

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

**Nordiazepam (“Panel 2”)
by Solid Phase Extraction and
Liquid Chromatography/Mass Spectrometry
(Selected Ion Monitoring)
in Blood or Serum**

PRINCIPLE

The purpose of this assay is to quantitate Nordiazepam and Oxazepam where Chlordiazepoxide has been detected by another laboratory technique, or where Chlordiazepoxide is indicated by case history. Chlordiazepoxide and/or one of its metabolites are benzodiazepine compounds that undergo thermal degradation to Nordiazepam and Oxazepam when derivatized by MTBSTFA and analyzed by either GC or GCMS, resulting in a falsely elevated concentration of Nordiazepam and Oxazepam. Nordiazepam and Oxazepam are extracted from blood or serum using 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 the elution of drugs from the column. The eluate is evaporated and reconstituted in LC/MS mobile phase. The resulting solution is analyzed by LC/MS.

Quantitative analysis is performed by SIM LC/MS using a five point calibration curve, with a matched deuterated internal standards, Oxazepam-d5 and Nordiazepam-d5.

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
Serum	1.0 mL of the undiluted specimen

REAGENTS AND MATERIALS

Chemicals should be analytical reagent (AR) grade or higher. The chemical reagents required for the extraction procedure are prepared as indicated.

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

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. **Acetonitrile** Fisher Scientific or equivalent.
6. **KHCO₃** Certified ACS. Fisher Scientific or equivalent.
7. **K₂CO₃** Certified ACS. Fisher Scientific or equivalent.
8. **Sodium acetate trihydrate** Certified ACS. Fisher Scientific or equivalent.
9. **Glacial acetic acid** Certified ACS. Fisher Scientific or equivalent.
10. **Negative Blood, Serum** Calf or sheep blood obtained from outside source or equivalent. Sodium fluoride is added as a preservative, and stored frozen (-10°C or lower). Human plasma/serum obtained from outside source or equivalent, and stored frozen (-10°C or lower). All matrices are validated as negative by in-house analysis.
11. **Trace-J Solid Phase Extraction Column**, SPEware.
12. **System 48 Processor**, connected to nitrogen source.
13. **Waste Rack, SPE Rack, Collection Tube Rack**.
14. **Concentrator (Turbovap or SPEware)**, connected to a nitrogen source.
15. **Sonicator**
16. **Vortex**
17. **Centrifuge**
18. **pH Meter**
19. **Vacuum filtration apparatus**
20. **Laboratory glassware as needed**
21. **HPLC Columns** Agilent Zorbax Eclipse XDB-C18 Narrow-Bore 2.1x150mm (5 micron).
22. **100 mM Sodium Acetate buffer (pH 4.5).**

Dissolve 11.72 g sodium acetate trihydrate in 1000 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: in glass; room temperature.
Stability: at least 6 months; inspect daily before use for contamination.
23. **pH 9.0 buffer**

Dissolve 40 g KHCO₃ in 800 mL DI H₂O; add 20 g K₂CO₃. Dilute to 2000 mL using DI H₂O. Mix. Adjust pH to 9.0 ± 0.1 with KHCO₃ to raise the pH, or K₂CO₃ to lower the pH. Storage: in glass; room temperature. Stability: at least 1 month; inspect for contamination each day of use.

24. **Elution solvent:** Ethyl Acetate:NH₄OH (98:2). Prepare fresh each day of use.

25. **Calibrators and controls:**

Oxazepam	1.0 mg/mL in acetonitrile
Nordiazepam	1.0 mg/mL in acetonitrile

A. 10 mg/L "Panel 2" 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 "Panel 2" Calibrator and Control

Pipet 10 mL 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.

C. UTAK Benzodiazepines Plus 100 ng/mL (Whole Blood Toxicology Control)
Prepare and store as per manufacturer's insert.

26. **Internal Standards:**

Reference Standard is obtained in a 1.0 mL ampule from Cerilliant or equivalent.

Oxazepam-d ₅	1.0 mg/mL in acetonitrile
Nordiazepam-d ₅	1.0 mg/mL in acetonitrile

A. 10.0 mg/L "Panel 2" internal standard

Pipet 1 mL of 1.0 mg/mL stock of internal standard into a 100 mL volumetric flask. Dilute to mark with acetonitrile. Transfer working internal standard solution to properly labeled containers.

27. **LCMS Mobile Phases**

Water (Fisher Scientific LCMS Optima, or equivalent)

Methanol (Fisher Scientific LCMS Optima, or equivalent)

Acetonitrile (Fisher Scientific LCMS Optima, or equivalent)

Ammonium Acetate (Crystalline; HPLC grade or equivalent)

TFA (Fisher, for Peptide/Protein Analysis)

“A” 1 mM Ammonium Acetate

1. Add 0.154 g ammonium acetate to a 2 L volumetric flask.
2. Add approximately 800 mL of water to the 2 L flask and mix.
3. Add 400 µL of TFA to the flask and mix.
4. Add 100 ml (5%) methanol to the flask and mix.
5. Q.S. to 2L mark with water and mix.
6. Filter the solution using vacuum filtration apparatus.
7. Transfer filtered mobile phase into an appropriate storage container.

“B” 2 mM Ammonium Acetate

1. Add 0.154 g ammonium acetate to a 1 L volumetric flask.
2. Add 500 mL of methanol to the 1 L flask and mix.
3. Add 200 µL of TFA to the flask and mix.
4. Q.S. to 1L mark with acetonitrile and mix.
5. Filter the solution using vacuum filtration apparatus.
6. Transfer filtered mobile phase into an appropriate storage container.

EXTRACTION PROCEDURE FOR BLOOD AND SERUM

1. Aliquot 1 mL of validated negative matrix into each 16 x 125 mm tube labeled calibrator(s) or control(s). Aliquot 1 mL of sample into each appropriately labeled 16 x 125 mm tube.2. Five calibrators are prepared as follows using negative matrix:

20 ng/mL -	add 20	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 10	µL of 10 mg/L calibrator solution
500 ng/mL -	add 50	µL of 10 mg/L calibrator solution
1000 ng/mL -	add 100	µL of 10 mg/L calibrator solution

Mix by Vortex.

3. Positive controls are prepared using negative matrix as follows:

10 ng/mL	Add 10	µL of 1.0 mg/L control solution
200 ng/mL	Add 20	µL of 10.0 mg/L control solution

5. A matrix matched negative control must be included for each matrix type in the batch.
6. For blood batches, an external control (UTAK Benzo Plus 100) is included. The approximate value of this control is 100 ng/mL.
7. Add 50 µL of 10 mg/L working internal standard solution to all tubes. The concentration of the internal standard in each sample is 500 ng/mL.

8. Add 2 mL 100 mM sodium acetate buffer pH 4.5. Additional buffer (up to 4 mL total) can be used for complex matrices.
9. Mix by vortex for 30 seconds.
10. Sonicate for 15 minutes.
11. Centrifuge sample for 20 minutes at 3000 rpm.
12. Trace J extraction column prewash
 - Pour 2 mL Ethyl Acetate onto column, flow by gravity (slight pressure if needed).
 - Pour 2 mL Methanol onto column, flow by gravity (slight pressure if needed).
 - Dry at 25 psi for 15 sec.
13. Decant the supernatant into the Trace-J column and apply nitrogen at a pressure of 2-4 psi.
14. Wash Column (with optional pressure to achieve 1-2 mL/min).
 - Pour 2 mL pH 9.0 buffer onto column.
 - Pour 2 mL DI H₂O onto column.
 - Dry column for 20 minutes at 60 psi.
15. Prepare Elution Solvent
 - Ethyl Acetate/NH₄OH (98/2).

Note: Prepare elution solvent each day of use. Mix well.

16. Elute Drugs
 - Place labeled 10 mL conical centrifuge tubes under each column to collect eluate by gravity. Elute with 2 mL.
17. Dry under nitrogen at 40 °C to absolute dryness.
18. Reconstitute with 100 µL of 65/35% mobile phase mixture (1A/1B). Mix by Vortex.
19. Centrifuge 10 minutes at 3000 rpm.
20. Label autosampler vials indicating aliquot and toxicology number (eg. 1-YY-xxxx), specimen type, dilution, analyst and date.
21. Transfer reconstituted extract to an insert placed in a labeled autosampler vial. Cap immediately to avoid possible contamination from other samples. Do not wait until all transfers have been made to seal the vials. Samples are ready for MS injection.
22. Create batch sequence as specified in Instrument Setup.
23. Enter the date extracted 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 LCMSD series 1100, with 1100 HPLC, G1313A Autosampler, and Agilent Chemstation with appropriate software. The method name for the analysis is LCMS1BZPL2A. This method

utilizes the Agilent ZORBAX Eclipse XDB-C18 Narrow-Bore 2.1x150mm (5 micron) analytical column.

The following ions are monitored for each drug:

Oxazepam-d ₅	236, 246
Oxazepam	231, 241, 269
Nordiazepam-d ₅	276, 213
Nordiazepam	271, 208, 243

TOP LEVEL PARAMETERS

Method Information For: C:\CHEM\1\METHODS\LCMS1BZPL2A.m

Run Time Checklist:

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

Method Comments:

1100 High Pressure Gradient Pump 1

Control	
Column Flow	0.500 ml/min
Stoptime	15.00 min
Posttime	3.00 min
Solvents	
Solvent A 1	65%
Solvent B 1	35%
Pressure Limits	
Minimum Pressure	0 bar
Maximum Pressure	400 bar
Auxiliary	
Maximal Flow Ramp	100.00 ml/min ²
Compressibility A	50*10 ⁻⁶ /bar
Minimal Stroke A	Auto
Compressibility B	115*10 ⁻⁶ /bar
Minimal Stroke B	Auto

Store Parameters	
Store Ratio A	Yes
Store Ratio B	Yes
Store Flow	Yes
Store Pressure	Yes

Timetable		
Time	Solv.B	Flow
0.00	35.0	0.500
11.00	44.5	0.500
13.00	100.0	

Agilent 1100 Diode Array Detector 1

Signals			
SIGNAL	STORE	SIGNAL,BW	REF,BW [NM]
A:	YES	254 4	250 100
B:	NO	254 16	360 100
C:	NO	210 8	360 100
D:	NO	230 10	500 100
E:	NO	300 100	500 100

Spectrum	
Store Spectra	All
Range from	190 nm
Range to	325 nm
Range step	2.00 nm
Threshold	1.00 mAU
Time	
Stoptime	As Pump
Posttime	Off
Required lamps	
UV lamp required	Yes
Vis lamp required	No
Autobalance	
Prerun balancing	No
Postrun balancing	No
Margin for negative Absorbance	100 mAU
Peakwidth	> 0.05 min
Slit	4 nm
Analog Outputs	
Zero offset ana. out. 1	5%
Zero offset ana. out. 2	5%
Attenuation ana. out. 1	1000 mAU
Attenuation ana. out. 2	1000 mAU
Timetable is empty	

Mass Spectrometer Detector

General Information	
Use MSD	Enabled
Ionization Mode	API - ES
Tune File	Atunes.tun
StopTime	13.50
Time Filter	Enabled
Data Storage	Full
Peakwidth	0.20 min

Signals					
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{Signal 1}					
Polarity	Positive				
Fragmentor Ramp	Not applicable				
Percent Cycle Time	90%				
SIM Parameters					
Time (min)	Group Name	Sim Ion	Fragmentor	Sim Resol.	Actual Dwell
3.00	Group 1	193.00	240	High	49
		208.00	240		49
		213.00	240	High	49
		228.00	240		49
		231.00	240		49
		233.00	240		49
		236.00	240		49
		238.90	240		49
		241.00	240	High	49
		243.00	240		49
		246.00	240		49
		255.00	240		49
		260.00	240		49
		269.00	240		49
		271.00	240		49
		276.00	240		49
		308.00	240		49
		312.90	240		49
		314.90	240	High	49
		330.90	240		49
		358.90	240		49
10.50	Group 2	193.00	250		212
		257.00	250		212
		262.00	250		212
		285.00	250		212
		290.00	250		212
[Signal 2]					
Polarity	Positive				
Fragmentor Ramp	Disabled				
Percent Cycle Time	10%				
Scan Parameters					
Time	Mass range low	Mass range hi	Fragmentor	Threshold	Step size
0.00	100.00	400.00	70	150	0.10

Spray chamber					
[MSZones]					
Gas temp	350 ° C		Max temp	350 ° C	
Drying Gas	11.0 L/min		Max DryGas	13.0 L/min	
Neb Pres	45 psig		Max Pres	60 psig	
VCap (Positive)	3500 V				
VCap (Negative)	3500 V				

Gain is a dynamic variable, and should be set accordingly as needed for analysis.

END OF MS ACQUISITION PARAMETERS

FIA Series

FIA Series in this Method : Disabled

Agilent 1100 Autosampler 1

Injection	
Injection Mode	Standard
Injection Volume	5.0 µL
Auxiliary	
Drawspeed	200 µL/min
Ejectspeed	200 µL/min
Draw Position	0.0 mm
Time	
Stoptime	As pump
Posttime	Off

Agilent 1100 Column Thermostat 1

Temperature settings	
Left temperature	40 ° C
Right temperature	Same as left
Enable analysis	When Temp is within setpoint ± 0.8 ° C
Store left temperature	Yes
Store right temperature	No
Time	
Stoptime	As pump
Posttime	Off

Column switching valve	Column 1
Timetable is empty	

END OF INSTRUMENT CONTROL PARAMETERS

INSTRUMENT SETUP

An acceptable Checktune must be obtained each day that samples are run. Refer to the SOP entitled "LCMS Autotune" for instructions.

Prepare a sequence using the following steps.

1. Click on the Easy Sequence Setup tab.
2. Load an appropriate Easy Sequence Setup template. If one does not exist, see #16.
3. Click on the "Samples" tab.
4. Edit the number of samples to be run (include all blanks, cases, controls, and control reinjections).
5. Edit the data file name under Data Information. The data file should read LCMS#mmddX<c>. # represents instrument number. X indicates the batch being run (e.g. A, B, C). Reset the counter.
6. Edit the sequence name under Sequence Information. The sequence should read LCMS#mmddyX<c>.
7. Save the updated easy sequence template.
8. Now, click on the Easy Sequence tab. Open the Easy Sequence Setup that was previously modified and saved (.est).
9. Click "Fill samples".
10. Fill in sample names and information. Indicate lot numbers, dilutions and any comments as needed for calibrators, blanks, controls and cases.
11. Save Easy Sequence as LCMS#mmddyX.es.
12. "Preview/Print Sequence" for loading of vials. Using the printed Sequence List, verify that the vials are loaded in their correct location.
13. Verify Sequence Queue is in play mode. Save and add Easy Sequence to the queue.
14. The complete sequence cannot be printed correctly before the sequence is started. Once the sequence is running, click "Sequence" from the main toolbar, then "Print Sequence". Select Sequence Parameters, Sample Info Part, and Method and Injection part. Click Print.
15. Date and initial the chain of custody label on the sequence printout, listing any comments, transfers or exceptions.
16. If an easy sequence template must be created or modified, follow these steps.

- a) Click on the calibration tab.
- b) Select cyclic calibration mode.
- c) Click on calibrant icon, drag and drop into sequence start box.
- d) Fill out calibrant information (vial info, injections/vial, calibration level). Select No Update for both response factor and retention time.
- e) Repeat for each calibrator.
- f) Enter Calibration Interval as 50 (or > number of injections needed for entire batch). The interval unit is injections.
- g) Drag blank icon to sequence end box.
- h) Review Easy Sequence template overview.
- i) Save Easy Sequence Setup template.
- j) Return to step 3 and continue through #15.

DATA TRANSFER

1. PARSE DATA

- a. After the batch acquisition has completed, the raw data files will reside on the local Chemstation and OpenLab ECM.
- b. Go to the acquiring instrument and log into a session of Enhanced Data Analysis. Select "Custom tool 3". Highlight all files from the appropriate subdirectory, changing the path if necessary. Data files are located in C:\Chem32\1\data. Click the → Arrow and Process. This custom tool parses the three types of data (SIM, Scan, and UV-VIS) into two separate batches (SIM/Scan and UV) and uploads them to ECM.

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 Manager. 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 finding and/or review case history

ANALYSIS – FIRST LEVEL REVIEW

PROCESSING USING ENHANCED DATA ANALYSIS

1. Log into a session of Enhanced Data Analysis using analyst's OCME login for the username and their password.

2. Download only the SIM/Scan data files for processing and reviewing.
 - a. Under ECM toolbar, click Retrieve Entire Sequence from ECM.
 - b. Double click LCMS, the appropriate instrument, month, and subdirectory (LCMS#MMDDYYXms) and click OK.
 - c. Data Files will download to the local processing terminal. The progress of this transfer will appear at the bottom of the data analysis screen. If the operation is cancelled, log in to OpenLab ECM again and repeat step 2.
 - d. Once the retrieval is complete, the batch folder is located in the C:\msdchem\1\DATA\ECM\retrieve folder.
3. A SIM GCMS method is used to process data, rather than the LCMS acquisition method. An appropriate processing method is located in the Method folder under the monthly folder of the appropriate acquiring instrument.
 - a. Select Load Method from the ECM drop down menu. Select the method from the Method folder in the monthly folder of the appropriate acquiring instrument. Click OK.
 - b. Save this method to the processing terminal by selecting Save method from the Method drop down menu. The path should be C:\msdchem\1\ecm\retrieve\LCMS#MMDDYYXms. Additionally, save to the correct location in OpenLab ECM.
4. 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.
5. Review the peak assignments of the targeted compounds for each calibrator checking that the ion peaks are present and integrated correctly (i.e., correct peak based on retention time is selected, and 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.
6. 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 (20 ng/mL). Click OK. Repeat for remaining calibration levels (50, 100, 500, 1000 ng/mL). Select level 3 when prompted to update retention times.
7. Load the file associated with level 3 (100 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.
8. 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.
9. Save Method locally before proceeding. Select Method from the toolbar, Save method, make sure that the path is correct. Do not save to OpenLab ECM at this time.

10. 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. Evaluate the responses, retention times and ion ratios in accordance with acceptance criteria.
 - a.) Peak shape should be Gaussian or symmetrical.
 - b.) Retention times are within $\pm 5\%$ of the analyte's retention time determined by the midpoint calibrator.
 - c.) Baseline is the most scientifically accurate one that can be drawn.
 - d.) Ion ratios must be within $\pm 20\%$ of the target ion ratio, as determined by the midpoint calibrator.
 - e.) Acceptance range for calibrators is $\pm 20\%$ of the target concentration for blood matrix, $\pm 30\%$ of the target concentration for non-blood matrices.
 - f.) Maximum two out of five 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.
11. Regression correlation coefficient (r^2) for each analyte must be equal to or greater than 0.99.
12. Process controls and cases. Select Tools from the toolbar, DoLIST, Quant, No Report (QT 1), Add, and OK. Select remaining files. Click the → Arrow and Process. Review with QEDIT. Review the peak assignments of the compounds, following Step 10, a-d. Verify multipliers/dilution factors are applied.
13. 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.
14. To print reports, select Tools from the toolbar, DoLIST, Full Quant w/o Calculations (QT 0,1,'F'), Add, and OK. Select files to print, click the → Arrow and Process.
15. 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.
16. Save files to ECM. Select ECM from the toolbar, select "Save multiple data files to ECM". Select all files.
17. Save method to ECM. Select ECM from the toolbar, Save Method to ECM. Make sure data path is correct.
18. Submit all processed data, including calibrators, negative and positive controls and cases for Second Level Review.
19. Batch cleanup 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.

SECOND LEVEL REVIEW

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

NOTE: To determine whether peaks have been properly identified and integrated, the processed data is readily accessible and can be viewed in ChemStation software by the second reviewer should additional analysis of the data be necessary. In addition, raw data is always available and readily accessible and can be viewed in ChemStation software if re-processing is necessary.

NOTE: If for any reason re-analysis is performed on the processed data (i.e. baseline edited, peak deleted, peak identified that was not previously identified, etc.) a copy of the processed data file should be created; the saved file will be termed "Version 3".

Upon completion, make sufficient copies of the controls (in-house and external), calibration report, and the sequence list, enough to attach a set for each case in the batch.

REPORTING

After the batch has undergone second level review, it must be reported by a secondary reviewer, using the following guidelines:

NOTE: Should a suitable second level reviewer not be available, consult a supervisor or manager.

1. Each case printout must have a copy of the sequence and all controls appended.
2. Blood and serum results are reported in ng/mL.
3. Concentrations greater than or equal to 10 ng/mL are reported in ng/mL.
4. Concentrations lower than 10 ng/mL but greater than 5 ng/mL and meeting all other criteria are reported as < 10 ng/mL.
5. Concentrations lower than 5 ng/mL are reported as "not detected". If the drugs are not detected or the reporting criteria are not met, the drug is reported as "not detected".
6. Sample concentrations greater than the highest acceptable calibrator must be re-extracted with suitable dilution to bring it within the limits of the calibration curve.
7. Schedule repeat or confirmatory analysis as needed.
8. Review other findings in the case, e.g. immunoassay and GCMS results, to see if they are consistent with the LCMS findings. If there are discrepancies, schedule additional testing to resolve them. If in doubt, consult with a supervisor.

9. If the positive matrix matched controls are greater than $\pm 30\%$ of target for blood matrices, the samples may be reported qualitatively, if they are “not detected”. If quantitative results are needed, the sample must be re-extracted.

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.

ACCEPTANCE CRITERIA – APPLIES TO ALL LEVELS OF REVIEW

For all samples:

Peak shape should be Gaussian or symmetrical.

Retention times are within $\pm 5\%$ of the analyte’s retention time determined by the midpoint calibrator.

Baseline is the most scientifically accurate one that can be drawn.

For calibrators:

Peak shape should be Gaussian or symmetrical.

Retention times are within $\pm 5\%$ of the analyte’s retention time determined by the midpoint calibrator.

Baseline is the most scientifically accurate one that can be drawn.

Acceptance range for calibrators is $\pm 20\%$ of the target concentration for blood/serum matrices.

Ion ratios must be within $\pm 20\%$ of the target ion ratio, as determined by the midpoint calibrator.

Maximum two out of five 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.

For positive controls:

Peak shape should be Gaussian or symmetrical.

Retention times are within $\pm 5\%$ of the analyte’s retention time determined by the midpoint calibrator.

Baseline is the most scientifically accurate one that can be drawn.

Blood controls must be within $\pm 20\%$ of the target value.

Ion ratios must be within $\pm 20\%$ of the target ion ratio, as determined by the midpoint calibrator.

For negative controls:

Must not contain detectable amounts of target analytes or significant interfering peaks.

For case samples:

Ion ratios

Ion ratios must be within $\pm 20\%$ of the target ion ratio, as determined by the midpoint calibrator, in blood/serum matrices.

- a) It is acknowledged that ion ratios of LCMS fragments may be less stable and can be concentration dependent. For compounds at low concentrations, acceptable ion ratios can be extended to $\pm 30\%$ of the target value. Ion ratios that are $\pm 20\%$ of the nearest calibrator or lowest QC may also be accepted, provided that all other chromatographic criteria are met.
- b) Sometimes ion ratios will be out in cases of coelutions. The analyst must evaluate this, and determine other appropriate actions as needed.

Internal standard monitoring

For quantitative analyses, internal standard responses that are less than 50% or greater than 150% relative to calibrators or controls must be evaluated carefully.

A low internal standard response can be due to matrix, which can be addressed, when sample volume allows, by re-extracting with a dilution (i.e. testing less matrix) and/or adding extra buffer to the case sample pre-extraction.

A high internal standard response, greater than 150% of a calibrator or control can be the result of a double spiking error, which can be determined by evaluating the other internal standard responses. In this case, re-extract the sample.

When sample volume does not allow for repeat analysis, report results for the affected analyte qualitatively, as detected or not detected.

Note: Multiple isotopically labeled internal standards are fortified as part of a pool and some internal standard responses may not meet acceptance criteria. In the event that an affected internal standard is associated with an analyte that is not detected, the case sample does not have to be re-extracted.

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REVISION HISTORY

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|------------|---|
| 03.09.2015 | <ol style="list-style-type: none">1. New analytical method.2. Transfer of methodology from GCMS to LCMS when Chlordiazepoxide is either suspected or confirmed. |
| 04.17.2015 | <ol style="list-style-type: none">1. Addition of dilution control language. |
| 09.14.2015 | <ol style="list-style-type: none">1. Incorporated First, Second, Third level review language.2. Added Internal standard monitoring language.3. Removed dilution control language.4. Quantitative analysis of Oxazepam added to method. |

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