FORENSIC TOXICOLOGY LABORATORY
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NEW YORK CITY

GC-NPD DERIVATIVES WITH TFAA

PRINCIPLE

Fluoxetine and norfluoxetine co-elute by our gas chromatographic methods unless chemically derivatized. Additionally, fluoxetine appears to break down on column. The TFAA (triflouroacetic anhydride) derivatives of fluoxetine and norfluoxetine are stable and are well resolved from one another.

This derivitization method is used in cases where there are co-eluteing compounds. For example, nortriptyline co-elutes with doxepin and desipramine co-elutes with cocaine. The resulting TFAA derivatives are then separated by routine gas chromatography analytical methods.

SAFETY

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

REAGENTS AND MATERIALS

All 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. **Distilled or Deionized water**
2. **Buffer, pH 9.8**

   Slowly add 212 g of Na_2CO_3 to 2500 mL of distilled water in a 4000 mL Erlenmeyer flask. Once this is completely dissolved, slowly add 168 g of $NaHCO_3$. Dilute to the mark with distilled water and mix on a stirring plate for one hour. Using a pH meter, verify that the pH is in fact 9.8. If the pH needs adjustment, use acid or base as needed to adjust to 9.8, document on the solution preparation sheet that the pH was checked. Date and initial preparation sheet.
3. **n-Butyl chloride (1-Chlorobutane)**. Available from most chemical companies in Analytical Reagent Grade.
4. **0.5 N HCl**.

Slowly add 164 mL of concentrated HCl to 3500 mL of distilled water in a 4000 mL graduated cylinder. Dilute to the mark with distilled water and mix on a stirring plate for one hour.

CAUTION: PREPARE THIS SOLUTION IN A FUME HOOD. USE APPROPRIATE SAFETY EQUIPMENT. ALWAYS ADD ACID TO WATER!

5. **Sodium carbonate**

   Slowly add 530 g of Na_2CO_3 to 3000 mL of distilled water in a 4000 mL Erlenmeyer flask. Dilute to the mark with distilled water and mix on a stirring plate for one hour.

6. **Toluene.** Available from chemical companies as AR grade.
7. **Triflouroacetic Anhydride (TFAA)**. Available from chemical companies.

CALIBRATORS AND CONTROLS

Calibrators and in-house controls are prepared according to the section “Preparation of GC Calibrators and Controls” with following drugs to be used in grouped pairs as needed.

Fluoxetine and norfluoxetine.

Amitriptyline and nortriptyline.

Doxepin and nordoxepin.

Imipramine and desipramine

Cocaine and ethylbenzoylecgonine.

SPECIMEN PREPARATION

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

Blood	2 mL of the undiluted specimen
Urine	2. mL for qualitative identification
If requested:	
Bile	2 mL of a 1:5 dilution
Brain	2 mL of a 1:3 homogenate
Gastric Contents	2 mL of a 1:10 dilution
Liver	2 mL of a 1:5 homogenate

Vitreous Humor	2 mL of a 1:5 dilution

DILUTION OF SPECIMENS

Specimens are diluted as follows:

Bile 1:5	1.0 mL of undiluted bile *q.s.* to 5 mL with 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 Contents 1:10	2.0 mL of liquid *q.s.* to 20 mL of distilled water, or 2.0 g of a solid specimen homogenized with 18 mL of deionized water.

Note: *Do not use homogenates older than two weeks unless low sample size requires it. Discuss with supervisor and note in case record.*

Note: *The entire submitted amount of gastric contents must be weighed prior to homogenizing and sampling.*

EXTRACTION PROCEDURE

1. Obtain a worklist of TFAA derivative 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 screw-cap culture tubes (two per aliquot) and Teflon-lined caps to extract one blank sample, calibrator samples and all requested cases. Label the tubes appropriately. Tubes should bear the entire toxicology number (e.g. 97-1234, *not* 1234), the specimen type and any appropriate dilution.
4. Obtain the required calibrator and control working solutions from the refrigerator and allow them to equilibrate to room temperature. Record the lot numbers of the solutions used on the lot sheet.
5. Pipet 2.0 mL of a matching, negative matrix into each of the blank and calibrator tubes (the negative matrix used for gastric and vitreous humor is 2.0 mL of deionized water). Lightly cap these tubes to avoid possible contamination from other specimens.
6. Mix each specimen container by gentle inversion two to four times and pipet 2.0mL of each specimen into the appropriate tube. Make certain that the toxicology number on the specimen bottle exactly matches the number on the tube. Lightly cap each tube after pipetting to prevent contamination from other cases. If a blood specimen is being pipetted and a clot is encountered, homogenize the specimen via the Polytron or a glass manual

homogenizer, document on the blood bottle that it was homogenized and annotate on sequence list. Continue in this fashion until all cases have been pipetted.

Note*:* Immediately re-file all case specimens and negative matrices. Do not leave this for a later time. Decomposition, especially in negative matrices, can affect subsequent results.

7. Enter the date extracted in the Dataease database for each case picked up in the batch.

NOTE: *DO NOT WAIT UNTIL THE FOLLOWING DAY TO COMPLETE THE ABOVE!*

8. Spike the tubes with various calibrator solutions as indicated. Each drug analyzed in the batch must have its appropriate calibrators.

Final Concentration (mg/L)	Stock Solution Concentration (mg/L)	Spiking Volume (µL)
0.05	100	1
0.2	100	4
1.0	100	20
3.0	100	60

9. Spike the tubes with various control solutions as indicated.

Final Concentration (mg/L)	Stock Solution Concentration (mg/L)	Spiking Volume (µL)
0.025	100	0.5
0.5	100	10.0
1.0	100	20.0

10. Add 40 µL of 50 mg/L Methapyrilene Internal Standard (or the required internal standard) to each tube.
11. Pipet 1.0 mL of the pH 9.8 buffer into each tube. Mix thoroughly contents of each tube by Vortex for five to ten seconds. Pipet 4.0 mL of n-butyl chloride into each tube and cap all tubes. Briefly invert each tube to check for leaks. If a leak is found, transfer the tube contents to another appropriately labeled tube. Place all tubes on the mechanical shaker for 10 minutes using a low setting. Remove the tubes from the shaker and centrifuge for 10 minutes at ≈ 3000 rpm. If an emulsion is noticed, break up the emulsion by shaking or using a clean rod, and re-centrifuge. If the emulsion still persists, see the supervisor for alternate methods.
12. Transfer the upper, organic layer of each tube to a new appropriately labeled tube which contains 2.0 mL of 0.5N HCl. Check that the number on the two tubes matches. Handle only one set of two tubes at once. Cap and briefly invert each tube to check for leaks. Place on the mechanical shaker for 10 minutes on a low setting. Remove the tubes from the shaker and centrifuge for 10 minutes at ~3000 RPM.

13. Upon removal from the centrifuge, uncap each tube and aspirate the upper, organic layer to waste using a suction/aspiration apparatus. Pipet 1.0 mL of the sodium carbonate solution into the lower acid layer. Vortex for five to ten seconds to mix.

    Note: *Addition of the sodium carbonate will cause release of CO_2 during the neutralization reaction which will result in foaming.*

14. Pipet 200 µL of toluene into each tube. Cap all tubes and place on the mechanical shaker for ten minutes on a high setting. Remove the tubes from the shaker and centrifuge for 10 minutes at ≈ 3000 rpm.
15. Transfer the toluene layer into a clean, appropriately labeled 10 mL conical test tube. Add 100 µL of TFAA to each tube via Eppendorf. Immediately cap each tube after the addition of TFAA. Incubate tubes for 15 minutes at 90° C in a heating block or an oven.
16. Remove tubes from heat and allow tubes to cool to room temperature. Add 1.0 mL of pH 9.8 buffer and vortex for 5 seconds. Centrifuge for 10 minutes at ~3000 RPM. Using a Pasteur pipet, transfer the upper toluene layer to a glass insert in an appropriately labeled vial. After each transfer, immediately seal each vial with an aluminum seal, by using a hand crimper, to avoid possible contamination from other samples. Samples may also be transferred into screw cap vials and capped immediately. Do not wait until all transfers have been made to seal the vials. Transfer the vials to the GC Instrument and continue with analysis by gas chromatography.

ANALYSIS BY GAS CHROMATOGRAPHY

Instrumentation

The instrumentations used for analysis is the Agilent 6890 gas chromatograph equipped with an HP-17 column, a 7683 Series autosampler and a Agilent 6890 Series nitrogen-phosphorous detector. A computer with HP Chemstation software is used to gather the data and Chemstation Enhanced Data Analysis software is used to process the data.

HP-17 Column

The primary column used in the GC section is a 10.0 m x 0.53 mm x 2 µm HP-17 (50% phenyl methylsiloxane) megabore capillary column. This column is used for most routine work, both qualitative and quantitative.

INSTRUMENT SETUP

6890 Series II Gas Chromatogram

1. Ensure that the selected GC is operational and not in need of repair. If maintenance is required, consult the appropriate manual and notify the supervisor.

2. Make sure GC power on.
3. Check that the NPD bead voltage is set to current value as listed on the instrument.
4. The Chemstation method file for quantitation is usually named GCxNQNT.M, where the x refers to the instrument number. There should be a method file with the appropriate name in each method subdirectory. Load the appropriate method for the analysis. Consult supervisor if correct method is unclear.
5. On Chemstation verify that all other GC parameters are set correctly; go to Method, Edit Entire Method. Wait for "Ready".

Method Section To Run:

(X) Save Copy of Method With Data

( ) Instrument Control Pre-Run Cmd/Macro =

( ) Data Analysis Pre-Run Cmd/Macro =

(X) Data Acquisition

( ) Data Analysis

( ) Instrument Control Post-Run Cmd/Macro =

( ) Data Analysis Post-Run Cmd/Macro =

Inlet and Injection Parameters

Sample Inlet: GC
Injection Source: GC ALS

GC Injector (7683 Series Injector)

Select Single or Dual
Injector Parameters (Front or Back or Both)

Sample Washes	1
Sample Pumps	4
Injection Volume	5.0 microliters
Syringe Size	10.0 microliters
PreInj Solvent A Washes	0
PreInj Solvent B Washes	0
PostInj Solvent A Washes	5
PostInj Solvent A Washes	5
Viscosity Delay	0 seconds
Plunger Speed	Fast
PreInjection Dwell	0 minutes
PostInjection Dwell	0 minutes

Inlet Parameters

Front Inlet	*(Split/Splitless)*	*Back Inlet*	*(Split/Splitless)*
Mode	Splitless	Mode	Splitless
Initial Temp	275 ^{0}C (on)	Initial Temp	275 ^{0}C (on)
Pressure	5.20 psi (on)	Pressure	5.20 psi (on)
Pulse pressure	10.0 psi	Pulse pressure	10.0 psi
Pulse time	0.60 min	Pulse time	0.60 min
Purge flow	30.3 mL/min	Purge flow	30.3 mL/min
Purge time	2.00 min	Purge time	2.00 min
Total Flow	48.8 mL/min	Total Flow	48.8 mL/min
Gas Saver	off	Gas Saver	off
Gas type	Helium	Gas type	Helium

Column Parameters

HP-17 COLUMN (Column 1 & Column 2)

Capillary column			
Model number			HP 19095L-121
HP-50+ 50% Phenyl Methyl Siloxane			
Max Temp			310 ^{0}C
Nominal length			10.0m
Nominal diameter			530.00 um
Nominal film thickness			2.00um
Mode			Ramped Pressure
Initial Pressure			5.20 psi
Initial Time			12.00 min
#	**Rate**	**Final Pressure**	**Final time**
1 2	3.20 0.0 (off)	8.20	0.00
Post flow		5.20 psi	
Nominal initial flow		15.4 mL/min	
Average velocity		131 cm/sec	
Inlet		Front, Back, or Both	
Outlet (detector)		Front, Back, or Both	
Outlet pressure		ambient	

Oven Parameters (6890)

Initial temp: 120 ^{0}C
Initial time: 2.00 min
Maximum temp: 280 ^{0}C
Equilibration time: 1.00 min

Ramps:			
#	**Rate (^{0}C /min.)**	**Final Temp. (^{0}C)**	**Final Time (min.)**
1	15.0	280	18.00
2	0.0 (off)		
Post temp.	120 ^{0}C		
Post time	1.00 min		
Run time	30.67		

Detector Parameters

Front detector (NPD)		*Back detector(NPD)*	
Temperature	325 ^{0}C mL/min(on)	Temperature	325 ^{0}C (on)
Hydrogen flow	3.0 mL/min (on)	Hydrogen flow	3.0 mL/min (on)
Air flow	60.0 mL/min (on)	Air flow	60.0 mL/min (on)
Mode	Constant column+makeup flow	Mode	Constant column+makeup flow
Combined flow	30.0 mL/min	Combined flow	30.0 mL/min
Makeup flow	On	Makeup flow	On
Makeup gas type	Helium	Makeup gas type	Helium
Adjust offset	30.00	Adjust offset	30.00
Electrometer	On	Electrometer	On
Bead	On	Bead	On
Equilibration time	0.00	Equilibration time	0.00

Signals

(Select Front, Back, or Both)

Signal 1		***Signal 2***	
Data rate	20 Hz	Data rate	20 Hz

Type	Front detector	Type	Back detector
Save Data	On	Save Data	On
Start Save Time	1.00 min	Start Save Time	1.00 min
Stop Save Time	30.00 min	Stop Save Time	30.00 min
Zero	0.0 (off)	Zero	0.0 (off)
Range	0	Range	0
Fast Peaks	Off	Fast Peaks	Off
Attenuation	0	Attenuation	0

6. After reviewing instrument parameters select a "Real Time Plot" for viewing on the instrument top screen while the sequence is running.
7. Make sure that under "Select Reports" all UNCHECKED.
8. Save the Method.

SEQUENCE

Prepare a sequence by using the following steps:

1. Make sure the desired instrument session is up and running. Each GC has an instrument session, such as GC1, GC2, etc.
2. From the top menu select **Sequence → Load Sequence → Default.S**. Select **Sequence** again, then **Edit**. The **Sample Log Table** will open.
3. In the upper left hand corner on the **Sample Log Table**, select **Data Path**. Click on **Browse** to create a new folder under the **Data** folder of the instrument to be used.
4. In the upper right hand corner on the **Sample Log Table**, by clicking on **Browse** select the instrument **Method** to be used for analysis.
5. Type in all information under each column (i.e. Type, Vial, Sample, Method/Keyword, Datafile, Comments/Keywordstring).
6. If the Sample is a calibrator, then under **Comments/Keywordstring** type-in the MIX or Filter it is for the batch; i.e. for all levels of CAL 1 type-in MIX [A], for levels of CAL 2 type-in MIX [B] etc. *This step will be helpful during processing the data.*
7. The **Datafile** name should be read GCXMMDD**x**00**1** ; where "X" is the instrument's number, "MM" is the month, "DD" is the day, and "**x**" is the prefix of the sequence, such as "a", "b" *etc.*
8. Once all the information is typed, select **OK** to close the **Sample Log Table**. Save the sequence by selecting **Sequence** followed by **Save Sequence As**. . . . Name the sequence as follows: GCXMMDDYY ; where "YY" defines the year.
9. Select **Sequence** then **Simulate Sequence**. In the window that opens, select **Full Method**, **Inject Anyway**, and **Overwrite Existing DataFiles**. For **Sequence Comment** enter the

instrument # and detector used, the date of injection, the analyst initials, and the type of batch being injected. Select **Run Sequence**, verify all keywords by clicking "OK".

10. Print sequence by selecting **Sequence** followed by **Print**. . .then select **Brief**. Add a "Chain of Custody", a "Vials Loaded" label and a ECM documentation label to the hard copy of the sequence.
11. Empty solvent wash bottles on the instrument and replace it with fresh methanol before starting the sequence.
12. Use the printed sequence list to load the vials in the correct position on the autosampler tray. Edit the sequence as necessary to correct any errors; save the sequence. Reprint sequence if necessary.
13. Document the "Chain of Custody" and the "Vials Loaded" labels.
14. Document the "Instrument Status Logsheet" that hangs on the instrument's oven door.
15. Start sequence by selecting **Sequence → Run Sequence**.
16. When the sequence is complete, remove all vials using the sequence. Once again document the "Vials Loaded" label.

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\2\DATA\GCXMMDDYY*x*** where "X" 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: GCXMMDD**x**00**1**; ***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\2\DATA\GCXMMDDYY*x*** 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\2\DATA\GCXMMDDYY*x***? → Click **Yes**.

9. A 3rd dialog box will pop-up: Sequence Verification Done! View it? → Click **Yes** or **No**.

10. Save and Print Sequence.

11. Start sequence.

DATA ANALYSIS

Retrieving Method from Data Acquiring Instrument

1. Save the method to the batch from the acquiring instrument Chemstation.
2. Save method to batch by clicking ECM on the tool bar, then select **Save Method to ECM**. Locate the batch, select ok to save a copy of the method into the batch to be processed.
3. A copy of the method has now been saved to ECM and will accompany the batch when retrieved from ECM on any Data Analysis Processing Station.
4. Attach and document "ECM label"

PROCESSING USING ENHANCED DATA ANALYSIS

On any processing station:

1. Click on the Processing Data Analysis icon on the desktop and Log-in by using: analyst's OCME login for the username and their password.
2. From the tool bar menu select **ECM → Retrieve Entire Sequence From ECM**. Browse to locate and select the batch. Each data file will be retrieved along with the method to be used for processing.
3. Once the retrieval is complete the batch folder is located in the C: \msdchem\ECM\retrieve folder.
4. Select the method under the batch by right clicking on it and **Load** the method.
5. Select **Calibrate →Clear**; clear all responses and all calibrator levels.
6. Select **Calibrate** from the tool bar, then **Update**, **Global Update**, **Set Curve Fit Type**, **Linear Regression Force (0, 0)**, select **OK**. If a different type of curve fit is used in place of Linear regression force (0,0), this must be annotated on the front page of the master copy of calibrators and controls that is appended to each case chromatogram.
7. Set up processing Filters (Filter A = Cal 1, Filter B = Cal 2, Filter C = Cal 3, Filter D = Cal 4, Filter E = Cal 5 etc.

a. Select **Calibrate** again, **Edit Compounds**; select **Name**, type in compound name in the space provided, click **Find Compound.**

b. Change **Compound Type** from "T" to the required filter letter for that compound. All compounds for pools or Cals 1 & 2 are already assigned in all master methods (i.e. the compound type for meperidine is always A, doxylamine is always B etc.) and should not have to be assigned when processing each batch.

c. Click **OK** to save all changes to the compounds that have been made.

8. In order to ensure that only compounds that are found are printed in the report select **Quantitate** in the menu bar**,** then **Report Options**. In the lower right hand box labeled "All Reports" make sure that the box labeled "Omit Target Compounds that are Missed" is checked.

9. Create the filters needed to process the batch:

a. Select **NYC_OCME** in the menu bar, then select **QDB Compound Type Filter**.

b. Select **Create** and type-in as many filters as need for the batch one at a time (i.e. A, B, C, D etc). Filters are case sensitive.

c. Once all filters are created a "qdb filter" window automatically pop-up with a list of all the compounds in the filters needed to process the batch.

10. Load the first calibrator at the highest level (i.e. 3.0 mg/L) by selecting **File → Load Data File**

11. Load the filter needed for the data file:
    a. Select **NYC_OCME** in the menu bar, then select **Compound Type Filter**
    b. Type the # for that filter, where 1 is the first assigned filter.

12. In the space provided type in **QT 1**, then click on **Execute**; this processes the data file.

13. Select the **View** from the top menu, select **Easy ID** (this function allows for Retention times to be assigned for the entire method and batch). ***Easy ID*** *should only be used for your first calibrator level.*

14. To select a compound from the list double click, use the right click to integrate a peak for the compound, use the left click to control zooming on the chromatogram.

15. Once all of the retention times have been assigned, click on **Exit** and click on **Yes** to "update retention times to the compound list".

16. Type in **QT 1**, then click on **Execute**.

17. Again select the **View** from the top menu, select **Q-Edit Quant Result** (this function to view the response and retention times for each compound.) *Cal 3.0 mg/L will not have an "x" next to each compound name because it is the first calibrator to be processed.* Click **Exit** when all compounds have been reviewed.

18. Assign the calibrator level:

a. Select **Calibrate**, then **Update**, select **Update 1 Level.**

b. Select **Add Level** and type in the concentration of the calibrator (i.e. 3.0, 1.0 etc.).

c. Type-in the internal standard concentration (1.0).

d. On the right hand side of the window type-in the Level ID i.e. 4A, 3A, and 2A, 1A; where the # is the calibrator level and the letter is the filter.

e. Click on **Do Update** then select **OK**.

19. Load the next data file, Cal 1.0 mg/L. Type **QT 1** then click on **Execute**.
20. Select the **View**, select **Q-Edit Quant Result**. *Easy ID is not needed for this step, as retention times have already been set for this calibrator.* Double check each of the compound's retention time and that each compound has a response. All auto- integrated compounds should have an "x" next to their name. Click **Exit** once when finished.
21. Assign the calibrator level.
22. Repeat steps 15-21 for the remaining levels (0.2 and 0.05mg/L) for the first calibrator.
23. Requant the first calibrator:

    a. Go to **Tools**, **DOLIST.**

    b. Remove any options that are on the right hand side of the box.

    c. On the left hand menu select "**Summary Quant No Report**" which is **QT 2**, with arrows move this command to the empty space on the right then click **OK**.

    d. Select the calibrators just processed and move them to the empty space on the right then click **Process**. Each file will be "requanted".

    e. Review all files through **Q-Edit Quant Result** to verify quant results.
24. Process the corresponding QC's for the calibrator that was just reviewed, with the same filter on:

    a. Go to **Tools**, **DOLIST.**

    b. Remove any options that are on the right hand side of the box.

    c. On the left hand menu select "**Quant No Report**" which is **QT 1**, with arrows move this command to the empty space on the right then click **OK**.

    d. Select the QC's to be processed and move them to the empty space on the right then click **Process**. Each file will be processed.
25. Review each QC by selecting **View**, select **Q-Edit Quant Result**.
26. With the filter still on, print the Cal's and QC's for that filter:

    a. Go to **Tools**, **DOLIST.**
    b. Remove any options that are on the right hand side of the box.
    c. On the left hand menu select "**Summary Quant w/o Calculations**" which is **QT 0, 1, "s"**, with arrows move this command to the empty space on the right then click **OK**.

d. Only select all Cal levels and QC's just reviewed and quanted and move them to the empty space on the right then click **Process**. Each file will print.

27. Load the 3.0mg/L of the next calibrator to be processed and follow steps 10-26.
28. Once all calibrators and QC's have been processed and printed, ***save the processing method to ECM*** then ***set the filter # to 0, which gives a list of all the compounds for the entire method,*** and process the all the blank injections and cases:

    a. Go to **Tools**, **DOLIST.**

    b. Remove any options that are on the right hand side of the box.

    c. On the left hand menu select "**Quant No Report**" which is **QT 1**, with arrows move this command to the empty space on the right then click **OK**.

    d. Select the QC's to be processed and move them to the empty space on the right then click **Process**. Each file will be processed.

29. Review each blank and case through **Q-Edit Quant Result**:

    a. Deselect any compounds that are not needed in each blank or case by double clicking on it and clicking on **QDel**.

    b. Check the responses and retention times of the needed compounds.

    c. If a compound is needed but not integrated, double click on the compound and integrate.

    d. Click **Exit** once when finished, and click "yes" to the save changes dialogue box.

30. If a compound is too low to be integrated or no peak is present include an overlay:

    a. Go to **Tools**, **Overlay Chromatogram.**

    b. Select the calibrator or standard to overlay on the case; multiple overlays can also be set.

    c. Select **Process.**

    d. Go to **File**, **Print** then select **Chromatogram**

31. Once all blanks and cases are processed, print as directed in step # 26.
32. Print an R^2 report by:

    a. Go to **Calibrate**, **List** then select **Calibration Report** and **OK**.

    b. Compounds that were not used in the batch can be deleted by highlighting them and simply pressing delete on the keyboard. *Caution should be used, do not remove compounds needed.*

    c. Right click on the list and select **Print**, choose the correct printer.

33. Once everything is printed, save the all the data files from the batch back to ECM:

    a. Select **ECM** from the top tool bar; select **Save Multiple Data Files to ECM.**

b. Select all data files on the left and move them to the right empty space, then click **Process**.

c. Allow each data file to be successfully copied to ECM before exiting Data Analysis.

34. After uploading the files to ECM, delete the sequence from the C: Drive of the processing station:

    a. Right click on the **Start** on the lower left hand corner and select **Explore**

    b. Open the **MSDCHEM** folder on the **C: Drive**, then select **1 → ECM → Retrieve**

    c. Locate the batch processed, right click on it and delete it.

    d. Document" ECM label" on sequence list.

RE-INJECTION CRITERIA

Occasionally, samples analyzed may need to be reinjected for a variety of reasons. The criteria for reinjection, other than poor chromatography or requests made by the appropriate supervisor, are listed below.

1. Individual blood samples may be re-injected once if internal standard (IS) area counts fall below that of the counts for the Cal's and QC's in the batch.

2. Analytes in a calibrator or control are not present in the chromatogram.

3. Additional peaks present in the chromatogram of a calibrator or control. See TROUBLESHOOTING OF AGILENT GAS CHROMATOGRAPHS section of the SOP manual.

4. QC failure: reinject the blank, the calibrator of interest and the associated QC.

5. Examine peak shape for signs of overload. If peaks are still overloaded after reinjection, re-extract samples with overloaded peaks using appropriate dilutions. If a calibrator peak is still overloaded after re-injection, the batch must be re-extracted.

CARRYOVER

Two types of carryover are possible with the methods employed by GC. One is the "carryover" of late-eluting peaks from one injection into the next (or even next two or three) injection. This is usually apparent from the shape of the peak (broad).

The other possibility is "carryover" due to contamination from an excessive amount of drug in the previous sample, either through the syringe or by drug being retained in the injection port or column. Any samples with large concentration of drug are rescheduled with dilutions, to reduce the amount to within the linear range of the method. If it appears that carryover of this type has

occurred, the reviewer will reschedule the case after the overload to verify that there was no contamination.

Frequent changing of the autosampler wash vials helps to control this type of potential carryover.

ACCEPTANCE CRITERIA

1. In-house controls must be analyzed in accordance with the standard laboratory procedures to determine the target and acceptable range before being incorporated into routine analysis of the specimens.
2. None of the target analytes should be detected in the negative control (blank).
3. Retention times of target analytes should match those of the calibrator or controls within ± 2%.
4. The positive controls should be within ± 20%, for blood batches, or ± 30%, for tissue batches, of the established target for all analytes detected in the samples. In other words, if fluoxetine and norfluoxetine are present in the samples, the respective components of the controls must meet the acceptance criteria, but it is not necessary for the other compounds in that same control to meet the acceptance criteria. If the QC fails for quantitation, but is correct qualitatively, a batch can be accepted for qualitative results only. Acceptance of controls outside of ± 20% blood or ± 30% for tissues, of the established target require authorization of the supervisor and documentation.

DATA REVIEW
(Second level review)

1. Check chromatography of all injections. Examine the peak shape and note if any peaks show non-Gaussian shape. Identify unresolved peaks and peaks with shoulders on either side. Address any noted problems before proceeding with data review. Consult a supervisor about any unusual events.
2. Check that all significant peaks in the chromatogram are integrated. If the printout allows, check if the baseline used to integrate is appropriate. The analyst should consult with a supervisor about any unusual events, such as the presence of overload peaks
3. Check that all components of each calibrator are present and that each peak is properly assigned. Correct any errors. If any calibration compounds do not extract, or if expected multiple peaks are detected for a calibration compound, the analyst must consult with a supervisor.
4. Check the blank for significant peaks that might co-elute with a compound of interest. There is some room for judgment in this step, but the analyst should consult with a supervisor if there are ANY questions. A blank may be scheduled for mass spectrometry to discover the identity of peaks in a blank. If any significant peaks are present in a blank for the batch, the run must be rejected.

5. Review QC samples, determine if the controls meet all acceptance criteria and verify the concentration of the components.
6. A copy of the sequence calibrators and controls are forwarded to the QC officer, who will enter the results of the QC sample in the QC database. If the batch fails, annotate sample chromatograms, listing the reason for failure.
7. Annotate the following information on the Cal chromatogram:
    a. Calibrators and blank reviewed and accepted (or not accepted). Any QC or calibrator failures are annotated on the proper chromatograms.
    b. If the files did not transfer, do so, and notify supervisor.
    c. Note any deviations from the SOP in a concise but detailed fashion.
    d. Initial and date.
    e. Regression coefficient (r^2) must be greater or equal to 0.98.

Note: *By initialing and dating this document, the analyst certifies that a complete and accurate review was done, to the best of the ability of the analyst.*

8. Make sure that each control has the appropriate target concentration range label.
9. A supervisor reviews calibrators and controls, dates and initials the results of the review before photocopying.
10. Photocopy a master copy of the sequence, calibration, control and any other data. Arrange this master copy as follows (from top to bottom): Calibrators, all QC samples and blanks, r^2 report (or the "Calibration Report"), sequence list and the lot sheet form. It is the responsibility of the analyst to verify that the master copy is legible and is an accurate copy of the originals, with no information cut off at the margins. Photocopies are made from this master, one each case in the batch. Attach a copy of the calibrator and control package to each original case chromatogram.

DATA REVIEW

(Third level review)

Quantitative Results

1. The reviewer should review the calibration, control, and sequence information received after the photocopying of the sequence. Use the same criteria as listed in batch review, with the additional criteria that the r^2 of the five-point calibration curve of each component present in any case in the batch must be equal to or greater than 0.98. Notify a supervisor about any errors and make sure they are corrected before proceeding.

2. Retrieve the case file for each for each sample in the batch, associate it with the chromatogram(s) and attach the copy of calibration, control, and sequence information.
3. Review the case chromatogram. Determine if the results are consistent with all the other applicable case data to date. For quantitation, it is important to compare the first chromatogram results with the final quantitative results. The ratio of peak to internal standard for both chromatograms should be examined. If the results are consistent, report the results on the Result Summary Sheet. Date and initial both the chromatogram and the Result Summary Sheet. If the results are not consistent, the inconsistency must be resolved. Consult a supervisor about rescheduling the sample, or other appropriate corrective action.
4. Repeating analysis may be necessary if the chromatogram shows overloaded peaks or poor recovery of the internal standard,. If the problem is an overload, reschedule with appropriate dilutions. In cases of poor recovery, the sample, a positive control, negative control, and a calibrator may be re-injected once. If the criteria as listed in batch review are still not met, the sample is re-extracted. If any cases have an unknown or unidentified peak not observed in previous scan results, the case vials must be transferred to GC/MS for identification. All chromatograms must be attached to the case file. Consult with the supervisor if there are any questions.

Note: *Do not discard any sample paperwork.*

REPORTING (QUANTITATIVE RESULTS)

1. All control acceptance criteria must be met.
2. Only specimens that have been run along with acceptable controls may be reported. Report concentrations equal to or higher than 50% of the lowest acceptable control or the lowest acceptable calibrator. Concentrations equal to or greater than 25% of the lowest control or lowest calibrator but lower than 50% of the lowest acceptable control or the lowest acceptable calibrator. are reported as “less than X” where “X” equals the concentration of the lowest acceptable calibrator or control. Concentrations lower than 25% of the lowest acceptable control or calibrator are reported as “not detected”.
3. All positive findings are reported to two decimal points (e.g., “fluoxetine 0.82 mg/L”).
4. Drugs detected at a concentration above the upper limit of quantitation must be reported as “greater than...”, unless the concentration was determined by repeat analysis using appropriate dilutions. Dilutions are performed until the results are within the linear range of the calibration curve. If the sample is insufficient or other considerations preclude dilution, report as above.
5. Drug facilitated sexual assault cases and driving under the influence cases are reported in ng/mL