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

ACETAMINOPHEN, SALICYLATES, THEOPHYLLINE, CAFFEINE by SOLID PHASE EXTRACTION and HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

PRINCIPLE

This procedure is used for both qualitative and quantitative analysis of acetaminophen, salicylates, theophylline and caffeine. Drugs are identified based on their retention times following separation by high performance liquid chromatography (HPLC) and by the ultraviolet (UV) spectra of the eluting peaks using a diode array detector.

This procedure is also used to quantify salicylamide and theobromine and to qualitatively identify metronidazole.

Acidic and neutral drugs are extracted from biological specimens (blood, urine, brain, liver and gastric) 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 then washed to remove interfering compounds, followed by the elution of the drugs from the column using an organic solvent. The eluate is evaporated to dryness and the residue containing the drugs is reconstituted with mobile phase and analyzed by HPLC. Separation of drugs is based on their affinity with the C-18 column and the mobile phase. The polar drugs such as acetaminophen and salicylic acid have lower affinity for the ODS-silica and hence elute relatively faster than less polar drugs such as caffeine.

SAFETY

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

REAGENTS AND MATERIALS

All reagents are HPLC grade or better.

1. **Deionized Water**
2. **Ethyl Acetate**. Fisher Scientific Optima or equivalent. HPLC grade or better.
3. **Methanol** Fisher Scientific or equivalent. HPLC grade or better.
4. **Acetic Acid**, Glacial, HPLC grade. Fisher Scientific or equivalent.
5. **Sodium Acetate** Certified ACS, Fused Anhydrous. Fisher Scientific or equivalent.
6. **Sodium Acetate Trihydrate** Certified ACS. Fisher Scientific or equivalent.
7. **Sodium Phosphate Monobasic** Certified ACS. Fisher Scientific or equivalent.

8. **Sodium Phosphate Dibasic** Certified ACS. Fisher Scientific or equivalent.
9. **O-Phosphoric acid 85%** Certified ACS. Fisher Scientific or equivalent.
10. **Beta-hydroxyethyltheophylline** (Internal standard) FW 224.2 $C_9H_{12}N_4O_3$. Sigma Chemical Company or equivalent.
11. **Acetaminophen** (4-acetaminophenol) FW 151.2 $C_8H_9NO_2$. Alltech-Applied Science Labs or equivalent.
12. **Salicylic Acid**. Analytical reagent. Alltech-Applied Science Labs or equivalent. F.W. 138.12 $C_7H_6O_3$.
13. **Theophylline**. Anhydrous Crystals. F.W. 180.17 $C_7H_8N_4O_2$. Sigma Chemical Company or equivalent.
14. **Caffeine**. Sandoz Pharmaceutical or equivalent. F.W 194.19 $C_8H_{10}N_4O_2$.
15. **Certified Negative Blood**. Previously found to have no interfering peaks. Freeze validated negative blood. Discard after 6 months.
16. **Certified Negative Liver**. Previously found to have no interfering peaks. Freeze validated negative liver. Discard after 6 months**.**
17. **Certified Negative Brain**. Previously found to have no interfering peaks. Freeze validated negative brain. Discard after 6 months.
18. **Ammonium Hydroxide**, Certified A.C.S. Plus. Fisher Scientific or equivalent.
19. **Polycrom Clin II** Solid Phase Extraction Column. CEREX.
20. **System 48 Processor** connected to nitrogen source.
21. **Waste Rack**, SPE Rack, Collection Tube Rack
22. **Concentrator**, connected to a nitrogen source. Zymark **TurboVap**, or equivalent.
23. **Sonicator**
24. **Vortex**
25. **Centrifuge**
26. **Vacuum Filtration Apparatus**
27. **Nicotinamide** FW 122.12. Sigma-Aldrich Chemical Company or equivalent.
28. **Nylon filter membranes,** 47 mm 0.45 µm. Agilent Technologies part number 9301-0895 or equivalent.

***Note**: It is recommended that the primary reference standards used for the preparation of controls and calibrators be obtained from different manufacturers, or that they be prepared from different lot number from the same manufacturer.*

PREPARATION OF CONTROLS

1. Label a 100 mL volumetric flask as follows: 2500 mg/L acetaminophen, salicylic acid: 1000 mg/L theophylline, caffeine **Control** Solution. Indicate date prepared, initials of the person who prepared the solution, lot number, solvent, and expiration date.
2. Accurately weigh **250 mg of acetaminophen** on a piece of weighing paper and transfer into a clean 100 mL volumetric flask.
3. Accurately weigh **250 mg of salicylic acid** on piece of weighing paper and transfer into the same 100 mL volumetric flask.
4. Accurately weigh **100 mg of theophylline** on a piece of weighing paper and transfer into the same 100 mL volumetric flask.
5. Accurately weigh **100 mg of caffeine** on a piece of weighing paper and transfer into the same 100 mL volumetric flask.
6. Add 40 mL of methanol into the 100 mL volumetric flask.
7. Mix solution by mechanical stirrer until dissolved.
8. Remove stirrer and rinse with methanol into the flask. Q.S. to 100 mL mark with methanol.
9. The control solution may then be transferred into appropriately labeled headspace vials and sealed with a Teflon septum and aluminum seal. Store at 2-8 °C. The headspace vials will now contain the **working** control solution. On the vials indicate date prepared, expiration date, initials of person who prepared the solution, lot number, solvent, storage condition and bottle number with total number of bottles. The solution is stable for one year.

PREPARATION OF NICOTINAMIDE CONTROL

1. Accurately weigh **25.0 mg of nicotinamide** and transfer into a clean labeled 25 mL volumetric flask.
2. Add 20 mL of methanol to the 25 mL volumetric flask.
3. Mix solution by agitation until dissolved.
4. Q.S. to 25 mL mark with methanol
5. Label volumetric flask as 1000 mg/L nicotinamide **Control** Solution. Indicate date prepared, initials of the person who prepared the solution, lot number, solvent, and expiration date.
6. The solution may then be transferred into appropriately labeled headspace vial and sealed with a Teflon septum and aluminum seal. Store at 2- 8 °C. Label the vial to indicate date prepared, expiration date, initials of person who prepared the solution, lot number, solvent, storage condition. The expiration date for the solution may be extended past one year as long as nicotinamide is qualitatively detected.
7. The nicotinamide is a qualitative control, to provide retention time and UV spectra to identify this compound which occurs frequently in case samples, but is not reported or quantitated.

PREPARATION OF CALIBRATORS

1. Label a 100 mL volumetric flask as follows: 2500 mg/L acetaminophen, salicylic acid; 1000 mg/L theophylline, caffeine calibrator reference solution. Indicate date prepared, initials of the person who prepared the solution, lot number, solvent, and expiration date.

2. Accurately weigh 250 mg of acetaminophen on a piece of weighing paper and transfer into a clean 100 mL volumetric flask.

3. Accurately weigh 250 mg of salicylic acid on piece of weighing paper and transfer into the same 100 mL volumetric flask.

4. Accurately weigh 100 mg of theophylline on a piece of weighing paper and transfer into the same 100 mL volumetric flask.

5. Accurately weigh 100 mg of caffeine on a piece of weighing paper and transfer into the same 100 mL volumetric flask.

6. Add 40 mL of methanol into the 100 ml volumetric flask.

7. Mix solution by mechanical stirrer until dissolved.

8. Remove stirrer and rinse with methanol into flask. Q.S. to 100 ml mark with methanol.

9. The calibrator solution may then be transferred into appropriately labeled headspace vials and sealed with a Teflon septum and aluminum seal. Store at 2- 8 °C. The headspace vials will now contain the working calibrator solution. On the vials indicate date prepared, expiration date, initials of person who prepared the solution, lot number, solvent, storage condition and bottle number with total number of bottles. The solution is stable for one year.

INTERNAL STANDARD (2000 mg/L β-hydroxyethyltheophylline)

1. Label a 100 mL volumetric flask. Indicate date prepared, initials of the person who prepared the solution, lot number, solvent and expiration date.
2. Weigh 200 mg of β-hydroxyethyltheophylline and transfer to a 100 mL volumetric flask.
3. Add 80 mL of methanol.
4. Stir by magnetic stirrer until dissolved.
5. Remove stirrer and rinse with methanol into flask. Q.S. to 100 mL mark with methanol.
6. Transfer samples to headspace vials labeled with the lot number, initials of person who prepared the solution, date prepared, expiration date, solvent, storage condition and bottle number with total number of bottles.
7. Stable for one year. Store at 2-8 °C.

PREPARATION OF EXTRACTION REAGENTS

Acetate Buffer, 100 mM (pH 4.5)

1. Add 400 mL of distilled water to a 500 mL volumetric flask.
2. Add 2.93 g sodium acetate trihydrate to the 500 mL volumetric flask.
3. Add 1.62 mL glacial acetic acid to the 500 mL volumetric flask.
4. Dilute to 500 mL mark with distilled water and mix.
5. Adjust pH to 4.5 with 100 mM acetic acid. Measure pH with electronic pH meter.
6. Label and store at room temperature in glass or plastic bottle. Stable for 6 months.

Phosphate Buffer, 100 mM (pH 3.0)

1. Add 1.700 g sodium phosphate monobasic to a 1 L volumetric flask.
2. Add 12.14 g sodium phosphate dibasic to the 1 L volumetric flask.
3. Dilute to 1 L mark with distilled water and mix.
4. Add 3.0 mL o-phosphoric acid 85% and mix. Measure pH with electronic pH meter
5. Adjust pH to 3.0 if needed with o-phosphoric acid 85%.
6. Refrigerate at approximately 5 °C in a glass or plastic bottle. Stable for 6 months as long as pH remains stable.

ELUTING SOLVENT

1. Add 98 mL ethyl acetate to a glass or plastic bottle with cover.
2. Add 2 mL concentrated ammonium hydroxide and by Vortex.

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.*

Mobile Phase A

1. Add 3600 mL of deionized water to a 4 L graduated cylinder.
2. Add 400 mL of methanol to the 4 L graduated cylinder while mixing by mechanical stirrer.
3. Add 20 mL of glacial acetic acid to the 4 L graduated cylinder while mixing by mechanical stirrer.
4. Add 20 g of sodium acetate to the 4 L graduated cylinder while mixing by mechanical stirrer.
5. Mix mobile phase for 30 minutes.
6. Transfer mobile phase to a 4 liter brown bottle and store at room temperature. The label on the storage container will include the lot number, initials of person who prepared the solution, and date prepared. Discard after 3 months.

7. Filter the solution using vacuum filtration apparatus before transferring to the solvent bottle on the instrument.

Pre-mixed Mobile Phase (for reconstitution)

1. Add 9 mL of mobile phase A to a 50 mL graduated cylinder.
2. Add 1 mL of distilled water.
3. Mix with mechanical stirrer.
4. Filter the reconstitution solution.
5. Transfer to a screw cap specimen container. Label the container with the lot number, initials of person who prepared the solution and date prepared. Store at room temperature. Discard after 4 weeks.

SPECIMEN PREPARATION

Blood	1.0 mL of the undiluted specimen
Urine	1.0 mL of 1:3 dilution
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

Dilution of specimens

Specimens are diluted as follows:

Urine 1:3	1 mL of urine diluted with 2 mL distilled water
Brain 1:3	3.0 g of brain homogenized with 6 mL of distilled water
Liver 1:5	2.0 g of liver homogenized with 8mL of distilled water
Gastric 1:10	2.0 mL of liquid *q.s.* to 20 mL of distilled water, or 1.0 g of a solid specimen homogenized with 9 mL of distilled water

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

Note: *Homogenates of other dilution factors than 1:3 or 1:5 may be used if available. If case is suspected to have a high concentration of analyte, additional dilutions may be analyzed. Record any exception on sequence list.*

Note: *Record the total weight of each gastric content.*

EXTRACTION PROCEDURE

1. All reagents, samples, controls and calibrators must equilibrate to room temperature prior to sampling.
2. Prior to sampling, label required amount of 16 by 125 mm test tubes. The test tube order in the rack must correspond to the order in which samples will be pipetted and injected. Each test tube must be labeled such that the specimen type, aliquot number, laboratory number and any factors unique to a given specimen are prominently written on the test tube. Handwriting must be legible.
3. Pipette 1 mL of a matching negative matrix into a 16 by 125 mm Pyrex test tube. Add **4** µL of the acid/neutral working calibrator solution to the test tube. This is Calibrator **I**. The acetaminophen and salicylic acid concentration is 10 mg/L, theophylline and caffeine concentration is 4 mg/L. Add **10** µL of the nicotinamide control solution to this calibrator.
4. Pipette 1 mL of a matching negative matrix into a 16 by 125 mm Pyrex test tube. Add **20** µL of the acid/neutral working calibrator solution to the test tube. This is **Calibrator II**. The acetaminophen and salicylic acid concentration is 50 mg/L, theophylline and caffeine concentration is 20 mg/L.
5. Pipette 1 mL of a matching negative matrix into a 16 by 125 mm Pyrex test tube. Add **40** µL of the acid/neutral working calibrator solution to the test tube. This is **Calibrator III.** The acetaminophen and salicylic acid concentration is 100 mg/L; theophylline and caffeine concentration is 40 mg/L.
6. Pipette 1 mL of a matching negative matrix into a 16 by 125 mm Pyrex test tube. Add **60** µL of the acid/neutral working calibrator solution to the test tube. This is **Calibrator IV.** The acetaminophen and salicylic acid concentration is 150 mg/L, theophylline and caffeine concentration is 60 mg/L.
7. Pipette 1 mL of a matching negative matrix into a 16 by 125 mm Pyrex test tube. This is the negative control (blank).
8. Pipette 1 mL of specimen(s). See specimen preparation for more information.

> ***Note:*** *Open specimen bottles* ***one at a time****.*

9. Both low and high control samples are run with each batch. Add **10** µL of the acid/neutral control working solution to a matching negative matrix. This is the **low control.** The acetaminophen and salicylic acid concentration is 25 mg/L, theophylline and caffeine concentration is 10 mg/L. Add **40** µL of the acid/neutral control working solution to a matching negative matrix. This is the **high control.** The acetaminophen and salicylic acid concentration is 100 mg/L, theophylline and caffeine concentration is 40 mg/L. If necessary, make up enough additional high or low QCs to make one QC for every 9 samples

> ***Note****: tissue samples may be quantitated against blood calibrators provided matrix matched blank and controls are included and pass all QC criteria.*

10. Add 25 µL of **β-hydroxyethyltheophylline** internal standard to each test tube.

11. Add 3 mL of 0.1 M sodium acetate buffer (pH 4.5) to each tube.
12. Sonicate for a minimum of 20 minutes.
13. Centrifuge at ≈ 3000 rpm for 20 minutes until all sediment is at the bottom of tube.
14. Label solid phase columns. This can be done during centrifugation.
15. Place the rack that holds the solid phase columns on top of waste rack of processor.
16. Check the labeling of each 16 x 125 mm test tube against the corresponding solid phase column before decanting. Decant the contents of the 16 x 125 mm culture tubes to the properly labeled corresponding solid phase column, then place solid phase column on rack.
17. Apply positive pressure to achieve a flow rate of ≈ 1mL/min.
18. Wash column with 1 mL of phosphate buffer (pH 3.0).
19. Apply positive pressure to achieve a flow rate of ≈ 1mL/ min.
20. Wash column with 1mL of deionized water.
21. Apply positive pressure to achieve a flow rate of ≈ 1mL/min.
22. Increase pressure to 25 psi to dry column for 30 minutes.

Note: *Use port plugs (PP-003) on unused ports for even drying.*

23. Remove waste rack.
24. Place rack of properly labeled conical centrifuge tube on processor. Check the labeling of each conical centrifuge tube against the corresponding solid phase column.
25. Place rack of solid phase extraction on top of the conical centrifuge tube rack.
26. Elute column with 2.0 mL of ethyl acetate: ammonium hydroxide (98:2) solution into a corresponding conical centrifuge tube. Elute by gravity, if possible. If column does not elute or elutes slowly, use squeeze bulbs to force eluting solvent through the cartridge. In very difficult cases, reduce Nitrogen pressure to ~5 psi and use the processor to force samples through.
27. Dry in concentrator with a gentle flow of nitrogen at ≈ 25 °C or other suitable drying apparatus.

Note*: It is very important that heat is not applied while drying as this could result in poor salicylate or theophylline recovery.*

28. Remove tubes once completely dry.

Note*: Do not leave tubes in concentrator once dry.*

RECONSTITUTE SAMPLES

1. With a calibrated Eppendorf Pipet or equivalent, add 200 µL of pre-mixed mobile phase to each tube (freshly degas for use on LC1 or LC2).

Note: *If the sample is significantly cloudy after mobile phase addition, the sample may be centrifuged up to 20 minutes at ≈ 3000 RPM.*

2. Mix contents of each tube by Vortex, at low speed, for 15 seconds. If there are significant solids suspended in the solution, centrifuge the conical tubes for 10 minutes at 3000 rpm. Transfer to an insert placed in an autosampler vial.
3. Label autosampler vials indicating aliquot and toxicology number (ex: 2-YY-xxxx), specimen type, dilution, analyst and date. Immediately seal each vial with an aluminum seal using a crimper to avoid possible contamination from other samples. Samples may also be transferred into screw cap vials and capped immediately. Physically check that the crimped seal is tight by attempting to rotate the seal. Crimp until tight, using a new seal if necessary. Do not wait until all transfers have been made to seal the vials. Transfer vials to HPLC section for analysis.

INSTRUMENTATION

Instrument # 3 Agilent LC 1100 HPLC with Autosampler equipped with a Diode-Array Detector.

Column: Supelco Sil LC-18. 7.5 cm x 4.6 mm. 3 micron particle size.

Integrator: Computer equipped with Chemstation software.

HPLC Method. ANSPE.M

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Integrator: Computer equipped with Chemstation software.

HPLC Method. ANSPE.M

INSTRUMENT SETUP

Information regarding the daily maintenance and standard operation of the LC1100 can be located in the Agilent instrument manuals, the HPLC Maintenance Standard Operation Procedure and the individual method Standard Operation Procedures. For screening and quantitation of HPLC samples, the following procedure must be followed.

1. All appropriate information must be annotated on the autosampler vials. This data will be transferred to the sequence list which will be compared to the data entered on the autosampler vials.
2. Calibrators are injected in order of increasing concentration. A blank is injected after the highest calibrator.
3. Postmortem samples are injected next.
4. The quality control samples are injected as every 10^{th} sample and as the very last injection.

INSTRUMENT PRE-RUN PROCEDURE

LC 1100 Instrument #3 and #4 ACMP Parameters

1. Pump (PV5):

Stop time	13.00 min
Post time	1.00 min
Flow	0.250 mL/min
Min. pressure	10 bar
Max. pressure	400 bar
Oven Temp	50 °C
Solvent A	90.0 % (acmp mob ph)
Solvent B	10.0 % (Bottle B, H_2O)

2. Injector:

Injection volume	10.0 µL
Draw speed	200 µL/min

3. Mobile Phase Time Table:

Time [min]	*Flow [mL/min]*
0.00	0.250
0.75	0.300
1.00	0.500
1.50	0.650
8.00	1.000
9.5	1.500
10.75	2.500

4. Signals:

	Sample,	*Bw*	*Reference,*	*Bw [nm]*
A:	270	14	550	6

5. Curve Type: power, **Origin:** forced

6. Spectrum:

Store	All
From	220 nm
To	340 nm
Step	2 nm
Threshold	1.0 mAU

TEST RUN

In order to ensure the instrument is in working condition, the analyst is required to perform a test run on the instrument. This ensures that the retention time is appropriate for all the target drugs and checks for contamination of the column (this may be observed by the peak shape in the test run, i.e., a tailing peak may indicate a contaminated column).

Click on **METHOD** and load the **ANSPE** method. Click on **RUNCONTROL**, then **SAMPLE INFO**. In **SAMPLE INFO SCREEN**, enter the **analyst initials** in operator field, verify **DATA File** path is: C:Chem\32\1\Data\. In **Prefix Subdirectory** update the **FILE NAME** to **LC3 (or 4) date of run (MMDDYY)T.** Update counter to 00001. Under **Sample Parameters** note the location of the vial and sample name of test sample (usually Cal 4-10 mg/L) . Under **comment field** note A/N Test run. Then click on **Run Method**.

If there is more than one set of calibrators in the current batch (say a batch that requires a quantitative result in two different matrices), then the "Easy Sequence" features must be used as only the Easy Sequence works with the Sequence Queue to run consecutive separate sequences. If there is only one set of calibrators, the below sequence entry procedure may be used.

Click on SEQUENCE and then SEQUENCE PARAMETER. If the instrument is running, analysts can prepare the sequence in the Offline system.

SEQUENCE PREPARATION PROCEDURE

> ***Note:*** *The following sequence preparation procedure is used when only one set of matrix calibrators are utilized in the sequence. See Easy Sequence Preparation SOP when there are more than one set of matrix calibrators in the sequence.*

Click on **SEQUENCE** and then **SEQUENCE PARAMETER**. If the instrument is running, analysts can prepare the sequence in the Offline system.

The sequence parameters screen appears and displays the eight fields that can be modified. In general, the sequence preparer will be concerned with three:

Operator, Subdirectory and Sequence Comment

1. **Prefix** field. This determines the name of the subdirectory where the result files will be stored. Except for special circumstances, use the instrument name and the date in the form of LC(3 or 4)MMDDYY e.g. LC4041411. Any additional sequences run on the same date on the same instrument will put a letter after the date. For example, the proper data subdirectory for the second batch run on 4/14/11 would be LC4041411A.
2. On labels to be attached to the sequence note who extracted the sequence, who created the sequence, who loaded and unloaded the sequence and who processed the sequence.
3. **Part of Method to be Run field**. "According to Runtime Checklist"
4. **Wait After Loading New Method field**. Usually zero, but may be changed if the sequence contains samples that need to be run on other methods.
5. **Post Sequence Command Macro**. Unchecked. The Shutdown Method now has the Shutdown Macro run in its Runtime Checklist.

6. **Not Ready Timeout field**. 10 minutes.
7. **Sequence Comment** field. Indicate the initials of the person performing the three steps of the analysis. It should take the form of E-XX, R-YY, S-ZZ, where E stands for extraction; R stands for reconstitution, S stand for sequence, and XX, YY and ZZ stands for the initials for the analyst performing that particular part of the analysis. Indicate the assay name, Mobile Phase lot number and any information specific to the batch.

Click on **Sequence** and then **Sequence Table**. The **Sequence Table** screen contains includes the following columns:

SEQ line	Vial #	Sample name	Method	Inj/vial	Sample type	Cal level	dil	Update RF	Update RT
1	1	cal 1 4/10 mg/L	anspe.m	1	calib	1		replace	replace
2	2	cal 2 20/50 mg/L	anspe.m	1	calib	2		replace	average
3	3	cal 3 40/100 mg/L	anspe.m	1	calib	3		replace	average
4	4	cal 4 60/150 mg/L	anspe.m	1	calib	4		replace	average
5	5	blank	anspe.m	1	control				
6	6	1-11-9998 hrt bld	anspe.m	1	sample				
7	7	QC 10/25 mg/L	anspe.m	1	control				
8	8	1-11-9998 fem bld	anspe.m	1	sample				
9	9	QC 40/100 mg/L	anspe.m	1	control				
10	10	1-11-9999 hrt 1:3	anspe.m	1	sample		3		
11	11	QC low or High	anspe.m	1	control				
			shutdown						

The Sample Name field should be modified according to the individual specifications for each sample. After the information of each sample is entered, type an appropriate value in the Dilution column if a dilution was made (if original concentration was used, skip the dilution field). If the sequence is the last sequence of the date, put the **SHUTDOWN** in the last Seq Line to clean up the column and stop the pump.

Note: **ALL** sequences' and subsequences **must** end with QC material.

Click on OK after all samples have been entered.

At this point, save the sequence into a file named the same as the data subdirectory. Click on **SEQUENCE** then **SAVE AS**. For example, 041411.S or any additional sequence run in the same date in the same instrument will put a letter after the date. For example, 041411A.S.

Click on **SEQUENCE**, **PRINT SEQUENCE**, highlight **SEQUENCE PARAMETERS**, **SEQUENCE TABLE**, **SAMPLE LOG TABLE AND PRINTER.** Then click **PRINT** to print the sequence on the printer.

BATCH ANALYSIS

1. Place sample vials in the sample tray (LC3 or LC4), using the printed copy of the sequence to insure that each vial is placed in the correct position, and that the sample name is checked against the vial and sequence. If the person loading the samples and comparing sample name is different from the person listed in the sequence, annotate by initialing and dating the sequence.
2. Click on RUN, then click RUN SEQUENCE. Observe the first injection to insure that the system is operating correctly.
3. After the sequence is finished, indicate who removed the samples.
4. After the sequence finishes, check that all data files are successfully transferred to ECM. If the files do not transfer successfully, notify the supervisor so proper corrective action can be taken.
5. After the run finishes, the data files will be in the data subdirectory on the local chemstation, and also will be automatically transferred to the ECM Server. The files will be in the LC Location, Instrument Name Cabinet, Data Drawer and Month Folder. See data processing SOP for data processing instructions.

DATA TRANSFER AND PROCESSING – FIRST LEVEL REVIEW

All processing and review should be performed on the Chemstation. The processed data files are then archived on the ECM.

Refer to the “Data processing” section of the SOP manual for the details on processing and review of data.

REINJECTION CRITERIA

Infrequently, analyzed samples (and very rarely sequences) may need to be reinjected for a variety of reasons. Subsequent to data review, use the criteria listed below to determine if reinjection of any sample from the sequence is necessary.

1. Poor chromatography
2. Apparent carryover or UID peak
3. Apparent interfering peaks: the sequence may be reinjected at 25 °C with 15 µL injection volume.
4. Evidence of a possible non-target drug: the sample may be reinjected with appropriate methanolic standards.
5. Requests made by the appropriate supervisor.

If reinjection or other unusual actions are required, annotate this on the sequence list. Any deviation from the standard procedure must be noted, initialed, and dated. If a reinjection fails, then repeat the analysis.

Note: *When a sample or samples are reinjected, in addition to the reinjected sample(s), reinject the blank, and the associated QC samples. This will give a qualitative result. If any of the unknowns are positive then reinject all calibrators, controls and unknowns under the modified conditions.*

ACCEPTANCE CRITERIA

Subsequent to HPLC analysis, all data is examined and reviewed according to the guidelines below.

1. A power regression curve is calculated by the processing method during calibration. Four calibrators and the origin are used to establish the calibration curve. All calibrators should quantitate at ± 20% of the weighed in target. One calibrator may be dropped if appropriate acceptance criteria are not met.
2. Regression correlation (r^2) must be equal to or greater than 0.98 for each analyte. If r^2 is below 0.98, only qualitative results may be reported.
3. Internal standard (IS) response comparable to the response for the matrix calibrators (internal standard recovery/response shall be ± 30% of the selected internal standard).
4. The upper limit of quantitation is equal to the highest valid calibrator when power calibration curves are utilized. Any specimens with concentrations exceeding that value must be re-extracted using appropriate dilution.
5. Positive blood controls must be within ± 20% of the weighed in target. Tissue positive controls must be within ± 30% of the weighed in target.
6. Negative matrix controls must have no interfering peaks.
7. Peak shape should be symmetrical and Gaussian in appearance and the retention times of the analyte peaks are ± 2 % of the calibrator retention times.
8. UV spectra of the analyte peaks in question must have the same absorbance curve as the equivalent peak in the respective calibrators. Samples in which co-eluting peaks (UV spectra which do not match the UV spectrum of the corresponding calibrator analyte) are detected may be confirmed by alternate methods (i.e., TLC or GCMS).
9. Additional significant peaks in the chromatogram may occur. They may be qualitatively determined by reference to the UV spectra and approximate retention time of controls previously analyzed for that compound, or the sample may be re-analyzed by GCMS to attempt to determine the identity of the peaks. If quantitation is required, other drugs may be quantitated by this method, with appropriate calibrators and controls processed as indicated for the usually analyzed compounds. Any such testing will be annotated as an exception to the SOP.

SECOND LEVEL REVIEW

The Second Level Reviewer will review the processed data in its entirety according to the acceptance criteria. The second level reviewer will also ensure the following;

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. 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 unexpected 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. If any significant peaks are present in a blank for the batch, the run must be rejected. Consult with a supervisor if there are ANY questions.
5. Review QC samples, determine if the controls meet all acceptance criteria and verify the concentration of the components.
6. A copy of the batch 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, ensure that the sample chromatograms are annotated, listing the reason for failure. Notify a supervisor and / or the QC manager.
7. Ensure the following information is annotated on the first calibrator's chromatogram or sequence list:
    a. Calibrators and blank reviewed and accepted (or not accepted). Any QC or calibrator failures are annotated on the proper chromatograms.
    b. Any deviations from the SOP are noted or any comments are stated in a concise but detailed fashion.
    c. Initial(s) and date
    d. Regression coefficient (r^2) of the four point calibration curve of each component present in any case in the batch must be greater than or equal to 0.98. Notify a supervisor about any errors and make sure they are corrected before proceeding.
8. Make sure that each control has the appropriate target concentration range label.
9. Review calibrators, controls, and blanks; ensure all acceptable criteria are met.
10. Initial and date the results.

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

11. Ensure the master copy of the sequence list includes, (from top to bottom): Calibrators, all QC samples and blanks, r^2 report, sequence list, internal standard recovery form 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.

12. Retrieve the case file for each sample in the batch and associate the case file with the corresponding chromatogram(s).

13. Review the case's initial chromatogram, if applicable. 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 second chromatogram 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.

14. If the chromatogram shows overloaded peaks, or poor recovery of the internal standard, then repeating the sample may be necessary. If the problem is an overload, re-schedule with appropriate dilutions. In cases of poor recovery, the sample, its control, blank, and a calibrator may be re-injected once. If the criteria as listed in batch review are still not met, the sample is re-scheduled. If a case has an unknown or unidentified peak not observed in previous scan results, the case must be transferred to the GCMS section for identification of that peak. All chromatograms must be attached to the case file. Consult with the supervisor if there is any question.

15. Submit the case file for third level review.

> ***Note:*** *Do not discard any sample paperwork.*

THIRD LEVEL REVIEW (FINAL REVIEW)

The third and final level review can only be performed by the Laboratory Manager. 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.

REPORTING

1. All results must be entered on the result summary form in the case file.
2. Copies of all the calibrators and controls along with a copy of the sequence worksheet must be attached to the original chromatogram of the case and placed in the case file folder.
3. All negative cases are reported on the result summary form as "acetaminophen, salicylates, theophylline and caffeine not detected".
4. If any case is positive for a targeted analyte, then attach an overlay of the analyte UV/Vis spectrum with either a library match or a spectrum of the analyte from the calibrator or

QC that is closest in concentration to the case in question. In case of severe detector saturation, taking the spectrum of the leading or trailing edge of the analyte in question and overlaying that may be necessary.

5. Only specimens that have been run along with acceptable controls may be reported. Report concentrations equal to or higher than that of the lowest acceptable control or the lowest acceptable calibrator. Concentrations less than the lowest control or lowest calibrator are reported as “less than X” where “X” equals the concentration of the lowest acceptable calibrator or control.
6. Concentrations are reported to 1 decimal place (e.g., acetaminophen 11.76 mg/L is reported as 11.7 mg/L). Concentrations less than the lowest acceptable control or the lowest acceptable calibrators are reported as “less than X” where “X” equals the concentration of the lowest acceptable calibrator or control.
7. Gastric contents, in addition to the quantitation as mg/kg, is also reported as mg total in the gastric content (concentration in mg/kg x weight in g/1000 g/kg = total drug in mg).
8. Positive results must be confirmed or substantiated by either repeat analysis or by positive results based on a different analytical principle or in an additional tissue.
9. Nicotinamide is frequently observed to be present in case samples. It is identified by reference to a qualitative control and indicated on the result summary form, but is neither reported nor quantitated.

REFERENCES

Agilent 1090 and Agilent 1100 Series II HPLC Systems. Installation Guide.

Agilent 1090 and Agilent 1100 Series II HPLC Systems. Users Guide.

Agilent 1090 and Agilent 1100 Series II HPLC Systems. Standard Operating Procedures.

SPEware Corp. Cerex Applications Manual.

System 48 Processor. Users Guide.

Zymark Turbovap. Users Guide.

REVISION HISTORY:

Ver 03.08.2013	1. Revision history implemented. 2. Nicotinamide has been moved from the low QC to the first calibrator. 3. The number of QC specimens was changed to every 10th sample and as the final sample to be run in a batch.
Ver 04.20.2015	1. Final sample in each sequence/subsequence must be a QC material.
Ver 08.31.2015	1. Inclusion of the UV spectra for compound identification. 2. Minor grammatical changes. 3. Defined roles for first, second and third level reviewers.