

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

**BARBITURATES, PHENYTOIN AND CARBAMAZEPINE  
by  
SOLID PHASE EXTRACTION and HIGH PERFORMANCE LIQUID  
CHROMATOGRAPHY**

## **PRINCIPLE**

This procedure is used for both qualitative and quantitative analysis of barbiturates (butabarbital, butalbital, phenobarbital, pentobarbital, amobarbital, and secobarbital) and commonly used anticonvulsants (phenytoin and carbamazepine). Drugs are identified based on their retention times following separation by high performance liquid chromatography (HPLC) and by the ultraviolet (UV) spectra of eluting peaks using a diode array detector.

This procedure is also used to quantify aldicarb, oxcarbazepine, oxcarbazepine metabolite (monohydroxy derivative, MHD), guaifenesin, glutethimide, mephobarbital, metaxalone, methocarbamol, primidone, thiopental and zonisamide, and to qualitatively identify fluconazole, carbamazepine 10,11-epoxide and carbamazepine 10,11-transdiol. Appropriate calibrators and controls are prepared for analysis of these drugs.

Barbiturates, phenytoin and carbamazepine 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 washed to remove interfering compounds, followed by the elution of the drugs from the column using an organic solvent. The eluate is evaporated and the residue containing the drugs is reconstituted with mobile phase and analyzed by HPLC. Separation of barbiturates, phenytoin and carbamazepine is based on their affinity for the reverse phase C-18 column and the mobile phase. The more polar drugs such as phenobarbital, butabarbital, butalbital have a lower affinity for the ODS-silica and therefore elute relatively faster than less polar drugs such as phenytoin, carbamazepine and secobarbital.

## **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. **Sodium Phosphate Monobasic.** Fisher Scientific or equivalent. FW 137.99  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
3. **Sodium Phosphate Dibasic.** Fisher Scientific or equivalent. FW 141.96  $\text{Na}_2\text{HPO}_4$

4. **Potassium Phosphate.** Baker Analyzed Reagent or equivalent. FW 174.18,  $K_2HPO_3$
5. **O-Phosphoric Acid 85%.** Fisher Scientific or equivalent. FW 98.00.  $H_3PO_4$
6. **Methanol.** Fisher Scientific or equivalent. HPLC grade or better.
7. **Sodium Hydroxide.** Certified ACS Fisher Scientific or equivalent. FW 40.00. NaOH
8. **Sodium Hydroxide Solution, 50% w/w.** Fisher Scientific or equivalent.
9. **Acetonitrile.** Fisher Scientific Optima or equivalent. HPLC grade or better.
10. **Heptane.** Fisher Scientific or equivalent. HPLC grade or better.
11. **Polycrom Clin II Solid Phase Extraction Column.** CEREX.
12. **Nylon filter membranes, 47 mm 0.45  $\mu$ m.** Agilent Technologies part number 9301-0895 or equivalent.
13. **pH meter**
14. **Hexobarbital** (Internal standard). FW 236.3  $C_{12}H_{16}N_2O_3$ . Sigma Chemical Company or equivalent.
15. **Phenobarbital.** FW 232.2  $C_{12}H_{12}N_2O_3$ . Sigma Chemical Company or equivalent.
16. **Butobarbital.** FW 212.3  $C_{10}H_{15}N_2O_3$ . Sigma Chemical Company or equivalent.
17. **Butalbital.** FW 224.25  $C_{11}H_{16}N_2O_3$ . Sandoz Research Institute or equivalent.
18. **Pentobarbital.** FW 226.3  $C_{11}H_{18}N_2O_3$ . Sigma Chemical Company or equivalent.
19. **Amobarbital.** FW 226.27  $C_{11}H_{18}N_2O_3$ . Sigma Chemical Company or equivalent.
20. **Secobarbital.** FW 238.3  $C_{12}H_{17}N_2O_3$ . Sigma Chemical Company or equivalent.
21. **Phenytoin** (diphenylhydantoin) FW 252.26  $C_{15}H_{12}N_2O_2$ . Warner-Lambert Company or equivalent.
22. **Carbamazepine.** FW 236.3  $C_{15}H_{12}N_2O$ . Warner-Lambert Company or equivalent.
23. **Certified Negative Blood.** Previously found to have no interfering peaks. Freeze validated negative blood. Discard after 6 months.
24. **Certified Negative Liver.** Previously found to have no interfering peaks. Freeze validated negative liver. Discard after 6 months.
25. **Certified Negative Brain.** Previously found to have no interfering peaks. Freeze validated negative brain. Discard after 6 months.

## PREPARATION OF CONTROLS

**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.

The control solution is prepared as follows:

1. Label a 50 mL volumetric flask with date prepared, initials of the person who prepared the solution, lot number, solvent and expiration date.

2. Accurately weigh **50.0 mg of phenobarbital** and transfer into a clean 50 mL labeled volumetric flask.
3. Accurately weigh **50.0 mg of butobarbital** and transfer into the same 50 mL volumetric flask.
4. Accurately weigh **50.0 mg of butalbital** and transfer into the same 50 mL volumetric flask.
5. Accurately weigh **50.0 mg of pentobarbital** and transfer into the same 50 mL volumetric flask.
6. Accurately weigh **50.0 mg of amobarbital** and transfer into the same 50 mL volumetric flask.
7. Accurately weigh **50.0 mg of phenytoin** and transfer into the same 50 mL volumetric flask.
8. Accurately weigh **50.0 mg of carbamazepine** on a piece of weighing paper and transfer into the same 50 mL volumetric flask.
9. Accurately weigh **50.0 mg of secobarbital** and transfer into the same 50 mL volumetric flask.
10. Add 40 mL of methanol into the 50 mL volumetric flask.
11. Mix solution by mechanical stirrer until dissolved.
12. Remove stirrer and using a squirt bottle rinse with methanol into the volumetric flask. Q.S. to 50 mL mark with methanol.
13. This 1000 mg/L barbiturate working control solution is transferred into appropriately labeled headspace vials and sealed with a Teflon septum and aluminum seal. The solution is stable for one year. Store at 2-8 °C. Vial labels include date prepared, expiration date, initials of person who prepared the solution, lot number, solvent, storage condition and bottle number with total number of bottles.
14. A qualitative control containing acetaminophen, salicylic acid, theophylline, and caffeine is extracted with each batch, to provide retention time and UV spectra to identify these compounds if present in case samples. If any of these drugs are qualitatively identified, they will be scheduled for quantitation by the appropriate method.

**Note:** See Acmp by SPE SOP for detailed information on materials and preparation of Control Solution and spiking of the low control in blank matrix.

## **PREPARATION OF CALIBRATORS**

### **Calibrator stock solution, 1000 mg/L**

Follow same steps for the preparation of the control solution.

### **Calibrator Working Solution, 100 mg/L**

Dilute 10 mL of 1000 mg/L calibrator stock solution to 100 mL volumetric flask with methanol. This 100 mg/L barbiturate working calibrator solution may then be transferred into an appropriately labeled headspace vial and sealed with a Teflon septum and aluminum seal. Solution is stable for one year. Store at 2-8 °C. Label with date prepared, expiration date,

initials of person who prepared the solution, lot number, solvent, storage conditions and bottle number with total number of bottles.

### **INTERNAL STANDARD (2000 MG/L)**

1. Label a 100 mL volumetric flask with date prepared, initials of the person who prepared the solution, lot number, solvent and expiration date.
2. Weigh 200.0 mg of hexobarbital and transfer to a 100 mL volumetric flask.
3. Add 80 mL of methanol.
4. Stir by magnetic stirrer until dissolved.
5. Remove magnetic stirrer and using a squirt bottle rinse with methanol into the volumetric flask. Q.S. to 100 mL with methanol.
6. Transfer the Internal Standard solution to headspace vials and label 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 10N SODIUM HYDROXIDE**

1. Add 400 gms of sodium hydroxide pellets to a 1000 mL volumetric flask.  
**CAUTION: This reaction is highly exothermic.**
2. Q.S to 1000 mL with distilled water and mix well.
3. Transfer the solution to a storage bottle labeled with who prepared the solution, date prepared and expiration date (one year after the date of preparation.)

### **PREPARATION OF EXTRACTION SOLVENT**

#### **Eluting Solvent**

1. Add 50 mL of ethyl acetate to a glass bottle.
2. Add 50 mL of heptane and mix well.
3. Prepare daily when needed.

#### **Mobile Phase**

The mobile phase is made up of 45% mobile phase C and 55% mobile phase D. See mobile phase preparation below.

#### **Mobile Phase C**

1. Add 2400 mL of deionized water to a 4 L beaker.
2. Add 13 mL of 85% phosphoric acid to the 4 L beaker while mixing with a mechanical stirrer for 5 minutes.

3. Add enough 10N sodium hydroxide (approximately 17-18 mL) to increase the pH to 3. Check pH with pH Meter.
4. Add 1500 mL of acetonitrile to the 4 L beaker.
5. Stir mobile phase for 30 minutes.
6. Store the mobile phase in a 4 liter brown bottle at room temperature. Label the storage reservoir with the lot number, initials of person who prepared the solution and date prepared. Discard after 3 months.
7. Filter before use.

#### **Mobile Phase D**

1. Add 3600 mL of deionized water to a 4 L beaker.
2. Add 19.5 mL of 85% phosphoric acid to a 4 L beaker while mixing by mechanical stirrer for 5 minutes.
3. Add enough 10N sodium hydroxide (approximately 28 mL) to increase the pH to 3. Check pH with pH meter.
4. Add 400 mL of acetonitrile to the 4 L beaker.
5. Stir mobile phase for 30 minutes.
6. Store the mobile phase in a 4 liter brown bottle at room temperature. Label the storage reservoir with the lot number, initials of person who prepared the solution and date prepared. Discard after 3 months.
7. Filter before use.

#### **Pre-mixed Mobile Phase (for reconstitution)**

1. Add 3.5 mL of filtered mobile phase C to a capped plastic specimen cup.
2. Add 6.5 mL of filtered mobile phase D to the capped plastic specimen cup.
3. Mix by vortexing

#### **Phosphate Buffer, 100mM, pH 6.0**

1. Add approximately 800 ml of deionized H<sub>2</sub>O to a 1 L volumetric flask
2. Weight 1.70 grams of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, sodium phosphate monobasic.
3. Weight 12.14 grams Na<sub>2</sub>HPO<sub>4</sub>, Sodium Phosphate Dibasic.
4. Mix by mechanical stirrer for at least 1 hour
5. Q.S. to 1 L with deionized H<sub>2</sub>O
6. Check with pH meter. If necessary, adjust pH to 6.0 plus or minus 0.1 with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH).
7. Transfer to a 1 L repipettor. Label the repipettor with the lot number, initials of person who prepared the solution and date prepared. Discard after 6 months.

#### **Wash solution, 25:75 Methanol:deionized H<sub>2</sub>O**

Add 15 mL methanol to 45 mL of deionized H<sub>2</sub>O in a graduate cylinder. Mix by shaking.

### Specimen Preparation

Blood	1.0 mL of the undiluted specimen
Urine	1.0 mL of the undiluted specimen
Brain	1.0 mL of a 1:3 homogenate
Gastric Contents	1.0 mL of a 1:10 dilution
Liver	1.0 mL of a 1:5 homogenate

### Dilution of specimens

Specimens are diluted as follows:

Brain 1:3	3.0 g of brain homogenized with 6 mL of deionized water.
Liver 1:5	2.0 g of liver homogenized with 8mL of deionized water.
Gastric 1:10	2.0 mL of liquid <i>q.s.</i> to 20 mL of deionized water, or 1.0 g of a solid specimen homogenized with 9 mL of deionized water.

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

**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 dilution factors other 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 exception on sequence list.

### EXTRACTION PROCEDURE

1. All reagents, samples, controls and calibrators must equilibrate to room temperature before 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 so 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. Pipet 1 mL of a matching negative matrix into a properly labeled 16 by 125 mm test tube. Add 10 µL of the 100 mg/L barbiturate working solution to the test tube. Add 4 µL of a control solution containing 2500 mg/L of acetaminophen and salicylic acid and 1000 mg/L of caffeine and theophylline to this calibrator (**Calibrator I**, 1 mg/L).

4. Pipet 1 mL of a matching negative matrix into a properly labeled 16 by 125 mm test tube. Add 5  $\mu$ L of the 1000 mg/L barbiturate working solution to the test tube. (**Calibrator II**, 5 mg/L).
5. Pipet 1 mL of a matching negative matrix into a properly labeled 16 by 125 mm test tube. Add 10  $\mu$ L of the 1000 mg/L barbiturate working solution to the test tube. (**Calibrator III**, 10 mg/L).
6. Pipet 1 mL of a matching negative matrix into a properly labeled 16 by 125 mm test tube. Add 25  $\mu$ L of the 1000 mg/L barbiturate working solution to the test tube. (**Calibrator IV**, 25 mg/L).
7. Pipet 1 mL of a matching negative matrix into a properly labeled 16 by 125 mm test tube. This is the negative control (blank).
8. Low and high control samples are run with each batch. Add 5  $\mu$ L of the 1000 mg/L barbiturate control working solution to a properly labeled test tube containing 1.0 mL of matching negative matrix. This is the 5 mg/L control. Add 15  $\mu$ L of the 1000 mg/L barbiturate control working solution to a properly labeled test tube containing 1.0 mL of matching negative matrix. This is the 15 mg/L control. Blank and controls for each matrix represented by case samples must be analyzed in the batch.

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

*Tissue samples may be quantitated by running against blood calibrators provided matrix matched negative and positive controls are included and pass all acceptance criteria.*

9. Add 7.5  $\mu$ L of the internal standard to each test tube.
10. Add 3mL of 100 mM Phosphate Buffer (pH 6.0) to each tube.
11. Mix by Vortex.
12. Sonicate for 20 minutes
13. Centrifuge at  $\approx$  3000 rpm for 30 minutes.

### Apply Sample

1. Label solid phase columns & conical test tubes. This can be done during sonication & centrifugation.
2. Place solid phase columns on rack, then place the rack on top of waste rack of processor.
3. Decant the contents of each 16 x 125 mm culture tubes into the corresponding solid phase column.
4. Apply positive pressure to achieve a flow of  $\approx$  1 mL/min.

### Column Wash

1. Add 1 mL of deionized water to column.
2. Apply positive pressure to achieve a flow of  $\approx$  1 mL/min.
3. Add 1 mL of 25:75 methanol:H<sub>2</sub>O to column.
4. Apply positive pressure to achieve flow of  $\approx$  1 mL/min.

5. Increase pressure to 20 psi to dry column for approximately 20 minutes.
6. Add 1 mL of heptane to column.
7. Apply positive pressure to achieve flow of  $\approx 1$  mL/min.
8. Increase pressure to 20 psi to dry column for approximately 20 minutes.

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

## Elution

1. Remove waste rack.
2. Place rack of properly labeled 10 mL conical centrifuge tubes on processor.
3. Place rack of solid phase extraction columns on top of the conical centrifuge tube rack. Verify that each column corresponds to its equivalent labeled test tube.
4. Elute column with 2.0 mL of 50/50 heptane/ethyl acetate solution into its corresponding conical centrifuge tube.

**Note:** prepare eluting solution fresh day of use.

5. 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  $\sim 5$  psi and use the processor to force samples through.
6. Dry in concentrator with a gentle flow of nitrogen at  $\leq 40$  °C.

**Note:** It is very important that temperature does not exceed 40 °C.

7. Remove tubes once dry.

## Reconstitute Samples

1. With a calibrated Eppendorf Pipet, add 200  $\mu$ L of premixed mobile phase.
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 or #4:** 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 with Agilent Chemstation software.  
HPLC Method. BARB.M

**Note:** The BARB.M procedure targets analysis of barbiturates (butabarbital, butalbital, phenobarbital, pentobarbital, amobarbital, secobarbital) and the anticonvulsants phenytoin and carbamazepine). The procedure may be utilized for qualitative and quantitative analysis of additional barbiturates and other drugs.

## 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 recorded on the autosampler vials. This data will be transferred to the sequence list, which will be compared with the data recorded on the autosampler vials.
2. Calibrators are injected in order of increasing concentration. A blank is injected after the highest calibrator.
3. Unknown samples are injected next.
4. The quality control samples are placed at every 10<sup>th</sup> sample and at the end of each sequence/sub sequence.

## INSTRUMENT PRERUN PROCEDURE

### LC 1100 Instrument #3 or #4 Barbiturate Parameters

#### 1. Pump (PV5):

Stop time	26.00 min
Post time	1.00 min
Flow	1.50 mL/min
Min. pressure	10 bar
Max. pressure	400 bar
Column temperature	50 °C
Solvent A	35.00 % (Mobile phase C)
Solvent B	0.00 % (Bottle B, H <sub>2</sub> O)
Solvent C	65.00 % (Mobile phase D)

#### 2. Injector:

Injection volume	50.0 µL
Draw speed	200 µL/min

### 3. Mobile Phase Time Table:

The run is isocratic. The flow is 1.5 mL/min for approximate 30 minute run time.

### 4. Signals:

	<u>Sample, Bw</u>	<u>Reference, Bw [nm]</u>
A:	205 14	550 6

### 5. Curve Type:

Power, using 4 points of calibration

### 6. Spectrum:

Store	All
From	190 nm
To	340 nm
Step	2 nm
Threshold	1.00 mAU

### 8. Diode Array Detector Setting

Stop time	26.00 min (as pump)
Post time	Off
Peak width	>0.05 min

## TEST RUN

In order to ensure that the HPLC instruments are in working condition, the analyst is required to inject 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 an aging or contaminated column).

Click on **METHOD** and load the **BARB** 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 1 mg/L) . Under **comment field** note Barb 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. Usually, 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.
8. Click on **Sequence** and then **Sequence Table**. The **Sequence Table** screen contains includes the following columns for instrument #1:

SEQ line	Vial #	Sample name	Method	Inj/vial	Sample type	Cal level	dil	Update RF	Update RT
1	1	1 mg/L cal bar	BARB.M	1	calib	1		replace	replace
2	2	5 mg/L cal bar	BARB.M	1	calib	2		replace	replace
3	3	10 mg/L cal bar	BARB.M	1	calib	3		replace	replace
4	4	25 mg/L cal bar	BARB.M	1	calib	4		replace	replace
5	5	Bld blank	BARB.M	1	control				
6	6	1-11-9998 hrt bld	BARB.M	1	sample				
7	7	QC 10/25 mg/L	BARB.M	1	control				
8	8	1-11-9998 fem bld	BARB.M	1	sample				
9	9	QC 15 mg/L	BARB.M	1	control				
10	10	1-11-9999 hrt bld	BARB.M	1	sample				
			shutdown						

The Sample Name field may 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, followed by instrument shutdown.) Click on OK after all samples have been entered.

Save the sequence into a file named the same as the data subdirectory. Click on **SEQUENCE** then **SAVE AS**. For example, 041711.S or any additional sequence run in the same date in the same instrument will put a letter after the date. For example, 041711A.S.

Click on **SEQUENCE, PRINT SEQUENCE**, highlight **SEQUENCE PARAMETERS, METHOD AND INJECTION INFO PART, CALIBRATION PART AND QUANTITATION PART**. Then click **PRINT** to print the sequence on the printer.

### BATCH ANALYSIS

1. Place sample bottles in the autosampler tray, using the printed copy of the sequence to ensure that each bottle is placed in the correct position and that the sample name is checked against the bottle 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

1. All processing and review should be performed on the Chemstation. The processed data files are then archived on the ECM.
2. 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 peak(s): the sequence may be reinjected at 25 °C.
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 reinjection fails, repeat analysis.

**Note:** When samples are reinjected, in addition to the reinjected sample, reinject a negative control, a calibrator and the associated positive controls.

## ACCEPTANCE CRITERIA

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

1. A power curve is calculated by the processing method during calibration. Four calibrators (1 mg/L, 5 mg/L, 10 mg/L, 25 mg/L) are used to establish the calibration curve. All calibrators should quantitate at  $\pm 20\%$  of the weighed in target. One calibrator may be dropped if the appropriate acceptance criteria are not met.
2. Regression correlation ( $r^2$ ) must be equal to or higher than 0.98 for each analyte. If  $r^2$  is below 0.98, only qualitative results may be reported.

1. Internal standard (IS) response comparable to the response for the matrix calibrators (internal standard recovery/response shall be  $\pm 30\%$  of the selected internal standard).
2. 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.
3. Positive blood controls must be within  $\pm 20\%$  of the weighed in target. Tissue positive controls must be within  $\pm 30\%$  of the weighed in target.
4. All negative matrix matched controls must have no interfering peaks in the area of the target analyte.
5. Peak shape should be symmetrical and Gaussian in appearance, and the retention times of the analyte peaks are  $\pm 2\%$  of the calibrator retention times.
6. 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 are confirmed by alternate methods (e.g., TLC or GCMS).
7. 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. If acetaminophen, salicylic acid, caffeine and/or theophylline are presumptively detected by corresponding retention time and UV spectra match, the detection of the analyte is noted on the report and the sample scheduled for the appropriate quantitative method for the analyte.

## **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;

8. 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.
9. 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
10. 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.

11. 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.
12. Review QC samples, determine if the controls meet all acceptance criteria and verify the concentration of the components.
13. 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.
14. 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 toxicology report sheet as "barbiturates, phenytoin and carbamazepine not detected."
4. Concentrations of any analytes below 1 mg/L are reported as "less than 1 mg/ L."
5. Concentrations greater than or equal to 1 mg/L are reported to 1 decimal place (e.g., pentobarbital 1.76 mg/L is reported as 1.7 mg/L).
6. Gastric contents, in addition to the quantitation as mg/kg, is also reported as total mg in the gastric content (concentration in mg/kg x weight in kg = total drug in mg).
7. 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.

### **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.

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## REVISION HISTORY

Ver 03.08.2013

1. Revision history implemented.
2. The semi quantitative solution for salicylates, acetaminophen, caffeine and theophylline has been moved to Calibrator I.
3. HPLC column length decreased from 15 cm to 7.5 cm.
4. The number of QC specimens was changed to every 10<sup>th</sup> sample and as the final sample to be run on a batch.

Ver 03.28.2014

1. Corrected the 10 N NaOH solution preparation formula.

Ver 04.20.2015

1. Changed position of QC materials from randomly throughout the sequence to every 10<sup>th</sup> sample.
2. Included an end of run QC for every sequence/sub sequence.

Ver 08.31.2015

1. Revised internal standard acceptance criteria.

Ver 09.14.2015

1. Inclusion of First, Second and Third level review.

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