SOP 401 – Acetaminophen Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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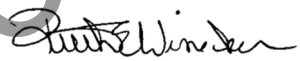
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:** 4 – Added standard prep instructions 6 – Updated instrument parameters 11 – Updated Validation Table	**Revision Date/Initials:** MSF – 05/25/2017 MSF – 06/05/2017 MSF – 01/22/2018

Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		08/25/2016
Ruth E. Winecker, Ph.D.		01/22/2018

SOP 401 – Acetaminophen Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. **Introduction and Principle of Assay**

1.1. This method is designed to confirm and quantitate acetaminophen in biological specimens by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-MS/MS). Acetaminophen is extracted from biological matrices by protein precipitation with acetone and identified by the retention time and ion ratio of product ions.

1.2. Acetaminophen (paracetamol, APAP) is a popular drug, sold worldwide as an analgesic for mild to moderate pain and as an antipyretic. In the United States, acetaminophen is available over-the-counter in generic and branded products (such as Tylenol), and in a multitude of multi-ingredient medications for the treatment of body aches, fever, and flu and cold symptoms (see https://www.drugs.com/ingredient/acetaminophen.html). Depending on the formulation, acetaminophen can range from 80 mg in chewable tablets to 650 mg (or greater) in extended release products. The most commonly observed amount, however, is 325 mg.

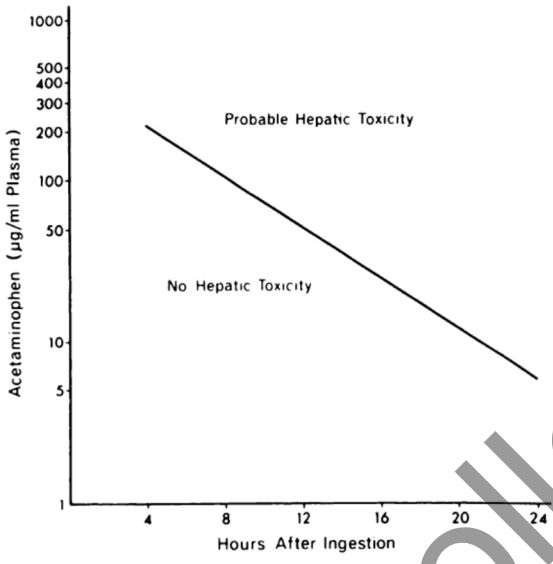
In addition to OTC products, acetaminophen is commonly prescribed in combination with the opioid analgesics oxycodone and hydrocodone, as Percocet and Vicodin, respectively. Percocet formulations contain 325 mg of acetaminophen, while Vicodin formulations contain 300 mg.

While generally regarded as safe when taken in moderate, therapeutic doses, hepatotoxicity due to accidental or deliberate overdoses is common, and is the primary cause of acute liver failure in the United States (1). In an effort to reduce liver injury, the FDA ordered prescription products to contain no more than 325 mg of acetaminophen per dose, and to include boxed warnings regarding the potential for severe liver failure.

Acetaminophen is a weak acid (pKa 9.5), which at physiological pH is rendered neutral and thus absorbed from the duodenum. Elimination takes place in the liver, with the majority of the drug glucuronidated or sulfated before being excreted in the urine (phase II metabolism). A more important metabolic fate of acetaminophen is mediated by cytochrome P450 (phase I metabolism). This process generates the reactive, and toxic, metabolite N-acetyl-p-benzoquinone imine (NAPQI). (2)

When therapeutic amounts of acetaminophen are ingested, toxic NAPQI metabolites are scavenged by glutathione. In overdoses, however, glutathione stores are quickly depleted. Excess NAPQI reacts with protein sulfhydryl groups in the liver. These adducts, in the mitochondria, disrupts the electron transport chain, generating oxidant stress and leading to cellular necrosis and potential liver failure. (3)

Treatment of acetaminophen overdoses includes the administration of N-acetylcysteine, which binds to and scavenges excess NAPQI when glutathione stores are diminished. To aid the clinician, a nomograph of plasma acetaminophen concentration relating to time post-ingestion and probable hepatotoxicity was generated (4). Forensic toxicologists have since adopted this tool as an aid in interpreting post-mortem acetaminophen concentrations.



The volume of distribution is low, 0.8 – 1.0 L/kg, and not expected to undergo postmortem redistribution, therefore quantitation of acetaminophen in this laboratory is generally accomplished in central specimens. Other laboratories, however, have observed mild postmortem redistribution with a ratio between heart/femoral blood of 1.3 (5).

As with any drug, particularly one commonly combined with opioid analgesics, interpretation of postmortem acetaminophen concentrations must rely not only on the drug concentration, but incorporate patient history, autopsy and scene findings.

2. **Specimens**
   2.1. This procedure is applicable to urine, blood, serum, *bile, *gastric contents, and properly prepared tissue specimens (typically 1:4 homogenates). A 0.1 mL (g) specimen amount (in duplicate) is generally employed unless a dilution is required so that the calibration curve encompasses the expected range of unknown specimens.

   2.1.1. *For non-typical matrices, an additional 0.1mL aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. **Reagents and Materials**
   3.1. DI water, HPLC grade
   3.2. Methanol, HPLC grade
   3.3. Deuterated Internal Standard Mix
   3.4. Calibration Standard
   3.5. QC Standard
   3.6. Drug Free Blood, Urine, Liver Homogenate
   3.7. Water with 0.1% formic acid

3.8. Methanol with 0.1% formic acid

4. **Standards, Controls, and Solutions**

4.1. Acetaminophen-d4 Internal Standard (100µg/mL)

4.1.1. Into a 10mL volumetric flask, add the contents of 1 ampule (~1mL) of Acetaminophen-d4 (Cerilliant - 1mg/mL).

4.1.2. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010.

4.2. Acetaminophen Calibrators and Positive Controls – these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.3. Water with 0.1% formic acid

4.3.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid

4.3.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

4.4. Methanol with 0.1% formic acid

4.4.1. To a 4L bottle of HPLC grade methanol, add 4 mL of formic acid

4.4.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

5. **Equipment and Special Supplies**

5.1. Test Tubes, 16 x 125 mm
5.2. LC autosampler vials, 12 x 32 mm
5.3. Polyspring inserts, 5 mm O.D.
5.4. Centrifuge 2000 x g
5.5. Vortex mixer

6. **Instrumentation and Parameters**

6.1. Windows PC with Thermo LCQuan and Xcaliber software
6.1.1. Instrument method (TSQ03): "Acetaminophen"
6.1.2. Click here for instrument parameters.
6.2.
6.3. Thermo Surveyor LC autosampler, or equivalent
6.4. Thermo Surveyor LC pump, or equivalent
6.5. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

7.1. Acetaminophen (**152** 110 65)
7.2. Acetaminophen-d4 (**156** 114 69)

7.2.1. Note: The precursor ion of each analyte is listed first and bolded, the first product ion- used for quantification-is second, followed by the second product ion-used for qualification/confirmation.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16x125 mm test tubes.

8.2. Add the appropriate quantity (according to the Standard & Control Worksheet) of Deuterated Internal Standard Mix to all the tubes.

8.3. Add the appropriate quantity (according to the Standard & Control Worksheet) of calibration standard and QC to the tubes labeled as standards and control, respectively, labeling test tubes as you go. Only internal standard should be present in the test tube labeled "Blank".

8.4. Add 0.1mL of blank blood to all standards, controls, and blank test tubes (0.1 mL blank urine/0.1g blank liver homogenate to urine/liver blank and QC test tubes respectively).

8.5. Add the appropriate amount of predetermined unknown specimen labeling test tubes as you go. (See Specimens section).

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 10 mL methanol to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Transfer approximately 1 mL of each extract to appropriately labeled autosampler vials and place in the autosampler tray of the Thermo TSQ triple-quadrupole LC/MS/MS.

8.10. Build and initiate sequence as directed in SOP 053.

9. **Calculations**

9.1. Quantification

9.1.1. The method for processing the data using the Thermo LCQuan software is "Acids Quant" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, concentration, and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's quantifying product ion.

9.1.2.1.2. Response Ratio = QN_a / QN_{istd}

9.1.2.1.2.1. QN_a = response of the quantitative ion of the analyte

9.1.2.1.2.2. QN_{istd} = response of the quantitative ion of the internal standard amount

9.2. Calibration

9.2.1. A linear regression resulting from the 6 standards is used to quantitate the analytes in the load. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.3. Dilution Factor

9.3.1. D = Total volume/Sample volume

9.4. Multiplier for homogenates, dilutions, and non-standard volumes

9.4.1. $M = (V_{curve} / V_{samp}) \times D$

9.4.1.1. M = Multiplier

9.4.1.2. D = dilution factor

9.4.1.3. V_{curve} = matrix volume of calibration curve

9.4.1.4. V_{samp} = matrix volume of specimen

9.5. Concentration

9.5.1. C = (A / V) * M

9.5.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.

9.5.1.2. A = Amount of drug in sample

9.5.1.3. V = Volume of sample

9.5.1.4. M = Multiplier

9.6. Max/Min

9.6.1. Percent Difference = $((R_h / R_l)-1) \times 100$

9.6.1.1. R_h = high result

9.6.1.2. R_l = low result

9.7. Average

9.7.1. Average = $(R_1 + R_2) / 2$

9.7.1.1. R_1 = first result

9.7.1.2. R_2 = second result

9.8. Qualifier Ion Ratios

9.8.1.1.1. Ratio 1 = QL_1/QN

9.8.2. QL_1 = response of the quantifying product ion

9.8.3. QN = response of the qualifying product ion

10. **Quality Control**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

10.1.1.3. Peaks sharing parent/product ions must have baseline resolution.

10.1.1.4. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.1.5. Retention time must not deviate outside ± 3% of target, based upon the retention time of the calibrators and controls.

10.1.2. Mass spectroscopy

10.1.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio, as determined by a mid-level calibrator (CAL 4).

10.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.1.3. Calibrators

10.1.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value.

10.1.3.3. Refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual.

10.1.4. Controls

10.1.4.1. Controls must calculate within ± 20% of the target value.

10.1.5. Blanks

10.1.5.1. Blanks should not contain any target analyte signal with an internal standard response ratio greater than 10% that of the lowest calibrator for the same analyte.

10.1.6. Any deviation from the above criteria must be approved by a senior chemist.

11. **Validation of Method**

Parameter	Result
Bias	Blood - L: -2.26% H: -0.19%
Precision	Blood - L: 2.41% H: 2.30%
Calibration model	10 - 500 ug/mL - Linear (1/x)
Carryover	No carryover observed following acetaminophen spiked at 1000 ug/mL
Interference Studies	No significant interfering signal from matrix, internal standard, common drugs of abuse (including metabolites), OTC drugs, and Prescription medications was observed.
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	N/A - IS coelutes with target analyte (0.1 min).

LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	2 ug/mL
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	10 ug/mL
Dilution Integrity	NA - Specimens not routinely diluted - will be evaluated on a case to case basis
Processed Sample Stability - (re-analyze after 8 days)	Extracts remain stable: 3-days - stored in TSQ autosampler 8-days - re-capped and refrigerated

12. **Reporting**

12.1. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section).

12.2. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 1

Scenario \ Dil	1	1	REPORT
A	In curve	In curve	Average
B	In curve	AQL or BQL	"In" value
C	In curve	ND *	Repeat
D	AQL/BQL	AQL/BQL	Less than/ Greater than
E	BQL	ND	ND

* ND = None Detected, due to IRC, S/N threshold, r.t., or other

12.2.1.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration range

12.2.1.2. AQL = Measured concentration (pre-multiplier) falls Above Quantitation Limit

12.2.1.3. BQL = Measured concentration (pre-multiplier) falls Below Quantitation Limit

12.2.1.4. ND = None Detected

12.3. Averaging reportable values

12.3.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.3.2. Enter calculated concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in milligrams per liter (mg/L).

13. **Preparation of Load**

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data - paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data – stapled

14. **References**

14.1. Fontana, Robert J. "Acute Liver Failure Including Acetaminophen Overdose." *Medical Clinics of North America* 92.4 (2008): 761-94.

14.2. Mcgill, Mitchell R., and Hartmut Jaeschke. "Metabolism and Disposition of Acetaminophen: Recent Advances in Relation to Hepatotoxicity and Diagnosis." *Pharm Res Pharmaceutical Research* 30.9 (2013): 2174-187.

14.3. Jaeschke, Hartmut. "Acetaminophen: Dose-Dependent Drug Hepatotoxicity and Acute Liver Failure in Patients." *Dig Dis Digestive Diseases* 33.4 (2015): 464-71.
14.4. Rumack, B.H., Peterson, R.G. "Acetaminophen overdose: incidence, diagnosis, and management in 416 patients." *Pediatrics* 62.5 (1978): 898-903.
14.5. Dalpe-Scott, M., M. Degouffe, D. Garbutt, and M. Drost. "A Comparison of Drug Concentrations in Postmortem Cardiac and Peripheral Blood in 320 Cases." *Canadian Society of Forensic Science Journal* 28.2 (1995): 113-21.

Other suggested reading:

14.6. Chambers, Erin, Diane M. Wagrowski-Diehl, Ziling Lu, and Jeffrey R. Mazzeo. "Systematic and Comprehensive Strategy for Reducing Matrix Effects in LC/MS/MS Analyses." Journal of Chromatography B 852.1-2 (2007): 22-34.