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

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1 INTRODUCTION

Aspirin, or acetylsalicylic acid, is one of the most common over-the-counter drugs and 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 300 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 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. Acetaminophen toxicity is due to a metabolite, NAPBQI (N-acetyl-p-benzoquinone imine).

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☒ Quantitation
Matrices	Blood
Analytes	Acetaminophen Salicylic Acid
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

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 SPECIMEN CRITERIA

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

5 EQUIPMENT

5.1 Equipment

A. Vortexer
B. Centrifuge
C. Calibrated pipettors with disposable tips
D. Volumetric glassware

5.2 Consumables

A. Centrifuge tubes
B. Autosampler vials with inserts and caps
C. 0.45 µm centrifuge filters

5.3 Instruments

A. Thermo LTQ Orbitrap XL
B. Shimadzu HPLC

5.3.1 Column

Xterra Phenyl LC column: 150 x 2.1 mm. 5 µm d_p or equivalent

5.3.1.1 Guard Column

7.5 x 2.1 mm

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1 / XP Professional
Mass Spectrometer	Foundation	1.0.2 or higher
	Xcalibur	2.1.0 SP1 / 2.0.7
	LTQ Tune Plus	2.5.5
	Shimadzu LC Controller	5.4 / 6.5

5.5 Chemicals/Reagents

Storage/stability determined by manufacturer unless otherwise noted.

5.5.1 Purchased

Acetonitrile	Optima grade
Formic Acid	Puriss grade or better
Methanol	HPLC grade
Water	Optima grade

5.5.2 Prepared

Mobile Phase 1 (Aqueous) 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 weeks.
Mobile Phase 2 (Organic) 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.

5.6 Standards/Controls

Storage/stability determined by manufacturer unless otherwise noted.

5.6.1 Purchased

5.6.1.1 Matrix

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.

5.6.1.2 Internal Standards

Typically purchased from Cerilliant or an equivalent supplier.

Acetaminophen-d_4	100 µg/mL
Salicylic Acid-d_4	100 µg/mL

5.6.1.3 Calibration/Control

Typically purchased from Cerilliant, Lipomed, Sigma or an equivalent supplier.

Acetaminophen	1.0 mg/mL
Salicylic Acid (sodium salicylate)	>HPLC grade

5.6.2 Prepared

5.6.2.1 Calibration/Control Stock

Salicylic Acid Control Stock (2.0 mg/mL)
Add 23.3 mg of sodium salicylate 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.

5.6.2.2 Calibrator Level Solutions

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		
	0.1 mL of each Calibrator Level is added to 0.1 mL Negative Control Blood		
	If only one analyte is calibrated, add acetonitrile to compensate for the stock volume missing. If alternate concentrations of calibrator solutions are used, adjust volumes accordingly.		

5.6.2.3 Control Level Solutions

Control Level (µg/mL)	Volume of Salicylic Acid Calibration Stock (µL)	Volume of Acetaminophen Calibration Stock (µL)	Volume of Acetonitrile (µL)
640/320	320	320	360
60/30	Dilute 0.375 mL Cal 640/320 with 3.625 mL acetonitrile		
	0.1 mL of each Control Level is added to 0.1 mL Negative Control Blood		
	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.		

5.6.2.4 Performance Standard

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.

6 PROCEDURE

	Step	Note	Reference/Lot
	A. Samples (in duplicate for quantitation)		
	1. To labeled centrifuge tubes:		
☐	i. Add 100 µL of blood		
	B. Control(s)		
	1. Prepare Negative Control(s)		
☐	i. Add 100 µL of blood		
	2. Prepare Positive Control(s) (in duplicate for quantitation)		
☐	i. Add 100 µL of blood		
☐	ii. Control Working Solution(s)		
	iii. Control Scheme		
	C. Calibrators		
	1. Prepare Calibrators		
☐	i. Add 100 µL of blood		
☐	ii. Calibration Working Solution(s)		
	iii. Calibration Scheme		
	D. Internal Standard(s)		
☐	1. Add 50 µL of each Internal Standard Working Solution		
	E. Extract		
☐	1. Add 100 µL of acetonitrile to case samples, negative control		
☐	2. Vortex and centrifuge at 10,000 rpm for 10 minutes		
☐	3. Transfer supernatant to 0.45 µm centrifuge filter tubes		
☐	4. Centrifuge at 10,000 rpm for 5 minutes		
	F. Prepare for Analysis		
☐	1. Transfer supernatant to labeled autosampler vial 2. Add 50 µL of water to each ALS vial		
☐	**G. Instrumental Analysis** 1. LC/MS: analyze 10 µL Analyze LC/MS performance standard prior to batch analysis Record: performance standard, mobile phases 1/2, LC Column		
	Note: salicylic acid is analyzed in negative ion mode and acetaminophen is analyzed in positive ion mode. Consult IOSS prior to changing Orbitrap polarity.		

7 ANALYTICAL PARAMETERS

7.1 Shimadzu HPLC

7.1.1 Gradient

Time (min)	Mobile Phase (mL/min)		Flow Rate
	1-Aqueous	2-Organic	(mL/min)
0	0.2	0.1	0.3
7	0.2	0.1	0.3

7.1.2 Conditions

Column Heater (°C)	40
Autosampler (°C)	15
Run Time (min)	7

7.2 Thermo LTQ Orbitrap XL

7.2.1 Source

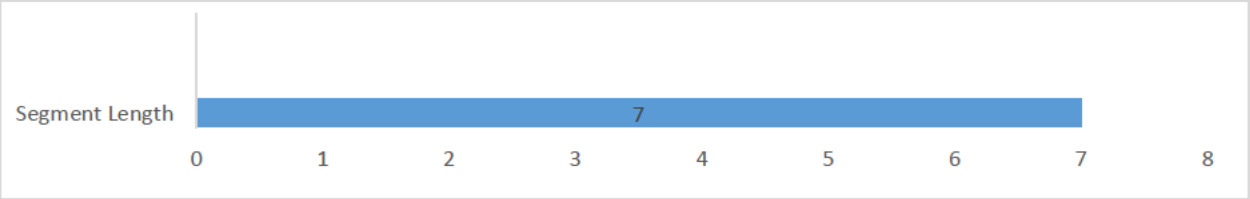
7.2.1.1 Salicylic Acid

Mode	ESI
Polarity	(-)
Spray Voltage (kV)	-5
Capillary Temperature (°C)	275
Sheath Gas	27
Aux Gas	6
Sweep Gas	3

7.2.1.2 Acetaminophen

Mode	ESI
Polarity	(+)
Spray Voltage (kV)	5
Capillary Temperature (°C)	275
Sheath Gas	34
Aux Gas	3
Sweep Gas	3

7.2.2 Segment(s)



7.2.3 Scan Events

7.2.3.1 Salicylic Acid

Event	Mode	Range (m/z)	Details	Isolation Width (m/z)	Collision Energy (rel)	Analyzer	Resolution
1	Full Scan	110-300				ITMS	Unit mass
2	MS^2 137	50-150		1.5	45	ITMS	Unit mass
3	MS^2 141	50-150		1.5	45	ITMS	Unit mass

7.2.4 Acetaminophen

Event	Mode	Range (m/z)	Details	Isolation Width (m/z)	Collision Energy (rel)	Analyzer	Resolution
1	Full Scan	110-300				ITMS	Unit mass
2	MS^2 152	50-160		1.5	45	ITMS	Unit mass
3	MS^2 156	50-160		1.5	45	ITMS	Unit mass

Full scan only (event 1) may optionally be used if MS^2 data are not needed for a given analysis.

8 DATA ANALYSIS

8.1 Decision Criteria

8.1.1 Performance Check

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.

8.1.2 Target Analytes

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

8.1.2.1 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.

8.1.2.1.1 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.

8.1.2.1.2 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.

8.1.2.2 Mass 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.

8.2 Calculations

8.2.1 Target Ions

Analyte	Molecular Ion	Internal Standard Molecular Ion
Salicylic acid	137	141
Acetaminophen	152	156

8.2.2 Calibration

Model	Linear
Weighting	
Salicylic Acid	1/x
Acetaminophen	Unweighted

Refer to TOX-101 for further guidance.

8.2.3 Software

Quantitative and qualitative calculations may be performed by one or more of the following software packages:

A. Thermo Xcalibur
    1. QualBrowser
    2. QuanBrowser
    3. Tracefinder

B. Microsoft
    1. Excel

9 Reporting

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.

9.1 Measurement Uncertainty

Refer to CHEM-100 and TOX-101.

10 Corrective Measures

Refer to TOX-101 for guidance on action steps in the event of a quality control failure.

11 PERFORMANCE CHARACTERISTICS

11.1 LOD

Analyte	Type	LOD µg/mL
Salicylic acid	Quant	10
Acetaminophen	Quant	5

11.2 LOQ-Linear Range

The LOQ is set as the lowest calibrator.

Analyte	Low/LOQ µg/mL	High µg/mL
Salicylic acid	20	800
Acetaminophen	10	400

11.3 Bias, Repeatability, Intermediate Precision

n=15

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%

11.4 Carryover

No significant carryover established during validation.

12 LIMITATIONS

12.1 Interferences

None known

12.2 Matrix Effects

Average matrix effects were 0.8% for salicylic acid and -42.8% for acetaminophen.

13 SAFETY

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

14 REVISION HISTORY

Revision	Issued	Changes
03	02/11/2022	Document reformat. Minor updates to Section 1 and 5.