

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

**CLENBUTEROL
by
LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

PRINCIPLE

Clenbuterol, is a sympathomimetic amine used by sufferers of breathing disorders (such as asthma) as a decongestant and bronchodilator. It is most commonly available as the hydrochloride salt.

Clenbuterol is a β_2 agonist with structural and pharmacological similarities to epinephrine and salbutamol. It differs in its effects in that it is more potent and longer-lasting as a stimulant and thermogenic (drugs which increase heat through metabolic stimulation) drug. It causes an increase in aerobic capacity, central nervous system stimulation, blood pressure, and oxygen transportation. It increases the rate at which body fat is metabolized while increasing the body's BMR.

There have been reports where persons have tested positive for clenbuterol after having consumed livestock that was illicitly treated with clenbuterol. It has recently become a drug of abuse.

This method is used to confirm the presence of clenbuterol in biological samples. The method uses a small sample volume fortified with methapyrilene which is used as the internal standard. Clenbuterol is extracted from biological matrices by solid phase extraction. The eluate is evaporated and then reconstituted with LC/MS mobile phase. The resulting solution is analyzed by liquid chromatography/mass spectrometry (LC/MS). Quantitative analysis is performed using a six point calibration curve.

SAFETY

The handling of all biological specimens and reagents is performed within the guidelines which are detailed in the Safety and Health manual.

SPECIMEN PREPARATION

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

Blood	2.0 mL of the undiluted specimen
Urine	1.0 mL of 1:5 dilution for qualitative identification
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	2.0 mL of the undiluted specimen

DILUTION OF SPECIMENS

Specimens are diluted as follows:

Urine 1:5	1.0 mL of urine + 4.0 mL of deionized water
Brain 1:3	5.0 g of brain homogenized with 10 mL of deionized water.
Liver 1:5	5.0 g of liver homogenized with 20 mL of deionized water.
Gastric 1:10	2.0 mL of liquid <i>q.s.</i> to 20 mL of deionized water, or 2.0 g of a solid specimen homogenized with 18 mL of deionized 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

All chemicals should be analytical reagent grade or better.

1. **Deionized water**
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 a pH 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. **100 mM phosphate buffer (pH 6.0)**
Dissolve 3.4 g Na₂HPO₄ and 24.28 g NaH₂PO₄ H₂O in 1600 mL DI H₂O.
Dilute to 2000 mL using DI H₂O. Mix. Adjust pH to 6.0 + 0.1 with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH).
Store at 5 °C in glass.
Stability: 1 month. Inspect each day before use for contamination.
5. **Eluting solvent, Ethyl acetate/NH₄OH (98/2)**. Prepare fresh each day of use.
6. **Ethyl Acetate**, Fisher Scientific Optima or equivalent.
7. **Acetic Acid**, Glacial, HPLC grade. Fisher Scientific or equivalent.
8. **Acetic Acid, 1.0 M**
To 400ml deionized H₂O add 28.6 ml glacial acetic acid. Dilute to 500 ml with deionized H₂O.
Store at room temperature in a glass container.
Stability: 6 months. Inspect daily before use for evidence of contamination.
9. **Acetic Acid, 100 mM**
Dilute 40 mL of 1.0 M acetic acid to 400 mL with deionized H₂O.
Store at room temperature in a glass container.
Stability: 6 months. Inspect daily before use for evidence of contamination.
10. **pH 9.0 buffer**
Dissolve 20 g KHCO₃ in 800 mL DI H₂O; add 10 g K₂CO₃. Dilute to 1 L using DI H₂O. Mix. Adjust pH to 9.0 ± 0.1 with KHCO₃ to raise the pH, or K₂CO₃ to lower the pH.
Store at room temperature in glass

Stability: 6 months. Inspect each day before use for contamination.

11. **Clenbuterol** hydrochloride, 95% or better Sigma-Aldrich, or equivalent.
12. **Negative blood, serum, brain, liver**
Calf or sheep blood obtained from outside source. Sodium fluoride is added as a preservative, and stored frozen (-10°C or lower). Human serum obtained from outside source or equivalent, and stored frozen (-10°C or lower). Calf brain and liver obtained from outside source or equivalent. Homogenized and stored frozen (-10°C or lower). All matrices are validated as negative by in-house analysis.
13. **Polycrom Clin II Solid Phase Extraction Columns**, CEREX
14. **System 48 Processor** connected to nitrogen source.
15. **Waste Rack, SPE Rack, Collection Tube Rack.**
16. **Concentrator (Turbovap or SPEWare)** connected to a nitrogen source.
17. **Vacuum Filtration Apparatus**
18. **HPLC Column**, Agilent Zorbax SB-C18 part # 823975-932, Rapid Resolution HT cartridge, 4.6 x 30 mm 1.8 micron.
19. **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)
Trifluoroacetic Acid (TFA) (Fisher, for Peptide/Protein Analysis)

1A 1 mM Ammonium Acetate

1. Add 0.077 g ammonium acetate to a 1 L volumetric flask.
2. Add approximately 400 mL of deionized water to the 1 L flask and mix.
3. Add 200 µL of trifluoroacetic acid to the flask and mix.
4. Add 50 ml (5%) methanol to the flask and mix.
5. Q.S. to 1L mark with deionized water and mix.
6. Filter the solution using vacuum filtration apparatus.
7. Transfer mobile phase back to the 1L volumetric.

1B 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 trifluoroacetic acid 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 mobile phase back to the 1L volumetric.

2B 50% Water/50% Methanol

1. Add 500 mL each of deionized water and methanol to a 1 L volumetric flask
2. Filter the solution using vacuum filtration apparatus.
3. Transfer the solution back to the flask.

CALIBRATOR AND CONTROL STOCK SOLUTIONS

Clenbuterol calibrator stock solutions

1. Weigh accurately 11.3 mg of clenbuterol HCl. Transfer to a 10 mL volumetric flask. Q.S. to mark with methanol. This is a 1.0 mg/mL (1,000 mg/L) solution.
2. Pipet 1.0 mL of the 1000 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 100 mg/L calibrator solution. Transfer to properly labeled headspace vial.
3. Pipet 1.0 mL of the 100 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 10 mg/L calibrator solution. Transfer to properly labeled headspace vial.
4. Pipet 5.0 mL of the 100 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 50 mg/L calibrator solution. Transfer to properly labeled headspace vial.
5. Pipet 1.0 mL of the 10 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 1.0 mg/L working calibrator solution. Transfer to properly labeled headspace vial.
6. Pipet 1.0 mL of the 1.0 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 0.1 mg/L working calibrator solution. Transfer to properly labeled headspace vial.
7. Seal all headspace vials upon completion of calibrator preparation.
Note: Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.
8. Store frozen at less than 0°C.

Clenbuterol control stock solutions

1. Weigh accurately 11.3 mg of clenbuterol HCl. Transfer to a 10 mL volumetric flask. Q.S. to mark with methanol. This is a 1.0 mg/mL (1,000 mg/L) solution.
2. Pipet 1.0 mL of the 1000 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 100 mg/L control solution. Transfer to properly labeled headspace vial.
3. Pipet 1.0 mL of the 100 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 10 mg/L control solution. Transfer to properly labeled headspace vial.
4. Pipet 1.0 mL of the 10 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 1.0 mg/L working control solution. Transfer to properly labeled headspace vial.
5. Pipet 1.0 mL of the 1.0 mg/L stock solution to a 10 mL volumetric flask. Q.S. to mark with methanol to produce 0.1 mg/L working control solution. Transfer to properly labeled headspace vial.
6. Seal all headspace vials upon completion of control preparation.
Note: Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.
7. Store frozen at less than 0°C.

INTERNAL STANDARD SOLUTION

Methapyrilene (1.0 mg/L in methanol) is used as the internal standard (IS) for the clenbuterol procedure.

Weigh 0.114 g methapyrilene HCl per 100 mL final volume (1000 mg/L) to produce a stock solution of 1000 mg/L. Store stock solution is stored frozen at -10⁰ C or lower and is stable for at least one year.

The stock 1000 mg/L solution is serially diluted to produce a working internal standard solution of 1.0 mg/L as shown below:

1. Pipet 10.0 ml of 1000 mg/L stock methapyrilene solution into a 100 mL volumetric flask. Q.S to mark with methanol to produce a 100 mg/L intermediate IS solution.
2. Pipet 10.0 ml of 100 mg/L intermediate IS solution into a 100 mL volumetric flask. Q.S to mark with methanol to produce a 10 mg/L intermediate IS solution.
3. Pipet 10.0 ml of 10 mg/L intermediate IS solution into a 100 mL volumetric flask. Q.S to mark with methanol to produce a 1.0 mg/L working IS solution.
4. Store intermediate and working solutions in appropriately labeled containers and store at 2-8 °C.

Note: Include identity, concentration, solvent, lot number, date prepared, and initials of analyst

EXTRACTION PROCEDURE (QUANTITATIVE)

1. Aliquot 2 mL of validated negative matrix in each tube labeled as calibrator or in-house control or sample into a 16 x 125 mm test tube labeled as to the contents.
2. Add appropriate amounts of standard calibrator solutions to negative matrix tubes as follows:

6 positive calibrators and one blank for each matrix present in the batch should be run with each batch of samples.

Positive calibrators are prepared as follows:

Calibrator 0.1 ng/mL - add 2 µL of clenbuterol calibrator solution 0.1 mg/L

Calibrator 0.25 ng/mL - add 5 µL of 0.1 mg/L working clenbuterol calibrator solution 0.1 mg/L

Calibrator 0.5 ng/mL - add 10 µL of clenbuterol calibrator solution 0.1 mg/L

Calibrator 0.75ng/mL - add 1.5 µL of clenbuterol calibrator solution 1.0 mg/L

Calibrator 1.0 ng/mL - add 2 µL of clenbuterol calibrator solution 1.0 mg/L

Calibrator 5.0 ng/mL - add 10µL of clenbuterol calibrator solution 1.0 mg/L

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

3. Add appropriate amounts of standard control solutions to properly labeled negative matrix tubes as follows:

2 positive controls and one blank for each matrix present in the batch should be run with each batch of samples. Positive controls are prepared as follows:

Quality Control 0.2 ng/mL - add 4 µL of clenbuterol control solution 0.1 mg/L

Quality Control 2.0 ng/mL - add 4 µL of clenbuterol control solution 1.0 mg/L

4. Add 10 µL of 1.0 mg/L working internal standard solution to all tubes. The concentration of the internal standard in each sample is 5 ng/mL
5. Add 4 mL 100 mM phosphate buffer pH 6.0. Mix by Vortex for 30 seconds then sonicate for 15 minutes. Centrifuge sample for 15 minutes at ≈ 3000rpm.
6. Decant the supernatant into the appropriately labeled Polychrom Clin II column and apply nitrogen at a pressure of 2-4 psi.
7. Wash Column (all wash steps are pressurized at 2-4psi).
 - Pour 2 mL pH 9.0 buffer onto column
 - Pour 4 mL 100 mM acetic acid onto column
 - Pour 2 mL CH₃OH onto column
 - Pour 4 mL ethyl acetate onto column
 - Dry column for 2 minutes at ≈ 25 psi

8. Elute Drugs
Place labeled 10 mL conical centrifuge under each column to collect eluate. Elute with 4 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 fresh elution solvent each day of use.

9. Dry under nitrogen at 25 °C to absolute dryness using concentrator.
10. Reconstitute with 100 µL of working mobile phase (75% 1A/25% 2B). Mix by Vortex. Transfer to an insert placed in an autosampler vial.
11. Label autosampler vials indicating aliquot and toxicology number (e.g., 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.
12. Transfer to auto-sampler vials for LCMS injection. Enter a copy of the sequence in the Sequence Logbook. Also enter the date completed in the Dataease database, so the samples are not duplicated by another analyst.

EXTRACTION PROCEDURE (QUALITATIVE)

Urine or other tissues may be screened for the presence of clenbuterol using a reduced sample volume, and extracting only one calibrator (single point), one blank for each matrix, and one control for each matrix. Any positive clenbuterol results must be repeated using the quantitative procedure. Screens are analyzed using the following modification of the procedure:

Sample preparation (usually urine)

1.0 mL of sample (urine) is diluted with 4.0 mL of deionized water. 1.0 mL of this 1:5 dilution is used for the sample volume. The single calibrator is fortified at 1.0 ng/mL, by adding 1.0 µL of clenbuterol calibrator solution 1.0 mg/L to 1.0 ml of deionized water. The control is fortified at 0.2 ng/mL by adding 2.0 µL of clenbuterol control solution 0.1 mg/L to 1.0 ml of deionized water. The matrix blank is 1.0 mL of deionized water. All samples are fortified with 5.0 µL of 1.0 mg/L methapyrilene working internal standard solution. 2.0 mL of 100 mM phosphate buffer pH 6.0 is added to all tubes in the batch in place of the 4.0 mL in step 5 above. Continue the procedure at step 5 as indicated above, except use ½ of the volume indicated for each step, until reconstitution with mobile phase, which is the same, at 100 µL. LCMS analysis is unchanged. When processing the data, the Target method is modified to include only one calibrator point, at 1.0 ng/mL, and otherwise processed as below.

INSTRUMENTATION

Agilent LCMSD series 1100, with 1100 HPLC, G1313A Autosampler, and Agilent Chemstation with appropriate software. The method name for this assay is LCMS1CLENBUT.M
The following ions are monitored for each drug:

Methapyrilene IS	262.1, 264.1
Clenbuterol	277.0, 278.1, 279.0, 281.0

Method Information For: C:\CHEM\1\METHODS\LCMS1CLENBUT.M

Run Time Checklist:

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

Method Comments:

Opiate method development
1100High Pressure Gradient Pump 1

Control	
Column Flow	0.400 ml/min
Stoptime	9.00 min
Posttime	5.00 min
Solvents	
Solvent A 1	75.0%
Solvent B 1	25%
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	20 µl
Compressibility B	115*10 ⁻⁶ /bar
Minimal Stroke B	20 µl
Store Parameters	
Store Ratio A	No
Store Ratio B	No
Store Flow	No
Store Pressure	No

Timetable			
Time	Solv.B	Flow	Pressure
1.00	25.0	0.400	
5.00	55.0	0.400	
6.00	100.0	0.400	
6.10	100.0	1.000	

Agilent 1100 Diode Array Detector 1

Signals			
SIGNAL	STORE	SIGNAL,BW	REFERENCE,BW [NM]
A:	YES	256 8	450 80
B:	NO	254 4	OFF
C:	No	380 60	OFF
D:	No	254 4	OFF
E:	No	254 4	OFF

Spectrum	
Store Spectra	EVERY 2 nd spectrum
Range from	200 nm
Range to	400 nm

Range step	2.00 nm
Threshold	2.00 mAU
Time	
Stoptime	7.00 min
Posttime	off
Required lamps	
UV lamp required	YES
Vis lamp required	YES
Autobalance	
Prerun balancing	YES
Postrun balancing	NO
Margin for negative	100 mAU
Peakwidth	> 0.1 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	7.00 min
Time Filter	Enabled
Data Storage	Condensed
Peakwidth	0.15 min
Scan Speed Override	Disabled

END OF MS ACQUISITION PARAMETERS

FIA Series

FIA Series in this Method : Disabled

Time Setting

Signals						
{Signal 1}						
Polarity	Positive					
Fragmentor Ramp	Not applicable					
SIM						

Parameters						
Time (min)	Group Name	Sim Ion	Fragmentor	Gain EMV	Sim Resol.	Actual Dwell
0.0	Group 1	262.10	70	2.0	High	145
		264.10	70			145
		277.00	70			145
		278.10	70			145
		279.00	70			145
		281.00	70			145
[Signal 2]						
Polarity	Positive					
Fragmentor Ramp	Disabled					
Percent Cycle Time	10%					
Scan Parameters						
Time	Mass range low	Mass range hi	Fragmentor	Threshold	Step size	Time
0.00	100.00	500.00	70	150	0.10	0.00
[Signal 3]						
Not active						
[signal 4]						
Not active						
Spray chamber						
[MSZones]						
Gas temp	350 ° C		Max 350 ° C			
Drying Gas	12.0 L/min		Max 13.0 L/sec			
Neb Pres	50 psig					
VCap (Positive)	3500 V					
VCap (Negative)	3500 V					
[Time Table]						
Time Table is	Empty.					

Time between Injections : 1.0 min

Agilent 1100 Autosampler 1

Injection	
Injection Mode	Standard

Injection Volume	20.0 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	50 ° C
Right temperature	Same as left
Enable analysis	When Temp is within ± 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	

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. 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. For LCMS1, vials are loaded in numerical sequence (i.e: 1-100). Verify Sequence Queue is in play mode. Save and add Easy Sequence to the queue.
13. 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.
14. Date and initial the chain of custody label on the sequence printout, listing any comments, transfers or exceptions.
15. If an easy sequence template must be created or modified, follow these steps.
 - a) Select extended parameters. Check shutdown and select standby from the drop down menu. Not ready timeout should be set to 0.0. Click OK.
 - b) Click on the calibration tab.
 - c) Select cyclic calibration mode.
 - d) Click on calibrant icon, drag and drop into sequence start box.
 - e) Fill out calibrant information (vial info, injections/vial, calibration level). Select No Update for both response factor and retention time.
 - f) Repeat for each calibrator.
 - g) Enter Calibration Interval as 50 (or > number of injections needed for entire batch). The interval unit is injections.
 - 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 (0.10 ng/mL). Click OK. Repeat for remaining calibration levels (0.25, 0.50, 0.75, 1.0, and 5.0 ng/mL). Select level 4 when prompted to update retention times.
7. Load the file associated with level 4 (0.75 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 (#4). 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 0.98.
 12. 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. 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, Profile Quant w/o Calculations (QT 0,1,'P'), Add, and OK. Select files to print, click the → Arrow and Process.
 15. Save files to ECM. Select ECM from the toolbar, select "Save multiple data files to ECM". Select all files.
 16. Save method to ECM. Select ECM from the toolbar, Save Method to ECM. Make sure data path is correct.
 17. Submit all processed data, including calibrators, negative and positive controls and cases for Second Level Review.
 18. 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. Concentrations greater than 0.1 ng/mL are reported in ng/mL. Results are reported to two decimal points (i.e., 0.275 ng/mL is reported as 0.27 ng/mL).
3. 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.
4. Schedule repeat or confirmatory analysis as needed.
5. Review other findings in the case, especially GC 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.
6. If the positive matrix controls are $\pm 30\%$ of target, the samples may be reported qualitatively, as detected or not detected. If quantitative results are needed, the sample must be repeated.

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 matrix, $\pm 30\%$ of the target concentration for non-blood 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. For tissues, the controls are acceptable up to $\pm 30\%$ 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

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

Non-blood samples: Ion ratios may be accepted within $\pm 30\%$, if the analyte has met acceptance criteria in a blood matrix.

Note: Sometimes ion ratios will be out in exceptionally low or high concentrations, or in cases of coelutions. The analyst must evaluate this, and determine other appropriate actions as needed, such as dilution or reinjection (using methods described on pages 15-16).

Internal standard monitoring

For each quantitative analysis, the response of the internal standard(s) shall be monitored. The analyst shall choose from the multipoint calibrators and controls a level at their discretion. All internal standard responses for that sequence shall be compared to the internal standard(s) at that chosen level. The acceptance criteria for the internal standard response shall be $\pm 30\%$ of the selected internal standard. If the internal standard response is outside the defined limits, the specimen shall be re-extracted. In the event that an internal standard does not meet acceptance criteria and is associated with an analyte that is not detected, the case sample does not have to be re-extracted. 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.

REFERENCES

R. J. Hoffman, R. S. Hoffman, C. L. Freyberg, R. H. Poppenga and L. S. Nelson, "Clenbuterol ingestion causing prolonged tachycardia, hypokalemia, and hypophosphatemia with confirmation by quantitative levels", *Clinical Toxicology*, 39 (4), 339-344, 2001
Agilent 1100 Series LCMS Systems. Installation Guide.
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SPEware Corp. Cerex Applications Manual.
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REVISION HISTORY:

Ver 06.06.2014

1. Revision history implemented.

Ver 04.17.2015

1. Dilution control added.

Ver 09.14.2015

1. Incorporated First, Second, Third level review language.
2. Added Internal standard monitoring language.
3. Removed dilution control language.