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**SOP 405 - Methadone Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)**

**SOP Name:**
Methadone Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

<table>
<thead>
<tr>
<th>Revision:</th>
<th>Revision Date/Initials:</th>
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</table>
| 11.1.1.5 – Updated RT acceptance range  
11.1.2.1 – Updated IRC acceptance range  
11.1.3.2 – Updated Calibrator acceptance range  
11.1.4.1 – Updated QC acceptance range  
11.1.2.1 – Updated IRC update criteria  
11.1.3.3 – Added reference to QA manual (Cal point exclusion)  
10.1 – Updated Validation table  
4 – Added standard prep instructions  
6 – Updated instrument parameters | MSF – 05/07/2015  
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**North Carolina Office of the Chief Medical Examiner Toxicology Laboratory**

**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. Introduction and Principle of Assay

1.1. This method is designed to confirm and quantitate Methadone in biological specimens by Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). The drugs are extracted from their biological matrix by protein precipitation with methanol and identified by the retention times of precursor ions and ion ratios of the product ions.

1.2. Methadone is a synthetic narcotic analgesic developed in Germany in 1937. Its long half-life and action at classical opioid receptors led to its popularity in use in opioid addiction treatment and has been in general use for that purpose in the US since the 1960’s. In the early 2000’s methadone gained acceptance in use as a medication for chronic pain. This move of methadone from the tightly controlled administration at MMT clinics to home use has caused an increase in diversion from legitimate use to recreational use and abuse. This has predictably led to a high prevalence of methadone-related overdose deaths. Because of the possibility of postmortem redistribution it is recommended that both peripheral blood and liver be analyzed for proper interpretation. As with any narcotic analgesic, interpretation of postmortem methadone 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, properly prepared tissue specimens (typically 1:4 homogenates), bile*, vitreous and gastric contents*.

2.2. A 0.1 mL (g) sample size (in duplicate) is generally employed for urine, blood, serum, bile, and gastric contents, and a 0.4g sample size (in duplicate) for tissue homogenate (unless a dilution is required) so that the calibration curve encompasses the expected range of unknown specimens.

2.2.1. *For non-typical matrices, an additional 0.1mL aliquot shall be taken (volume permitting), spiked with 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. Acetonitrile, HPLC grade
3.4. Deuterated Methadone Internal Standard

3.5. Methadone Standard

3.6. Methadone QC Standard

3.7. Drug Free Blood, Urine, Liver Homogenate

3.8. Water with 0.1% formic acid

3.9. Acetonitrile with 0.1% formic acid

3.10. Methanol with 0.1% formic acid

4. Standards, Controls, and Solutions

4.1. Methadone-d9 Internal Standard (10µg/mL)

4.1.1. Into a 10mL volumetric flask, add the contents of 1 ampule (~1mL) of Methadone-d9 (Cerilliant – 100µg/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. Methadone 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. Centrifuge 2000 x g

5.3. Vortex mixer

5.4. GC autosampler crimp cap vials, 12 x 32 mm

5.5. GC autosampler crimp caps

5.6. Polyspring inserts, 5 mm O.D.

6. Instrumentation and Parameters

6.1. Windows PC with Thermo LCQuan and Xcaliber software
   6.1.1. Instrument method (TSQ01 & TSQ02): “Methadone”
   6.1.2. Click here for instrument parameters.

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer
   6.4.1. =

7. Target Ions (± 1 nominal mass)

7.1. Methadone-d9 (319 268 105)
7.2. Methadone (310 265 105)

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.

8.1.1. **Note: follow the tube labeling and tape transfer procedure located in the Quality Assurance and Quality Control manual.**

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

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Methadone Standard and the Methadone QC Standard to the tubes labeled as standards and control, respectively, labeling test tubes as you go. Only internal standard should be spiked into 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/0.4g blank liver homogenate to urine/liver blank and QC test tubes if applicable).

8.5. Add the appropriate amount of unknown specimen, labeling test tubes as you go.

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 1.5 mL methanol to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Transfer ~600 µL of supernatant 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. Quantitative Ion ratios

9.1.1. The method for processing the data using the Thermo LCQuan software is “Methadone” (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:
9.1.2.1.1. Response Ratio:

9.1.2.1.1.1. Response Ratio = response of the analyte’s quantifying product ion compared to that of the internal standard

9.1.2.1.1.2. Response Ratio = \( \frac{Q_N}{Q_{N_{\text{std}}}} \)

9.1.2.1.1.3. \( Q_N \) = response of the quantitative ion of the analyte

9.1.2.1.1.4. \( Q_{N_{\text{std}}} \) = 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 case. 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. \( D = \frac{\text{Total volume}}{\text{Sample volume}} \)

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

9.4.1. \( M = \left( \frac{V_{\text{curve}}}{V_{\text{samp}}} \right) \times D \)

9.4.1.1. \( M \) = Multiplier

9.4.1.2. \( D \) = dilution factor

9.4.1.3. \( V_{\text{curve}} \) = matrix volume of calibration curve

9.4.1.4. \( V_{\text{samp}} \) = matrix volume of specimen

9.5. Concentration

9.5.1. \( C = \left( \frac{A}{V} \right) \times M \)

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) x 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. Validation of Method

10.1. The method validation plan was written and is stored with the validation data. The plan was followed to determine this assay’s linearity, precision, limit of detection, limit of quantitation, and carryover threshold. The validation results are as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Methadone</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.008 µg/mL</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.050 µg/mL</td>
</tr>
<tr>
<td>Curve Linearity</td>
<td>0.05–25 µg/mL- Linear 1/x</td>
</tr>
<tr>
<td>Upper limit of quantification</td>
<td>25 µg/mL</td>
</tr>
<tr>
<td>Carryover</td>
<td>&gt;200 µg/mL</td>
</tr>
</tbody>
</table>
11. Quality Control

11.1. Acceptance criteria

11.1.1. Chromatogram
   11.1.1.1. Peaks must be Gaussian shaped (symmetrical).
   11.1.1.2. Peaks sharing precursor/product ions must have baseline resolution.
   11.1.1.3. The peak of interest is inspected visually for the presence of unresolved co-eluting peaks. The maximum allowable valley between adjacent peaks must not exceed ~10% by visual inspection of the analyte peak height.
   11.1.1.4. 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.
   11.1.1.5. Retention time must not deviate outside ± 3% of target, based upon the retention time of the calibrators and controls.

11.1.2. Mass spectroscopy
   11.1.2.1. The ion ratio of all analytes must not be greater than ± 20% of the target ratio, as determined by a mid-level calibrator (CAL 4).
   11.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

11.1.3. Calibrators
   11.1.3.1. Analytical curves must have a coefficient of determination ($R^2$) of 0.992 or greater.
11.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (± 25% at LOQ).

11.1.3.3. Refer to “Calibration curve point exclusion guidelines” section of the QA/QC Manual.

11.1.4. Controls

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

11.1.5. Blanks

11.1.5.1. Matrix specific negative controls (blanks) must not contain the analyte of interest at a response greater than 1/10th of the signal obtained from the lowest positive calibrator.

11.1.5.1.1. If an analyte of interest is detected with a response greater than 1/10th of the signal obtained from the lowest positive calibrator, all cases specimens in which that analyte is present shall be repeated and a senior chemist notified.

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

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

<table>
<thead>
<tr>
<th>DIL Scenario</th>
<th>1</th>
<th>1</th>
<th>REPORT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>In curve</td>
<td>In curve</td>
<td>Average</td>
</tr>
<tr>
<td>B</td>
<td>In curve</td>
<td>AQL or BQL</td>
<td>“In” value</td>
</tr>
<tr>
<td>C</td>
<td>In curve</td>
<td>ND *</td>
<td>Repeat</td>
</tr>
<tr>
<td>D</td>
<td>AQL/BQL</td>
<td>AQL/BQL</td>
<td>Less than/ Greater than</td>
</tr>
<tr>
<td>E</td>
<td>BQL</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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

12.2.1.
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

12.2.2. Periodically, the assay will not achieve the reported range of linearity. In this situation, consult the Quality Assurance and Quality Control section of the Standard Operating Procedure manual.

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 mg/L (maximum of 3 decimal places e.g. 0.009 mg/L).

13. Reinjection

13.1. An extract may be reinjected due to ALS failure, apparent low recovery, to check for carry-over or to meet ion ratio and/or retention time criteria. Additional solvent may be added to the ALS vial if necessary due to excessive analyte concentration. The reinjected extract must be evaluated against the existing run. If any parameters have to be changed (e.g. thresholds due to response, smoothing due to split peaks, or windows etc.) then the control must be reinjected in addition to the patient samples under those criteria and must meet all QA/QC criteria. Finally, the entire batch may be reinjected (calibrators, controls and unknowns) in order to re-establish acceptance criteria. If ion ratio and retention time criteria for a specimen are not met, the specimen must be re-extracted. The data from the original injection and reinjection must be included in the data pack.
14. Preparation of Load

14.1. Enter case specimen data into LIMS in accordance with the Quality Assurance and Quality Control section of the Standard Operating Procedure manual.

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

14.2.1. Assignment sheet
14.2.2. Comments or note to file if applicable
14.2.3. Load summary
14.2.4. Specimen worklist
14.2.5. Chain of custody (Specimen)
14.2.6. Aliquot chain of custody
14.2.7. Standard and control worksheet
14.2.8. Sequence summaries/calibration reports – paper clipped
14.2.9. Calibrator data - paper clipped
14.2.10. Blank matrix data - paper clipped
14.2.11. Control data - paper clipped
14.2.12. Specimen data – stapled

15. References


