IDENTIFICATION AND CONFIRMATION OF ACETAMINOPHEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

32.1 POLICY

This test method may be used to identify and quantify acetaminophen in biological samples. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

32.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and confirmation/quantification of acetaminophen present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of acetaminophen.

32.3 PRINCIPLE

The targeted compound, acetaminophen (ACT) and internal standard, phenacetin (PCN), are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, the specimens, now termed extracts, are injected into a high pressure liquid chromatograph (HPLC) where they are separated between a liquid mobile and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time, by which acetaminophen is identified.

32.4 SPECIMENS

32.4.1 The specimen volume is 0.5 mL.

32.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

32.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

32.4.4 Analysis of larger specimen volumes must be approved and documented.

32.5 REAGENTS, MATERIALS AND EQUIPMENT

32.5.1 REAGENTS

32.5.1.1 Acetic acid, glacial

32.5.1.2 0.2% acetic acid

Add 2 mL of glacial acetic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Store the solution in a glass bottle at room temperature for up to one year. Filter this solution prior to use on the HPLC. Adjustments to final volume are permitted as long as the proportions are maintained.

32.5.1.3 Acetonitrile

32.5.1.4 Certified blank blood

32.5.1.5 Deionized water (DI H_2O)

32.5.1.6 Ethyl acetate

32.5.1.7 Heptane

32.5.1.8 Methanol

32.5.1.9 Reconstitution solution, 50:50 methanol:0.2% acetic acid

Add 2 mL of methanol to 2 mL of 0.2% acetic acid in a glass tube, cap and mix. Adjustments to final volume are permitted as long as proportions are maintained. The solution is for use on date of preparation only.

32.5.1.10 0.1M sodium acetate buffer (pH 4.5)

Dissolve 2.93 g sodium acetate trihydrate in 400 mL DI H_2O. Add 1.62 mL glacial acetic acid. Dilute to 500 mL with DI H_2O and mix. Check pH and, if necessary, adjust to 4.5 ± 0.2. Store the buffer in glass or plastic bottle at room temperature for up to one year.

32.5.1.11 Sodium acetate trihydrate

32.5.2 MATERIALS

32.5.2.1 Autosampler vials, inserts and caps

32.5.2.2 Disposable 16 x 100mm tubes with safety closures

32.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

32.5.2.4 Disposable pipette tips

32.5.2.5 Disposable serological pipettes

32.5.2.6 HPLC Column (Zorbax Eclipse XDB-C8, 100 mm x 3.0 mm, d_p=3.5 µm or equivalent)

32.5.2.7 Laboratory glassware (graduated cylinders, flasks)

32.5.2.8 Volumetric glassware (flasks)

32.5.3 EQUIPMENT

32.5.3.1 Agilent HPLC (Agilent 1100 series or equivalent)

32.5.3.2 Calibrated, adjustable air-displacement pipettes

32.5.3.3 Centrifuge

32.5.3.4 Evaporator (Caliper LS, formerly Zymark, TurboVap)

32.5.3.5 pH Meter and/or indicating pH paper

32.5.3.6 Rotary mixer

32.5.3.7 Vacuum aspirator

32.5.3.8 Vortex mixer

32.6 STANDARDS AND CONTROLS

32.6.1 STANDARDS

32.6.1.1 Reference materials (RMs, also referred to as stock standards) are used for the preparation of the working standard, which in turn is used to produce calibrators, and the stock internal standard. Certified reference materials (CRMs) are used directly to produce positive controls.

32.6.1.2 Stock standards and stock internal standard are purchased from an approved reference material supplier and include the following:

a.	Acetaminophen	1.0 mg/mL and solid RM
b.	Phenacetin	solid RM

Note: Phenacetin internal standard and acetaminophen working standard RMs are purchased as solid materials, and are weighed at the time of standard preparation. The acetaminophen CRM (1.0 mg/mL) is used directly to produce the positive controls.

32.6.1.3 Working standard (1.0 mg/mL)

a. Using a calibrated balance, weigh 25 mg of acetaminophen and add to a class-A 25 mL volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 1.0 mg/mL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

32.6.1.4 Working control standard (1.0 mg/mL)

a. The CRM from an approved reference material supplier is the working control standard.

32.6.1.5 Stock internal standard (5.0 mg/mL)

a. Using a calibrated balance, weigh 50 mg of phenacetin and add to a class-A 10 mL volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the stock internal standard is 5.0 mg/mL. The stock working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

32.6.1.6 Working internal standard (0.1 mg/mL)

a. Using a calibrated pipette, measure 1.0 mL stock internal standard into a class-A 50 mL volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 0.1 mg/mL. The working internal standard is stored in the freezer in an amber bottle and

expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

32.6.2 CONTROLS

32.6.2.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

32.6.2.2 Positive Controls

a. Three positive whole blood controls are tested with every confirmation batch and one positive whole blood control is tested with every screening batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The preparation of the positive whole blood controls is detailed in 32.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
d. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

32.7 SAMPLE PREPARATION

NOTE: If phenobarbital or salicylates have been identified in a case sample, an alternative method must be used for quantification of acetaminophen (See 32.11.5).

32.7.1 Label a clean 16 x 100mm tube for each member of the test batch (i.e. calibrator, control, case sample).

32.7.2 Place 1 mL sodium acetate buffer (pH 4.5) in to each tube.

32.7.3 Add 0.5 mL of certified blank blood into each of the calibrator and positive and negative control tubes.

32.7.4 Prepare a 1:2 dilution of the working standard. (0.5 mg/mL)

a. Using a calibrated pipette, combine 0.3 mL of the working standard with 0.3 mL of acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

32.7.5 Prepare a 1:20 dilution of the working standard. (0.05 mg/mL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:2 dilution with 0.9 mL of acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

32.7.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator Description	Volume Added (µL)	Standard Source
Calibrator 1 (5.0 mg/L)	50	0.05 mg/mL
Calibrator 2 (10 mg/L)	100	0.05 mg/mL
Calibrator 3 (25 mg/L)	25	0.5 mg/mL
Calibrator 4 (50 mg/L)	50	0.5 mg/mL
Calibrator 5 (100 mg/L)	50	1.0 mg/mL

32.7.7 Prepare a 1:2 dilution of the working control standard (CRM, 0.5 mg/mL).

a. Using a calibrated pipette, combine 100 µL of the working control standard with 100 µL of acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

32.7.8 Using the working control standard and the prepared dilution, spike the controls according to the following table.

Control Description	Volume Added (µL)	Standard Source
Control 1 (15 mg/L)	15	0.5 mg/mL
Control 2 (30 mg/L)	30	0.5 mg/mL
Control 3 (80 mg/L)	40	1.0 mg/mL

32.7.9 Sample 0.5 mL of each case sample into its respective tube.

32.7.10 Add 50 µL of the working internal standard to each tube. The final concentration of phenacetin is 10 mg/L.

32.7.11 Briefly vortex mix and let stand for 5 minutes.

32.7.12 Add 3 mL ethyl acetate to each tube.

32.7.13 Cap the tubes and place on rotary mixer for a minimum of 10 minutes.

32.7.14 Centrifuge the tubes for 10 minutes at 3500 rpm.

32.7.15 Transfer the supernatant (organic) layer to clean, labeled 10 mL centrifuge or screw cap tubes.

32.7.16 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

32.7.17 Add 100 µL methanol and wash with 500 µL heptane.

32.7.18 Briefly vortex mix.

32.7.19 Centrifuge the tubes for 5 minutes at 2000 rpm.

32.7.20 Aspirate the top layer (heptane) to waste.

32.7.21 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

32.7.22 Reconstitute extracts by the addition of 100 µL reconstitution solvent and briefly vortex mix.

32.7.23 Centrifuge at 3500 rpm for 10 minutes.

32.7.24 Transfer the extracts to labeled autosampler vials and cap.

32.8 INSTRUMENTAL PARAMETERS

The instrumental parameters for the HPLC can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is ACET.M for each line.

32.9 DATA ANALYSIS

32.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.

32.9.2 Quantitative calculations are generated by internal standard, multi-point, liner regression with equal weighting, and origin ignored. Calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

32.9.3 Printed reports for each vial in the batch, including calibration curves are generated for review.

32.9.4 Technical review of the batch is conducted according to the criteria listed below.

32.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

32.10.1 Calibrators and calibration curves

32.10.1.1 Chromatographic peaks for acetaminophen and phenacetin shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders.)

32.10.1.2 Retention times shall be within ±5%, and the ratio of the absorbance measured at two wavelengths shall be within ±20%, of those in calibrator 4. These are inclusive ranges.

Note: Acetaminophen and phenacetin must have an “X” under the “IS Q” heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

32.10.1.3 Quantitative results for acetaminophen in each calibrator shall be within ±20% of the target value with the exception of calibrator 1, where results shall be within ±25% of the target. These are inclusive ranges. Result comparisons will use values truncated to one decimal place.

32.10.1.4 The calibration curve for acetaminophen shall have a correlation coefficient ≥0.99.

NOTE: Verify equal weighting with origin ignored/linear curve fit on page 1 of the calibration table/curve printout.

32.10.2 Controls

32.10.2.1 The negative control(s) shall not identify acetaminophen above the limit of detection. Identification is based on a) acceptable retention time matching,

b) acceptable wavelength absorbance ratio match, c) criteria listed in 32.10.1.1 above.

32.10.2.2 Positive controls

a. Chromatographic peaks for acetaminophen and phenacetin shall appear symmetrical.

b. Retention times shall be within ±5%, and the ratio of the absorbance measured at two wavelengths shall be within ±20%, of those in calibrator 4. These are inclusive ranges.

Note: Acetaminophen and phenacetin must have an “X” under the “IS Q” heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

c. Quantitative result for acetaminophen in each control shall be within ±20% of the target value. These are inclusive ranges. Result comparisons will use values truncated to one decimal place.

d. At least two positive controls must meet these criteria for acetaminophen for the batch to be accepted.

32.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

32.11.1 Any chromatographic peak for acetaminophen identified shall appear symmetrical.

32.11.2 The retention time for acetaminophen and phenacetin are within ±5%, and the ratio of the absorbance measured at two wavelengths shall be within ±20%, of those in calibrator 4. These are inclusive ranges.

Note: Acetaminophen and phenacetin must have an “X” under the “IS Q” heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

32.11.3 The quantitative result for acetaminophen must be within the dynamic range for the test method.

32.11.4 When dilutions of case samples are tested, the quantitative result before multiplication shall be within the dynamic range of the test method.

32.11.5 The presence phenobarbital or salicylates in a sample has been shown to interfere with phenacetin (see NOTE in 32.7). In these cases, an alternative confirmation/quantitation method may be used to quantify acetaminophen.

32.12 REPORTING

32.12.1 Quantitative Reporting

32.12.1.1 Results are reported in units of milligrams per liter (mg/L), to no more than two significant figures.

32.12.1.2 When multiple dilutions are analyzed, the smallest dilution within the dynamic ranges is reported.

32.12.1.3 If acetaminophen is initially identified using this method, the result must be confirmed using this or another test method on a separate sampling before reporting (e.g. EMIT, Rapid Color Test, or FID).

32.12.2 Qualitative Reporting

32.12.2.1 To appropriately identify and report acetaminophen as present in a case sample, the following must be demonstrated:

a. Chromatography must meet criteria for acceptance found in 32.11.1, and 32.11.2 above, and be ≥ the LLOQ for the batch.

32.12.2.2 If acetaminophen is initially identified using this method, the result must be confirmed using this or another test method on a separate sampling before reporting. (e.g. EMIT, Rapid Color Test, or FID) on a separate sampling before reporting.

32.13 METHOD PERFORMANCE

32.13.1 Limit of detection (LOD): 2.0 mg/L

32.13.2 Lower limit of quantitation (LLOQ): 5.0 mg/L

32.13.3 Dynamic Range: 5.0 -100 mg/L

32.13.4 Upper limit of quantitation (ULOQ): 100 mg/L

32.14 TRACEABILITY

Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

APPENDIX A
INSTRUMENTAL PARAMETERS

HIGH PRESSURE LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.500 mL/min
Solvent A	0.2% Acetic Acid
Solvent B	Methanol
Initial Composition	70% (A), 30% (B)
0 - 2.0 min	%B increased to 50%
Run Time	8.0 min
Re-equilibration Time	5.0 min
Column Temp	35°C
Autosampler	
Injection Volume	5.0 µL
Needle Wash	Water
DAD	
Signals (λ)	250 nm (primary)
	240 nm (secondary)

LIST OF CHANGES

Revision Date	Description	Page Number
2/23/15	Method approved by Washington State Toxicologist. See DRA dated 2/5/15. Method released for use in evidentiary testing on 2/23/15.	All
3/16/15	Revised to change “pressure” in title to “performance,” and to include storage and expiration information for 0.2% acetic acid in 32.5.1.2 and the addition of 0.5 mL case sample to its respective tube in 32.7.9.	1, 5