SOP 413 - Loperamide Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

Table of Contents

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SOP Name:	SOP #:
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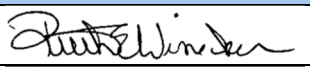
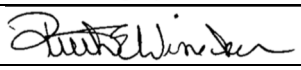
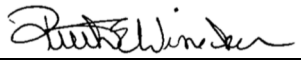
	Revision:	Revision Date/Initials:
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	8.7 – changed acetone to methanol to reflect lab practice. 8.10 – changed 500µL to 300µL to reflect lab practice. 10.1.1.5 – Updated RT acceptance range 10.1.2.1 – Updated IRC acceptance range 10.1.3.2 – Updated Calibrator acceptance range 10.1.4.1 – Updated QC acceptance range 8.9, 8.10 – Updated extraction procedure 10.1.3.3 – Added reference to QA Manual (Cal point exclusion) 4 – Updated Standard prep instructions 8.9 – 8.11 Updated extraction procedure. 11 – Updated Validation table	4/28/2015 MSF 4/28/2015 MSF MSF – 05/08/2015 MSF – 01/28/2016 MSF – 04/04/2016 MSF – 07/20/2017

Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

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1. **Principle of Assay**

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

Loperamide is a mu-opioid receptor agonist used to treat diarrhea stemming from indications such as inflammatory bowel disease or gastroenteritis. Antidiarrheal activity stems from the absorption of loperamide into the gut membranes where it activates opioid receptors and increases intestinal transit time. After being absorbed by the gut, loperamide is extensively metabolized by cytochrome P450 (CYP3A4) in the liver, leaving little for systemic circulation. While thought to be only peripherally active, loperamide does in fact cross the blood-brain barrier, but is a substrate for P-glycoprotein, the primary efflux transporter for removing substances from the brain. (2)

At high doses, loperamide was shown to be devoid of CNS effects, contributing to its high safety profile and low potential for abuse. As such, loperamide is available over-the-counter in both name brand and generic formulations. However, there is anecdotal evidence in online drug forums that one can experience opioid-like CNS effects (euphoria) upon massive ingestion of loperamide, particularly if taken with P-glycoprotein inhibitors, such as quinine, St. John's Wort, or a variety of other medications. Further, the on-line drug forums indicate that doses patients are ingesting are much higher than the clinical high dose studies.

In this laboratory, screening for loperamide is typically done in central blood specimens (e.g. aorta, inferior vena cava) via the organic bases screen (SOP 102). The volume of distribution for loperamide is unknown, but its structure suggest that it should be high (Vd >5 L/kg) and readily distributed in perfused organs such as the liver, lung, heart, and kidneys. Loperamide could be subject to postmortem redistribution, in which drugs diffuse from areas of high drug concentration, such as organ tissue, into the blood. Accordingly, confirmation and quantitation of loperamide is done in peripheral blood specimens (e.g. femoral, iliac) and liver, to more accurately reflect loperamide concentrations at the time of death and assist in interpretation.

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 all 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. Acetone, HPLC grade
   - 3.4. Acetonitrile, HPLC grade
   - 3.5. Deuterated Loperamide Internal Standard Mix
   - 3.6. Loperamide Standard
   - 3.7. Loperamide QC Standard (Standard & Control Worksheet)
   - 3.8. Drug Free Blood, Urine, Liver Homogenate
   - 3.9. Water with 0.1% formic acid
   - 3.10. Acetonitrile with 0.1% formic acid
   - 3.11. Methanol with 0.1% formic acid

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

4.1. **Loperamide-d6 Internal Standard Solution (1000ng/mL)**

4.1.1. Into a 10mL volumetric flask, add 0.1ml of Loperamide -d6 Stock Solution – 100µg/mL (Prepared by QA/QC Chemist or appointee).

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. **Loperamide 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. **Acetonitrile with 0.1% formic acid**

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

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

4.5. **Methanol with 0.1% formic acid**

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

4.5.2. Label bottle as “LC/MS” and “with 0.1% formic acid”.

5. **Equipment and Special Supplies**
   - 5.1. Test Tubes, 13 x 100 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
   - 5.6. Nitrogen evaporator

6. **Instrumentation and Parameters**
   - 6.1. Windows PC with Thermo LCQuan and Xcaliber software
   - 6.2. Thermo Surveyor LC autosampler, or equivalent
   - 6.3. Thermo Surveyor LC pump, or equivalent
   - 6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions (± 1 nominal mass)**

   - 7.1. Loperamide (**477** 266 210)
   - 7.2. Loperamide-d6 (**484** 273 217)

   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 13x100 mm test tubes.
   - 8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of Deuterated Loperamide Internal Standard to all the tubes.
   - 8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of Loperamide Standard and Loperamide 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, control, and blank test tubes (0.1 mL blank urine/0.1g blank liver homogenate to urine/liver blank and QC test tubes).
   - 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 3.5mL methanol to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top methanol layer into clean and labeled 13x100 test tubes, place in nitrogen evaporator, and evaporate under a stream of nitrogen at 55° C, to dryness.

8.10. Remove dried specimens from nitrogen evaporator and reconstitute with 500µL of methanol. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.11. Transfer ~100 µL of each extract into appropriately labeled autosampler vials fitted with 100 µL polyspring insert and place in the autosampler tray of the Thermo TSQ triple-quadropole LC/MS/MS (TSQ 04).

8.12. 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 "Loperamide" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, and concentration. It is also used to calculate the qualifier 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

10.1.2.1.1. 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 ± 25% at LOQ).

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.

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11. Validation of Method

Parameter	Result
Bias	**QC Low Blood: -0.78 % Liver: 3.48%**
	QC High Blood: -8.29% Liver -7.97%
Precision	**QC Low Blood: 4.29% Liver: 3.26%**
	QC High Blood: 4.06% Liver: 3.53%
Calibration model	**10 to 1000ng/mL Linear with 1/x weighting**
Carryover	**Tested to 5X high calibrator (5000ng/mL) - no detected carryover in MeOH blanks injected immediately after.**
Interference Studies	**No interference detected with the exception of observed contribution of signal from internal standard (Loperamide-d6) to target analyte (Loperamide). The contribution results in a signal response equal to approximatally 0.5% of the total signal of the IS. The target Area:IS area ratio of this response is slightly below 10% of the area ratio of the low calibrator. The siganl to noise threshold for loperamide will initially be set to 250 to mitigate this issue but may be adjusted in the future if needed to compensate for variation in instrument performance.**
Ionization Suppression/Enhancement	**Not evaluated - Loperamide and Loperamide-d6 elute within 0.05 min. of each other.**
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	**Blood/Liver 5 ng/mL**
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**Blood/Liver: 10ng/mL**
Processed Sample Stability - (re-analyze after 3 & 8 days)	**Loperamide average % diff. (Day1 vs. Day8) = -2% Methadone-d9 average % diff. (Day1 vs. Day8) = -3.29% Extract is stable for up to 1 week. (See original validation data)**
Recovery	**Not determined**
Dilution Integrity	**Not determined. Will be evaluated on a case-by-case basis.**

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

Dil / Scenario	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. 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.

14.2. Vandenbossche, J., Huisman, M., Xu, Y., Sanderson, D., Soons, P. "Loperamide and P-glycoprotein inhibition: assessment of the clinical relevance." *Journal of Pharmacy and Pharmacology* 62 (2010): 401-412.