially with:
 - 2mL ethyl acetate
 - 2mL methanol
 - 2mL water
11. Pour the supernatant into the column and apply positive pressure slowly until you see liquid come out of the column tip. At this point turn gas pressure off and allow sample to soak in column bed for 5 minutes. After soaking, turn gas pressure on and apply nitrogen to achieve a flow through the column of \approx 1.0 mL/min.
12. Wash Column (All wash steps are pressurized at 2-4psi).
 - Add 2 mL of pH 9.0 buffer.
 - Add 2 mL distilled or de-ionized water.
 - Dry columns for 15 min at \approx 40 psi.
 - Add 2 mL hexane.
 - Dry for 5 min at \approx 40 psi.
13. Elute Drugs
 - Place labeled 10 mL conical centrifuge under each column to collect eluate. Elute with 3 mL ethyl acetate: NH_4OH (98:2). Collect eluate at 1 to 2 mL/minute. Apply pressure as necessary to achieve the desired flow rate.

Note: Prepare elution solvent daily.

14. Dry under nitrogen at 40 $^{\circ}\text{C}$ to absolute dryness.
15. Derivatization
 - Add 50 μL ethyl acetate and 25 μL MTBSTFA (with 1% TMCS). Cap tubes. Vortex for 15 secs.
 - Incubate 20 minutes at 70 $^{\circ}\text{C}$. Remove from heat source to cool.

Note: Do not evaporate MTBSTFA solution.

16. Label autosampler vials indicating aliquot and toxicology number (ex: 2-YY-xxxx), specimen type, dilution, analyst and date. Immediately seal each vial with an aluminum seal using a crimper to avoid possible contamination from other samples. Samples may also be transferred into screw cap vials and capped immediately. Physically check that the crimped seal is tight by attempting to rotate the seal. Crimp until tight, using a new seal if necessary. Do not wait until all transfers have been made to seal the vials.

17. Transfer the vials to the GCMS section for analysis. Be sure to enter a copy of the sequence in the Sequence Logbook. Also document into LIMS the usage log. Enter the date completed in the Dataease database, so the samples are not duplicated by another analyst.

IMPORTANT: DO NOT WAIT UNTIL THE FOLLOWING DAY TO DO THIS!

INSTRUMENTATION

Agilent MSD (6890 GC with 5973 Mass Spectrometer), Autosampler (Agilent 7673 or 7683), and Agilent Chemstation with current revision of software. The method names for this assay are MSNBZQ.M for quantitative batches, and MSNBZSCR.M for urine batches, where N = instrument number. The methods are identical except for name. Each MSD used for benzodiazepine analysis will have methods with these names in the method directory.

The following ions are monitored for each drug:

Alphahydroxyalprazolam-d ₅	386.1, 388.1
Oxazepam-d ₅	462.2, 464.2
7-Aminoflunitrazepam	283.1, 284.1, 282.1
7-Aminoclonazepam	342.1, 344.1, 399.1
Alphahydroxyalprazolam	381.1, 383.1, 382.1
Alphahydroxytriazolam	415.0, 417.0, 416.0
Alprazolam	279.0, 281.1, 273.1
Clonazepam	372.1, 374.1, 326.1
Diazepam	256.0, 257.1, 258.1
Lorazepam	491.1, 493.1, 513.2
Nordiazepam	327.1, 329.1, 383.1
Oxazepam	457.2, 459.2, 513.2
Temazepam	357.1, 283.1, 359.1
Triazolam	313.1, 342.1, 344.1

TOP LEVEL PARAMETERS

Method Sections To Run:

- Save Copy of Method With Data
- Pre-Run Cmd/Macro =
- Data Acquisition
- Data Analysis
- Post-Run Cmd/Macro =

Method Comments:

HP 5973 MS Benzodiazepine method

END OF TOP LEVEL PARAMETERS

INSTRUMENT CONTROL PARAMETERS

Sample Inlet	GC
Injection Source	GC ALS
Mass Spectrometer	Enabled

HP6890 GC METHOD

OVEN

Initial temp	200 °C (On)	Maximum temp	320 °C
Initial time	5.00 min	Equilibration time	0.50 min

<i>Ramps</i>			
#	Rate	Final temp	Final time
1	15.00	295	9.00
2	0.0(Off)		
Post temp	295 °C		
Post time	5.00 min		
Run time	25.33 min		

<i>Front Inlet (Unknown)</i>		<i>Back Inlet ()</i>
Mode	Pulsed Splitless	
Initial temp	260 °C (On)	
Pressure	11.96 psi (On)	
Pulse pressure	30.0 psi	
Pulse time	0.50 min	
Purge flow	7.5 mL/min	
Purge time	1.50 min	
Total flow	11.5 mL/min	
Gas saver	On	
Saver flow	20.0 mL/min	
Saver time	3.00 min	
Gas type	Helium	

<i>Column 1</i>		<i>Column 2</i>
Capillary Column	Restek RTX-50	(not installed)
Nominal length	15.0 m	
Nominal diameter	250.00 um	
Nominal film thickness	0.25 um	
Mode	Ramped flow	
Initial flow	1.5 mL/min	
Initial time	13.50 min	

#	Rate	Final flow	Final time
Post Flow		2.0 mL/min	
Nominal init pressure		11.96 psi	
Average velocity		67 cm/sec	
Inlet		Front Inlet	
Outlet		MSD	
Outlet pressure		vacuum	

1	1.50	2.0	3.00
2	off	off	0.00
3	0.0(off)		

FRONT DETECTOR (NO DET)	BACK DETECTOR (NO DET)
COLUMN COMP 1	COLUMN COMP 2
(No Detectors Installed)	(No Detectors Installed)

Thermal Aux 2	
Use: MSD Transfer Line Heater	
Description	
Initial temp	280 °C (On)
Initial time	0.00 min
# Rate Final temp Final time	
1 0.0(Off)	

Post Run	
Post Time	5.00 min
Oven Temperature	295 °C
Column 1 Flow	2.0 mL/min

Time Table		
Time	Specifier	Parameter & Setpoint
7673 Injector		
<i>Front Injector:</i>		
Sample Washes	1	
Sample Pumps	2	
Injection Volume	4.0 microliters	
Syringe Size	10.0 microliters	
PretInj Solvent A Washes	3	
PretInj Solvent B Washes	3	
PostInj Solvent A Washes	3	
PostInj Solvent B Washes	3	
Viscosity Delay	0 seconds	
Plunger Speed	Fast	
PreInjection Dwell	0.00 minutes	
PostInjection Dwell	0.00 minutes	
<i>Back Injector:</i>		
No parameters specified		

MS ACQUISITION PARAMETERS

<i>General Information</i>	
Tune File	atune.u
Acquisition Mode	SIM
<i>MS Information</i>	
Solvent Delay	5.0 min
EM Relative	False
EM Offset	0
Resulting EM Voltage	(Varies)

[Sim Parameters]

<i>Group 1</i>			
Group ID	1		
Resolution	High		
Group Start Time	7.0 min		
Plot 1 Ion	462.2		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(256.0, 40)	(257.1, 40)	(258.1, 40)
	(283.1, 75)	(327.1, 40)	(329.1, 40)
	(357.1, 50)	(359.1, 50)	(383.1, 50)
	(457.2, 40)	(459.2, 40)	(462.2, 40)
	(464.2, 40)	(491.1, 50)	(493.1, 50)
	(513.2, 40)		
<i>Group 2</i>			
Group ID	2		
Resolution	High		
Group Start Time	11.22 min		
Plot 1 Ion	386.1		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(273.1, 120)	(279.0, 100)	(281.0, 110)
	(282.1, 40)	(283.1, 75)	(284.1, 75)
	(313.1, 75)	(326.1, 75)	(342.1, 75)
	(344.1, 75)	(372.1, 75)	(374.1, 75)
	(381.1, 75)	(382.1, 75)	(383.1, 75)
	(386.1, 75)	(388.1, 75)	(399.1, 75)
	(415.0, 75)	(416.0, 100)	(417.0, 100)

[MSZones]

MS Quad: 150 C maximum 200 °C
 MS Source: 230 C maximum 250 °C

END OF MS ACQUISITION PARAMETERS

END OF INSTRUMENT CONTROL PARAMETERS

INSTRUMENT SETUP

An acceptable autotune must be obtained prior to batch analysis. Refer to the SOP entitled "GCMS Tuning" for instructions.

All autosampler syringe wash vials are filled with ethyl acetate.

Prepare a sequence using the following steps.

When Chemstation is opened, the **Openlab ECM Login** screen appears. Enter the instrument name (ms2, ms4, etc.) as appropriate for username and the current password. Verify that Account field says production and Domain field says Built-In. If Chemstation is already running, it may be necessary to log out and relog in. Using the Chemstation software, at the top Method and Run toolbar under **ECM**, select **Logon to ECM**. Follow the instructions above to log on.

1. On the Method and Run toolbar, under Sequence, select Load Sequence. Select default.s. Click on Select.
2. In the Method and Run toolbar, under Sequence, select Edit Sequence. At the top of the screen under Data Path, click on Browse. Under Select Data Path, click on the msdchem folder. The click 1 and then click on the Data folder to highlight it. In lower left of dialog box, select Make New Folder. A folder with the name New Folder is created under DATA. Right click on New Folder and Rename or double click to highlight the folder name and change the entry. Rename the file using the format MSNMMDDYYx, where N is instrument number, MM = month, DD = day, YY= year and x = a letter indicating the batch being run, e.g. MS2041111a. Click OK which will take you back to the sample log table.
3. In the Sample column verify the correctness of pre-loaded entries. Starting at the first empty field, enter sample or QC information. For samples this would include aliquot number, laboratory number, specimen source, dilution if any (i.e., 2-11-2432 fem). If the sample has been diluted, enter the appropriate dilution factor in the Multiplier column. For QC samples use an appropriate designator. Enter the next sample in the batch in the next open field down the column.
4. In the Type column, select the corresponding sample type for each vial: Sample, Blank, Calibration or QC.
5. In the Vial column, click in the cell with the number 1, hold down the left mouse button and drag to the last vial number in the sequence (cells will be highlighted). Right click and select Fill Column and Increment. Verify that the vial numbers are correct.
6. In the Method column, verify that the correct method is loaded in the first cell. Then click on the first cell containing the method name, hold down the left mouse and drag to the last vial number in the sequence (cells will be highlighted). Right click and select Fill Column, No Increment. Verify that the method for each vial is correct.
7. In the Data File column, in the cell corresponding to the first vial, enter the data file name in the format MSNMMDDx001, where N = instrument number, MM = month, DD = day, and x = a letter indicating the batch being run, i.e. MS20411a001a. Click on this cell, hold down the

left mouse and drag to the last vial number of the sequence (the cells will be highlighted). Right click and select Fill Column and Increment. Verify that the data file information for each vial is correct.

8. In the Comment column, enter any additional information for the vials.
9. In the Multiplier column, enter any sample dilutions.
10. In the Level column, verify that the correct level numbers are entered for calibrators in this batch.
11. Verify No Update is selected for all vials under Update Rf and Update Rt.
12. Review the information typed for the sequence. Correct any information as needed. Verify that the Data Path is C:\MSDCHEM\1\Data\current sequence name. Verify that the Method Path is C:\MSDCHEM\1\METHODS. Then Click ok.

Note: Occasionally, it will be desirable to run several subsequences in one batch. Use the instructions below to accomplish this.

Setting-Up a Subsequence

On the Sample Log Table:

1. Select "**Keyword**" for "Type"
2. Select "**DataPath**" for "Method/Keyword"
3. Under "Comment/KeywordString" type in the *new data path* for your subsequence ie: **C:\MSDCHEM\1\DATA\MSNMMDDYYx** where "N" is the instrument's number and "x" is the letter designated to the subsequence (**it must be different than that of the original sequence**).
4. The suffix of the data files must be different from that of the original; ie: MSNMMDDY001; **the subsequence data files must start with 1 again.**
5. After typing in the entire sequence, save sequence accordingly.
6. Go to Sequence → Simulate Sequence → Run Sequence.
7. A dialog box will pop-up: DataPath **C:\MSDCHEM\1\DATA\MSNMMDDYYy** does not exist. Edit Sample Log Table? → Click **No** if the sequence was set up correctly.
8. A 2nd dialog box will pop-up: Create **C:\MSDCHEM\1\DATA\MSNMMDDYYy**? → Click **Yes**.
9. A 3rd dialog box will pop-up: Sequence Verification Done! View it? → Click **Yes** or **No**.

Setting-Up a Subsequence with a Different Method

On the Sample Log Table:

1. After inserting the DataPath keywords and Commands, Insert a Row.
 2. Select "**Keyword**" for "Type"
 3. Select "**MethodPath**" for "Method/Keyword"
 4. Check that the method for each sample is changed to the new method.
 5. Follow Steps 5-9 in the "**Setting-Up a Subsequence**" Section (see previous section).
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13. On the Method and Run toolbar, under Sequence, select Run Sequence. In the dialog box under Sequence Comment enter the initials of the individual who has entered the sequence in the Operator Field (e.g., MPM). Under Data File Directory verify that the data file path is C:\MSDCHEM\1\DATA\batch name.
 14. Under Sequence, select Save Sequence As. Under File Name, type in the name of the folder that the batch will be saved to under DATA, MSNMMDDYYx (e.g. MS2041211a) Select Save. The extension ".s" will automatically be added.
 15. Under Sequence select Print Sequence. Verify that Brief Format is selected and click on OK. The sequence will be printed. Apply the preprinted labels for documenting verification of process steps to the printed sequence list.
 16. Use the printed sequence list to load vials into the appropriate autosampler positions as indicated by the order on the printed sequence list. Check vial information against the sequence list and ensure that the vial is inserted in the correct numeric position in the autosampler as indicated on the sequence list. Document correct positioning by dating and initialing the appropriate line on the sequence list.
 17. Under Sequence select Run Sequence. Verify that the Sequence comments and Data Field information are correct (i.e. verify that the proper sequence is loaded. If not, load the proper sequence). Click on Run Sequence.
 18. After the batch is finished, unload the vials. Compare the vial information to the sequence list as they are removed, to verify that the correct vial was in the correct position. Date and initial the sequence list when this is completed. Annotate discrepancies if necessary

DATA TRANSFER AND PROCESSING - EXPORTING RAW DATA FOR PROCESSING

All processing and review of the processed data should be performed on a processing computer.

SAVE METHOD TO ECM

1. After the run finishes, the data files will be in the data subdirectory on the local chemstation and also automatically transferred to ECM. From the acquiring computer, make sure the proper method, the one used to acquire the data, is loaded. On the top toolbar under ECM click on Save Method to ECM.

2. Click on GCMS, the correct instrument name folder, the appropriate month and batch to which the method will be saved.

RETRIEVE BATCH FROM ECM - RAW DATA FOR PROCESSING

1. At the processing computer, click on Processing Data Analysis. Log on using your OCME network username and password.
2. On Enhanced Data Analysis screen, click on ECM at the top toolbar and select Retrieve entire sequence from ECM.
3. This will open up Openlab ECM screen. Select GCMS, then the appropriate instrument, the month, and, finally the batch to be retrieved. On the status line at the bottom of the screen that the batch is being retrieved. The batch will be downloaded to the following location: C:\msdchem\1\ECM\Retrieve\”batch name”

DATA REVIEW

There are three levels of review; the first level of review is the transference and processing of the raw data, this may be performed by any trained analyst; the second level of review is performed by an experienced analyst who is trained and signed off in data review, he / she will review the processed data; the third level of review is considered the final level of review, this can only be performed by the Laboratory Managers. He/she will review the data for the entire case ensuring that screening, confirmatory and quantitative analysis on the case have been completed and reported accurately. As needed, he/she will also schedule additional analysis and contact the Medical Examiner on the case to discuss any findings and / or review case history.

LOAD METHOD AND BATCH – FIRST LEVEL REVIEW

1. On the left screen under the C drive, open C:\msdchem\1\ecm\retrieve
2. Under retrieve, click on the batch that was retrieved. When all files have been downloaded to the processing computer, verify that the appropriate method is present in the batch.
3. To load the method, right click on the method under the batch being processed and select load. This will bring up “Be sure changes are saved. Load now?” Click yes. If the method is not present, load the method by retrieving the method from ECM.
4. Click on any file in the batch to load it.

PERFORM BATCH CALIBRATION – PROCESSING – FIRST LEVEL REVIEW

Under enhanced data analysis:

1. Process the calibrators. Select Tools from the toolbar, DoLIST, and Quant, No Report (QT 1). Press Add, and OK. Select the files for this action to be performed on, in this case, calibrators only. Verify that the selected files are located in the correct subdirectory. Change the path if necessary. Click the → Arrow and Process..
2. Review the integrations of the targeted compounds for each calibrator, checking that the ion peaks are present and integrated correctly (i.e. the baseline is the most scientifically accurate one that can be drawn). Select View from the toolbar, QEDIT. Answer appropriately when prompted to save changes made to quantitation results when moving from file to file. Return to Data Analysis by selecting View from the toolbar, return to Data Analysis.
3. Update the existing calibration table (all levels). Select Calibrate, Update, Quick Levels Update. When prompted to clear responses, select YES. When asked to requant files before update, select NO. Select single data file/level option. Select the appropriate data file to associate with calibration level 1 (10.0 ng/mL). Click OK. Repeat for remaining calibration levels (25.0, 50.0, 100.0, 500.0, 1000.0 ng/mL). Select level 3 when prompted to update retention times.
4. Load the file associated with level 3 (50 ng/mL), by selecting File, Load Data File. Select Calibrate, Update One Level. Do NOT requant. Select Update One Level, select only Replace Qualifier Ion Relative Responses, and choose the corresponding existing level ID (#3). Click Do Update.
5. Review the Compound database. Double click on the internal standard listed on the left to reveal the compounds quantitated with it. Select the calibration tab to reveal compound responses, calibration curves, and r^2 . To disable a point on the calibration curve for a compound, delete its response from the table. Click OK or Cancel when review is complete
6. Save Method before proceeding. Select Method from the toolbar, Save method, make sure that the path is correct. Save to OpenLab ECM at this time. On Update Calibration screen, select Update Level. Then click on Responses and Replace and on Retention Times and Replace. Then under Existing level ID, select the cal level to update and click on Do Update.
7. Requantitate the calibrators with the updated calibration curve. Select Tools from the toolbar, DoLIST, Requant, no report (QT 2), Add, and OK. Remove any existing commands. Select files to process. Click the → Arrow and Process. Review with QEDIT. Check the responses, retention times and ion ratios.
8. Regression correlation coefficient (r^2) for each analyte must be equal to or greater than 0.99.
9. Process controls and cases. Select Tools from the toolbar, DoLIST, Quant, No Report (QT 1), Add, and OK. Select appropriate files. Click the → Arrow and Process. Review with QEDIT. The blank must not contain detectable amounts of target analytes or significant interfering peaks.
10. When review is complete, return to Data Analysis. Select report format by choosing Quantitate from the toolbar, Report Options. Check SIM style report and uncheck Internal Standards. Press OK.
11. To print reports, select Tools from the toolbar, DoLIST, Profile Quant w/o Calculations (QT 0,1,'P'), Add, and OK. Select files to print, click the → Arrow and Process.

12. Print the calibration table for the current batch by clicking Calibrate on the command line. Select List, Calibrate Report and click OK. The Calibration report will print to the screen. Review the r^2 values, then right click on the screen report to print it.
13. Save files to ECM. Select ECM from the toolbar, select "Save multiple data files to ECM". Select all files.
14. Save method to ECM. Select ECM from the toolbar, Save Method to ECM. Make sure data path is correct.

BATCH CLEAN UP

1. Select my computer. Find the batch on the C drive at C:\msdchem\1\ecm\retrieve\batch. Right click on the batch to be deleted and select delete. Do not delete a batch that has not been successfully uploaded to ECM.
- 2.

PROCESSING SCREEN BATCHES

A single-point calibration at 500 ng/mL is used for screen batches. A 200 ng/mL control is used to verify the calibration. This control should be within $\pm 20\%$ of the target concentration. As the screen procedure is qualitative, if the analyte is detected in the control, that analyte can be reported as detected. If quantitation is required, the sample must be repeated in a quantitative batch. A 200 ng/mL oxazepam glucuronide control is used to verify hydrolysis. The hydrolysis control must quantitate the free drug, oxazepam, at the target concentration $\pm 20\%$. The r^2 criterion is not used. Check that the ion peaks are present and integrated correctly (e.g., that the baseline is the most scientifically accurate one that can be drawn), that the ion ratios are $\pm 20\%$ of the average of the calibrator, that the peaks are $\pm 2\%$ of the calibrator retention times and the peaks meet chromatography criteria (e.g., Gaussian). The negative control must not contain detectable amounts of target analytes.

ACCEPTANCE CRITERIA

1. Review the entire batch, checking that the ion peaks are present and integrated correctly (e.g., that the baseline is the most scientifically accurate one that can be drawn), that the ion ratios are $\pm 20\%$ of the average of the calibrator, that the peaks are $\pm 2\%$ of the calibrator retention times and the peaks meet chromatography criteria.
2. **Note:** *In some instances ratios will be off in exceptionally low or high concentrations. The operator must evaluate this and schedule proper dilutions or other methodologies, as needed. See REPORTING section. Acceptance range for calibrators is $\pm 20\%$ of the target. Maximum two out of six calibrators may be dropped if outside of the acceptable range. However, the remaining acceptable calibrators must be re-processed and quantitative values for cases reported within the dynamic range of the acceptable calibration range.*
3. The negative control must not contain detectable amounts of target analytes or significant interfering peaks. The blood controls must be within $\pm 20\%$ of the target value. For tissues, the controls are acceptable up to $\pm 30\%$.

4. The analyst shall choose from the standard levels a level at their discretion. All internal standard areas/peak heights for that sequence shall be compared to the internal standard(s) in that level. The acceptance criteria for the internal standard recovery/response shall be $\pm 30\%$ of the selected internal standard. If the internal standard recovery/response is outside these limits the specimen shall be repeated unless the analyte in question is not related to the internal standard that is outside the acceptable limits. If the internal standard is still unacceptable in the repeat analysis in the same way (i.e. both times too high or both times too low), then the sample shall be repeated a third time using a "blank and spike" method. The "blank and spike" run shall be viewed as acceptable if the following conditions are met: All standards and QC materials are within acceptable ranges, the "blank and spike" samples internal standards are within $\pm 20\%$ of their average responses, the calculated amount of the spiked sample is $\pm 20\%$ of the theoretical amount. If any of these conditions are not met than consult with a toxicology manager for further direction.

Make copies of all controls, the r^2 report and the sequence list, enough to attach a set to each case in the batch.

SECOND LEVEL REVIEW

The Second Level Reviewer will review the processed data in its entirety according to the acceptance criteria section of this SOP.

REPORTING After the batch has undergone second level review and has been printed, either the first level reviewer or the second level reviewer may report the data in the appropriate case file. **Report using the following guidelines:**

1. Each case printout must have a copy of the sequence and all controls appended.
2. Concentrations greater than or equal to 10 ng/mL are reported in ng/mL. Results are truncated and reported in whole numbers (i.e., 12.71 ng/mL is reported as 12 ng/mL), Concentrations less than 10.0 ng/mL but meeting all other criteria are reported as "less than 10.0 ng/mL". If the drug is not detected or the criteria are not met, the drug is reported as "not detected".
3. Sample concentrations greater than the highest acceptable calibrator must be re-extracted with suitable dilution(s) to bring the concentration below the ULOL.
4. Review other findings in the case, especially GCMS and GC results to see if they are consistent with these GCMS findings. If there are discrepancies, schedule additional testing to resolve them. If in doubt, consult with a supervisor.
5. If the positive matrix matched controls are greater than $\pm 30\%$ of target for non-blood matrices, the samples may be reported qualitatively, as "detected". If quantitative results are needed, the sample must be re-extracted.
6. When clonazepam and or 7- aminoclonazepam is detected in the urine or blood sample, schedule the case for quantitation by LCMS.
7. When nordiazepam is detected in the blood by GCMS SIM, the blood sample is scheduled for basic screen to rule out chlordiazepoxide. If chlordiazepoxide is detected, the blood

sample is quantitated by LCMS for nordiazepam and a send out may be necessary for quantitation of chlordiazepoxide.

8. Compounds detected in urine are reported as "detected". If quantitative results are required in a urine sample, it must be repeated in a quantitative batch.
9. Submit the case file with all reports for Third Level Review.

THIRD LEVEL REVIEW (FINAL REVIEW)

The third and final level review can only be performed by the Laboratory Managers. He/she will review the data for the entire case according to all established criteria. They will ensure that screening, confirmatory and quantitative analysis on the case have been completed and reported accurately. As needed, they will also schedule additional analysis and contact the Medical Examiner on the case to discuss any findings and / or review case history.

REFERENCES

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SPEware Corp. Cerex Applications Manual.

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REVISION HISTORY

- | | |
|----------------|--|
| Ver 03.08.2013 | 1. Revision history implemented. |
| Ver 04.20.2015 | 1. Changed validation of buffer
2. Changed drying time and pressure
3. Added dilution qc
4. Added usage log to LIMS
5. Scheduling clonazepam,7-aminoclonazepam,nordiazepam for LCMS. |
| Ver 08.31.2015 | 1. Added Group 1 and Group 2 analyte information.
2. Added internal standard criteria.
3. Defined the level of review(s) |

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