IDENTIFICATION AND CONFIRMATION OF ACETAMINOPHEN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

32.1 METHOD

This test method may be used to identify or confirm the presence of acetaminophen (ACT) in biological specimens. ACT and phenacetin internal standard (PCN) are isolated from biological matrices by liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a diode-array detector (DAD).

32.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Specimens that contain phenobarbital and/or salicylates, in addition to acetaminophen, must be analyzed using an alternative test method for quantitation of acetaminophen.

NOTE: Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in serum/plasma and liver (tissue) homogenate specimens (see 32.4.2 and 32.4.3).

32.3 REAGENTS, MATERIALS AND EQUIPMENT

32.3.1 REAGENTS

NOTE: Organic solvents used are reagent grade.

- Acetic acid, glacial
- 0.2% Acetic acid

  Add 2 mL glacial acetic acid to 800 mL DI H_2O in a 1 L glass flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the solution in an amber glass bottle at room temperature for up to one year.
- Certified blank blood and/or other biological matrices
- Deionized water (DI H_2O), laboratory general-use
- Ethyl acetate (EtAC)
- Heptane
- Methanol (MeOH)
- Reconstitution solution, 50:50 methanol:0.2% acetic acid

  Add 2 mL MeOH to 2 mL 0.2% acetic acid in a glass tube, cap and mix. The solution is for use on date of preparation only.

- 0.1M Sodium acetate buffer (pH4.5)

  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 with glacial acetic acid. Store the buffer in a glass bottle at room temperature for up to one year.

- Sodium acetate trihydrate

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

32.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Disposable serological or Pasteur pipettes
- Glass autosampler vials with inserts and caps
- HPLC Column, Agilent Zorbax Eclipse Plus XDB-C8, 100 x 3.0 mm, dp = 3.5 µm, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

32.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter or pH indicating paper, rotary mixer, solvent filtration apparatus, vacuum aspirator, vortex mixer)

32.4 STANDARDS, CALIBRATORS AND CONTROLS

32.4.1 STANDARDS

- Working standard (WS): 1 mg/mL
- Working control standard (CRM): 1 mg/mL
- Stock internal standard (PCN): 5 mg/mL
- Working internal standard: 0.1 mg/mL

NOTE: Working standard solution is prepared from solid certified reference material. Alternatively, a certified reference material (CRM) may be used directly as the working standard. A CRM is used directly as the working control standard. Where a CRM is used as the working standard, it must be a different lot number than the CRM used in positive control preparation.

32.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 32.5 SAMPLE PREPARATION. Quantitation in serum/plasma or liver (tissue) specimens requires that a full calibration curve be prepared in blank matrix. If testing only serum/plasma or only liver (tissue) homogenate specimens, a whole blood calibration curve is not required.

32.4.3 CONTROLS

32.4.3.1 At least one negative whole blood control and three positive whole blood controls are included in the batch, prepared as described in 32.5. For quantitative analysis of serum/plasma specimens or liver (tissue) homogenate specimens only, whole blood controls are not required.

32.4.3.2 Controls (positive and negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls.

32.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch.

32.4.3.4 For quantitative analysis of serum/plasma specimens or liver (tissue) homogenate specimens, matrix-matching of the full calibration curve, negative control and positive controls (to meet 10% and bracket specimens in that matrix) is required.

32.5 SAMPLE PREPARATION

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

32.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

32.5.2 Add 1 mL sodium acetate buffer (pH4.5) into each tube.

32.5.3 Using a calibrated pipette, add 0.5 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes and the negative control tube(s).

32.5.4 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 MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

32.5.5 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 MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

32.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 5.0 mg/L	50	0.05 mg/mL	1:20
Calibrator 2 – 10 mg/L	100	0.05 mg/mL	1:20
Calibrator 3 - 25 mg/L	25	0.5 mg/mL	1:2
Calibrator 4 - 50 mg/L	50	0.5 mg/mL	1:2
Calibrator 5 - 100 mg/L	50	1.0 mg/mL	WS

32.5.7 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 CRM with 100 µL MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

32.5.8 Using a calibrated pipette, spike the positive controls according to the following table, using the CRM and prepared dilution.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of CRM (or CRM)
Control 1 – 15 mg/L	15	0.5 mg/mL	1:2
Control 2 – 30 mg/L	30	0.5 mg/mL	1:2
Control 3 - 80 mg/L	40	1.0 mg/mL	CRM

32.5.9 Using a calibrated pipette, sample 0.5 mL of each case sample into its respective tube.

32.5.10 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the phenacetin internal standard is 10 mg/L.

32.5.11 Briefly vortex mix and let stand for 5 minutes.

32.5.12 Add 3 mL ethyl acetate to each tube.

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

32.5.14 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

32.5.15 Transfer the supernatant (organic) layer to a new, labeled centrifuge or screw-cap tube.

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

32.5.17 Add 100 µL MeOH and vortex mix.

32.5.18 Add 500 µL heptane and vortex mix.

32.5.19 Centrifuge the tubes for 5 minutes at 2000 rpm.

32.5.20 Aspirate the heptane (top) layer to chemical waste.

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

32.5.22 Reconstitute the extracts with the addition of 100 µL reconstitution solvent (50:50 MeOH:0.2% acetic acid) and briefly vortex mix.

32.5.23 Centrifuge the tubes at 3500 rpm for 10 minutes.

32.5.24 Transfer the extracts to labeled glass autosampler vials with inserts and cap.

32.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – ACET (instrumental parameters in Appendix A)
- Calibration curve – linear, equal weighting

  NOTE: Verify equal weighting with origin ignored/linear curve fit on page 1 of the calibration table/curve printout.
- Updating calibrator (retention times ±5%, wavelength absorbance ratios ±20%) – Cal 4

  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.
- Result comparisons – truncated to one decimal place in units of mg/L

32.7 REPORTING

Results are reported in units of milligrams per liter (mg/L), truncated to two significant figures.

NOTE: If acetaminophen is initially identified using this method, the result must be confirmed using a test method that employs mass spectrometry, on a separate sampling of the specimen, in order to report results from this test.

32.8 METHOD PERFORMANCE

- Limit of detection: 2.0 mg/L

- Lower limit of quantification: 5.0 mg/L
- Dynamic range: 5.0 – 100 mg/L
- Upper limit of quantitation: 100 mg/L

32.9 REFERENCES

- A. Black, B.E. O'Reilly, in-house method development
- E. Pufal, M. Sykutera, A. Dafalla, G. Rochholz, H.W. Schutz, K. Sliwka, H.J. Kaatsch, *Determination of paracetamol (acetaminophen) in different body fluids and organ samples after solid-phase extraction using HPLC and an immunological method*, Fresenius J Anal Chem. **367**: 596-599 (2000).
- J. West, *Rapid HPLC analysis of paracetamol (acetaminophen) in blood and postmortem viscera*, J Anal Tox. **5**: 118-121 (1981).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH – DAD DETECTOR

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.2% Acetic acid
Solvent B	MeOH
Initial composition	70% A, 30% B
0 – 2.0 min	% B increased to 50%
Run time	8.0 min (50% B)
Re-equilibration time	5.0 min
Column temp	35°C
Autosampler	
Injection volume	5.0 µL
Flush-port solvent	DI H_2O
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	"Performance" replaced "Pressure" in title of procedure to match original DRA. Expiration and storage information was added for 0.2% acetic acid and a step was added to 32.7 for addition of 0.5 mL specimen sample to its respective tube.	1, 5
9/30/16	Added "Printed Copies are Uncontrolled" to the footer. Added note regarding working internal standard expiration date in 32.6.1.6.c. Edited 32.12.1.1 to reflect that two significant figures are used for reporting. Other minor edits throughout.	All
7/10/17	Added wording to 32.4.3 regarding dilution and standard volume testing. Added expiration specifications for RMs and CRMs in 6.1.3.c, 6.1.4.a and 6.1.5.c. Added section 32.6.2 CALIBRATORS. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout SAMPLE PREPARATION in 32.7 and use of methanol only for dilution preparation in 32.7.4, 32.7.5 and 32.7.7. Edited 32.10.2.2.d to indicate all positive controls must pass to report quantitative results. Other minor edits throughout.	1-8
11/12/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 32.2 SPECIMENS, 32.4.2 CALIBRATORS and 32.4.3 CONTROLS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Added references in 32.9. Formatting and minor edits throughout.	All