Analysis of Salicylic Acid and/or Acetaminophen in Blood by LC/MS

1 Introduction

Aspirin, or acetylsalicylic acid, is one of the most common over-the-counter drugs that is taken for its analgesic, anti-inflammatory, antipyretic and/or anticoagulant effects. Acetylsalicylic acid is rapidly metabolized in the body to salicylic acid, with a half-life of ~15 minutes. Blood concentrations of less than 100 micrograms per milliliter (µg/mL) of salicylic acid are usually considered to be therapeutic. In cases of potential aspirin overdose, blood concentrations of salicylic acid may reach several hundred µg/mL.

Acetaminophen is another common over-the-counter drug. It is taken for its analgesic and antipyretic effects; it is also available in combination with many other prescribed analgesics such as codeine and hydrocodone. Acetaminophen overdose can cause liver toxicity. A single blood concentration of acetaminophen may be of limited use when trying to determine acetaminophen toxicity, unless the time of ingestion is known.

2 Scope

This procedure is used to analyze salicylic acid and acetaminophen in blood specimens qualitatively or quantitatively. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Blood samples are extracted with acetonitrile using isotopically labeled salicylic acid and acetaminophen as internal standards, filtered, and analyzed by liquid chromatography/mass spectrometry (LC/MS). Salicylic acid is analyzed in negative ion mode, and acetaminophen is analyzed in positive ion mode.

4 Specimens

This procedure uses 0.1 mL blood per replicate to analyze a specimen. (Quantitative analysis is typically prepared with duplicate samples.)

5 Equipment/Materials/Reagents

a. Acetonitrile (Optima grade)

b. Methanol (HPLC grade)

c. Centrifuge tubes

d. Autosampler vials with inserts and caps

e. Vortexer

f. 0.45 µm centrifuge filters

g. Centrifuge

h. Calibrated pipettors with disposable tips

i. Volumetric glassware

j. Liquid Chromatograph equipped with a mass spectrometer and a Xterra Phenyl LC column: 150 x 2.1 mm. 5 µm d_p, with 7.5 x 2.1 mm guard column (or equivalent)

k. Water (Optima grade)

l. Formic Acid (Puriss grade or better)

m. 0.1% Formic acid in acetonitrile:
Combine 500 mL acetonitrile and 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

n. 0.1% Formic acid in water:
Combine 500 mL water and 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

6 Standards and Controls

a. Salicylic Acid Calibration Stock (2.0 mg/mL):
Add 23.3 mg of sodium salicylate (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Add approximately 1 mL methanol and swirl to completely dissolve the salicylic acid. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass or plastic. Stable at least two years.

b. Salicylic Acid Control Stock (2.0 mg/mL):
Add 23.3 mg of sodium salicylate (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Add approximately 1 mL methanol and swirl to completely dissolve the salicylic acid. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass or plastic. Stable at least two years.

c. Acetaminophen Calibration Stock (1.0 mg/mL): [1]
Add 10 mg of acetaminophen (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass or plastic. Stable at least two years.

d. Acetaminophen Control Stock (1.0 mg/mL): [1]
Add 10 mg of acetaminophen (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass or plastic. Stable at least two years.

e. Negative Control Blood:
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Store refrigerated, frozen, or obtain fresh. Stability determined by manufacturer. A Negative Control Blood sample is extracted and analyzed with every assay.

f. Positive Control Blood:
Prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). For in-house prepared controls, recommended control concentrations are 30 and 320 µg/mL for acetaminophen and 60 and 640 µg/mL for salicylic acid. Prepared fresh on the day of extraction as described in Section 6 Table 2.

g. Salicylic Acid-d_4 (100 µg/mL):
Purchased from Cerilliant or another approved vendor. Storage and stability determined by the manufacturer.

h. Acetaminophen-d_4 (100 µg/mL):
Purchased from Cerilliant or another approved vendor. Storage and stability determined by the manufacturer.

i. LC Column Check Mix (5 µg/mL each salicylic acid and acetaminophen):
Combine 25 µL of the Salicylic Acid Calibration or Control Stock (2.0 mg/mL) and 50 µL of the Acetaminophen Calibration or Control Stock (1.0 mg/mL) in a 10-mL volumetric flask. Bring to the mark with acetonitrile. Store refrigerated in glass or plastic. Stable for at least 2 years.

This procedure may be used quantitatively via construction of a multi-point calibration curve for the analyte(s) of interest following the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101). Table 1 shows the concentrations and volumes used for preparation of calibration samples.

[1] Alternatively, Standard Stock Solutions (1 mg/mL) may be purchased from Cerilliant (typically used for calibrators), Lipomed (typically used for controls) or another approved supplier.

Table 1: Typical Calibrator Preparation (0.1 mL of each Calibrator Level is added to 0.1 mL Negative Control Blood as described in Section 9.)

Calibrator Level (µg/mL)	Volume of Salicylic Acid Calibration Stock (µL)*	Volume of Acetaminophen Calibration Stock (µL)*	Volume of Acetonitrile (µL)
800/400	400	400	200
600/300	300	300	400
400/200	200	200	600
200/100	100	100	800
100/50	Dilute 0.5 mL Cal 200/100 with 0.5 mL acetonitrile		
50/25	Dilute 0.5 mL Cal 100/50 with 0.5 mL acetonitrile		
20/10	Dilute 0.1 mL Cal 200/100 with 0.9 mL acetonitrile		

*If only one analyte is quantitated, add acetonitrile to compensate for the stock volume missing. If alternate concentrations of calibrator solutions are used, adjust volumes accordingly.

Table 2: Typical Control Preparation (0.1 mL of each Control Level is added to 0.1 mL Negative Control Blood as described in Section 9.)

Control Level (µg/mL)	Volume of Salicylic Acid Control Stock (µL)*	Volume of Acetaminophen Control Stock (µL)*	Volume of Acetonitrile (µL)
640/320	320	320	360
60/30	Dilute 0.375 mL 640/320 Control with 3.625 mL acetonitrile		

*If only one analyte is calibrated, add acetonitrile to compensate for the stock volume missing. If alternate concentrations of control solutions are used, adjust volumes accordingly.

For qualitative analysis, analyze a negative control and at least one positive control or calibrator listed in Tables 1-2.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

a. Measure 100 µL of each case sample into properly labeled centrifuge tubes. Prepare case samples in duplicate for quantitations.

b. Measure 100 µL Negative Control Blood into 12 labeled centrifuge tubes for the calibrators, Positive Controls (in duplicate), and a Negative Control.

c. Add 100 µL acetonitrile to each case sample and the Negative Control.

d. Add 100 µL of each Calibrator and Control (prepared as directed in Section 6) to the calibrator and positive control samples, respectively.

e. Add 50 µL each Salicylic Acid-d_4 and Acetaminophen-d_4 to all samples.

f. Vortex tubes well. Centrifuge at 10000 rpm for 10 minutes.

g. Filter the top layer through 0.45 µm centrifuge filters by centrifuging at 10000 rpm for 5 minutes.

h. Transfer extracts to labeled autosampler vials. Add 50 µL of water to each ALS vial. Analyze 10 µL by LC/MS using the conditions in Section 10 after verifying that the results of the column check mix are acceptable. (Note: salicylic acid is analyzed in negative ion mode and acetaminophen is analyzed in positive ion mode.)

9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters		Column
A: 0.1% Formic acid in water	Flow	0.2 mL/min	Phenyl (Xterra)
C: 0.1% Formic acid in acetonitrile		0.1 mL/min	Oven set at 40°
Total Run time: 7 minutes	Autosampler Temp: 15°C		

9.2 Mass Spectrometer Parameters – Salicylic Acid

Source Parameters		
Mode: Electrospray, Negative	Spray Voltage: -5 kV	Capillary Temperature: 275ºC
Sheath Gas: 27 (arb units)	Aux Gas: 6 (arb units)	Sweep Gas: 3 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Event #1	full scan m/z 110 - 300, unit mass resolution	
Event #2	MSMS 137→50-150	
	1.5 amu isolation width	45% collision energy
Event #3	MSMS 141→50-150	
	1.5 amu isolation width	45% collision energy

9.3 Mass Spectrometer Parameters – Acetaminophen

Source Parameters		
Mode: Electrospray, Positive	Spray Voltage: +5 kV	Capillary Temperature: 275ºC
Sheath Gas: 34 (arb units)	Aux Gas: 3 (arb units)	Sweep Gas: 3 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Event #1	full scan m/z 110 - 300, unit mass resolution	
Event #2	MSMS 152→50-160	
	1.5 amu isolation width	45% collision energy
Event #3	MSMS 156→50-160	
	1.5 amu isolation width	45% collision energy

10 Decision Criteria

10.1 Column Check Mix Performance Criteria

The performance of the LC/MS is demonstrated each day samples are analyzed. The LC Column Check Mix effectively evaluates system suitability. Depending upon the MS parameters used, the salicylic acid or acetaminophen peak should be present with reasonable peak shape.

10.2 Analyte Performance Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The peak shape of salicylic acid is known to tail. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following criteria should be met.

10.2.1.1 Retention Time

The retention time of the peak should be within ±5% of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

10.2.2 MS Spectra

For salicylic acid (fragments of 137), the only peaks present in the MS/MS spectrum above 10% should be m/z 93 and the precursor. Due to the stability of salicylic acid, the precursor ion may be the base peak.

For acetaminophen (fragments of 152), the base peak in the MS/MS spectrum should be m/z 110 with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 134.

10.3 Reporting Cut-offs for College of American Pathologists (CAP) T Series and FTC Series:

See Quality Control for Toxicology Examinations (TOX101) for guidance on estimating the amount of an analyte in a specimen. When analyzing CAP T-Series or FTC specimens, if all decision criteria for an analyte of interest are met, but the concentration of acetaminophen is estimated to be below 5 µg/mL and/or the concentration of salicylic acid is estimated to be below 10 µg/mL in two independent analyses, the analyte will not be reported. Note: the second analysis may be a repeat of this procedure or via another validated procedure. A Positive Control at the Cut-off Level is recommended for the second analysis.

11 Calculations

Analyte	Molecular Ion	Internal Standard Molecular Ion	Calibration Weighting
Salicylic acid	137	141	1/x
Acetaminophen	152	156	unweighted

See the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101) for acceptable practices for calculating quantitative results.

12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

13 Limitations

a. Linearity: 20-800 µg/mL for salicylic acid
10-400 µg/mL for acetaminophen

b. Limit of Detection: 10 µg/mL for salicylic acid
5 µg/mL for acetaminophen

c. Bias, Repeatability, and Intermediate Precision (n=15 per level):

Salicylic Acid	Bias	Repeatability	Intermediate Precision
60 µg/mL	-1.04%	4.07%	5.48%
300 µg/mL	-0.12%	3.98%	4.60%
640 µg/mL	+3.19%	2.62%	4.90%

Acetaminophen	Bias	Repeatability	Intermediate Precision
30 µg/mL	-4.24%	2.31%	3.41%
150 µg/mL	-1.28%	2.37%	2.37%
320 µg/mL	-2.61%	1.65%	2.63%

d. Matrix Effects: Average matrix effects were 0.8% for salicylic acid and -42.8% for acetaminophen.

e. Interferences: None known.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Quality Control for Toxicology Examinations (TOX101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

Chemistry Unit Procedures for Estimating Measurement Uncertainty (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Rev. #	Issue Date	History
1	10/09/18	Updated SOP title to "analysis" (vs. quantitation). Section 1: added "overdose" for clarification. Updated Section 2 scope language to reflect current staff requirements as well as include qualitative analysis. Section 4 and 8a: updated to account for one replicate. Section 6: updated to account for qualitative examinations, and updated TOX101 reference; moved some information to Section 11 Calculations. Updated 8f to include addition of 0.050 mL of water to improve peak shape. Updated 9.2 to enable sweep gas to reduce adduct formation. Removed "Calibration" section and renumbered sections. Updated 12 title phrase to "Measurement Uncertainty", and updated title of CUQA 13. Updated Section 15 TOX101 and CUQA 13 titles.
2	11/15/19	In Sections 6 c and d, added footnote to allow purchase of stock standards. In Sections 6 and 8 revised to see Section 6 table 1-2, not Section 7. In Tables 1 and 2, added language to adjust volumes for alternate concentrations. Added 10.3 reporting cut-offs for proficiency testing. Revised bench sheet calibrator and control section.

Approval

Redacted - Signatures on File

Acting Toxicology Technical Lead: Date: 11/14/2019

Chemistry Unit Chief: Date: 11/14/2019

Appendix 1: Abbreviated version of the Procedure for bench use.

Redacted - Form on File

Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.

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