SOP 418 – U-Compounds by Solid Phase Extraction (SPE) for Quantification by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-ES/MS/MS)

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1. **Principle of Assay**

    1.1. This method is designed to confirm and quantitate U-47700, U-49900, and U-51754, and qualitatively identify U-48800 in biological specimens by Liquid Chromatography tandem-electrospray Mass Spectrometry (LC/MS/MS). The drugs are extracted from their biological matrix by solid-phase extraction and identified by the retention time and ion ratio of product ions.

    1.2. (Need Additional Intro)

2. **Specimens**

    2.1. This procedure is applicable to blood, urine, serum, vitreous humor, *bile, properly prepared tissue specimens (typically 1:4 homogenates), and *gastric contents. A duplicate sample size of 0.1 mL(g) for all specimen types is generally employed - unless otherwise noted on the load worksheet - so that the calibration curve encompasses the expected range of unknown specimens.

        2.1.1. *For non-typical matrices, an additional 0.1mL(g) 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. 100 mM Phosphate Buffer pH 6.0
    3.3. Methanol, HPLC grade
    3.4. Isopropanol, HPLC grade
    3.5. Concentrated ammonium hydroxide
    3.6. Methylene Chloride, HPLC grade
    3.7. 100mM Acetic Acid
    3.8. 100 ng/mL U-47700-d6 Internal Standard
    3.9. Mixed U-Compound Standard Mix Standard and Control Worksheet.
    3.10. Mixed U-Compound Positive Control (QC) Mix
    3.11. Drug Free Blood, Urine, Liver Homogenate
    3.12. Water with 0.1% formic acid
    3.13. Acetonitrile with 0.1% formic acid
    3.14. Methanol with 0.1% formic acid

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

4.1. **U-47700-d6 Internal Standard Stock Solution A (100 µg/mL)**

4.1.1. Into a vial containing 1mg U-47700-d6 (Cayman Chemical PN19219), add approximately 2mL methanol - cap and shake to dissolve.

4.1.2. Transfer U-47700-d6 solution to a 10mL volumetric flask and repeat from step 4.1.1 to rinse the vial.

4.1.3. Fill the 10 mL volumetric flask 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. **U-47700-d6 Internal Standard Stock Solution B (1 µg/mL)**

4.2.1. Into a 10mL volumetric flask, add 0.1mL of U-47700-d6 Stock Solution A (4.1) with a micropipette.

4.2.2. 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

4.3. **U-47700-d6 Internal Standard Working Solution (100 ng/mL)**

4.3.1. Into a 10mL volumetric flask, add 1mL of U-47700-d6 Internal Standard Stock Solution B (1µg/mL) with a micropipette.

4.3.2. 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

4.4. **U-Compound 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.

4.5. **100 mM Acetic Acid Solution**

4.5.1. To a 1000 mL Erlenmeyer flask, add 5.7 ml of glacial acetic acid, and fill to the 1000 mL mark with deionized water, and mix well. Alternatively, a stock reagent at 4 Liters can be prepared by making appropriate multiplier of 4 for all volumes used.

4.6. **100mM Phosphate Buffer pH 6.0**

4.6.1. To a newly opened 4L bottle of DI H_2O, add 48.8 grams of potassium phosphate (KH_2PO_4) and 1.64 grams sodium hydroxide (NaOH). Swirl until dissolved. Check pH. If necessary, adjust with NaOH or phosphoric acid (H_3PO_4) to a final pH of 5.5-6.5.

4.7. **Column Elution Solution (make fresh daily)**

4.7.1. To a 200 mL graduated cylinder add 40 mL of isopropyl alcohol, 4 mL of concentrated ammonium hydroxide, and fill to the 200 mL mark with dichloromethane.

4.8. **Water with 0.1% formic acid**

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

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

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

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

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

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

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

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

5. Equipment and Special Supplies

5.1. Test tubes, 16 x 100mm (or equivalent)
5.2. Centrifuge 2000 x g
5.3. Vortex mixer
5.4. Nitrogen evaporator
5.5. Positive Pressure Extraction Manifold
5.6. UCT Clean Screen® extraction columns (CSDAU206)
5.7. LC autosampler vials, 12 x 32 mm
5.8. Polyspring inserts, 5 mm O.D.

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

6.4.1. Click here for instrument method parameters: (TSQ04 – U-Compounds_).

7. **Target Ions (± 1 nominal mass)**

7.1.	U-47700	(**329** 284 173)
7.2.	U-47700-d6	(**335** 284 173)
7.3.	U-49900	(**357** 284 173)
7.4.	U-51754	(**343** 298 218)
7.5.	U-48800	(**343** 298 218)

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

8. **Prepare Samples**

8.1.1. Add 50µL internal standard to appropriate number of 16X125mm test tubes.

8.1.2. Add the U-compound standards and quality control solution to appropriately labeled tubes according to Standard and Control Worksheet.

8.1.3. Add 0.1mL blank blood to each of the standards and controls. Include a urine blank and QC and/or a liver homogenate blank and QC by adding 0.1 mL(g) of blank urine or blank liver to appropriately labeled test tubes (as appropriate).

8.1.4. Aliquot specimens in duplicates described in the Specimens section (2.1).

8.1.5. Add 3 mL of 100mM phosphate buffer (pH=6) to each test tube, vortex for 10 seconds.

8.1.6. Centrifuge samples for 5 minutes at 2000 x g.

8.1.7. Load the appropriate number of labeled, new Clean Screen® extraction columns (CSDAU206) for each sample onto the positive pressure manifold.

8.2. Prepare SPE Columns:

8.2.1. Introduce 3mL of methanol into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.2.2. Introduce 3mL of deionized water into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.2.3. Introduce 2mL of 100mM phosphate buffer (pH=6) into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.3. Apply Samples:

8.3.1. Introduce samples into columns while simultaneously placing clean and labeled 13x100mm test tubes in the corresponding position of the collection rack. Allow to drip by gravity or under 2 PSI (positive pressure). If the matrix of the specimen prevents the sample from moving through the column by gravity alone, positive pressure (2-5 PSI) can be applied via manifold or pipette bulb.

8.4. Wash SPE Columns:

8.4.1. Introduce 2mL of deionized water into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.2. Introduce 2mL of 100mM acetic acid into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.3. Introduce 3mL of methanol into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.4. Dry for 5-10 minutes under full positive pressure.

8.4.5. Prepare extraction manifold for specimen collection. Wipe tips clean and place secured SPE columns onto collection rack, verifying that SPE tips are securely inside collection tubes prepared in step 8.3.1.

8.4.6. Introduce 3mL of column elution solution (methylene chloride/IPA/Ammonium hydroxide 78:20:2) into columns and allow to drip by gravity.

8.4.6.1. Note: After column elution solution has completely eluted, apply full pressure to force through any remaining solution into the collection tubes.

8.4.7. Evaporate each specimen to dryness in a nitrogen evaporation apparatus at 40°C; 15 psi.

8.4.8. Reconstitute with 200µL methanol, vortex and transfer at least 100µL to LC autosampler vial.

8.4.9. Build and initiate sequence as directed in SOP 053 (TSQ04).

9. **Calculations**

9.1. Quantification

9.1.1. The method for processing the data using the Thermo LCQuan software is "U-Compounds" Method (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.

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 (1/x weighting) 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 = 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. Ratio 1 = QL_1/QN
9.8.1.1. QL_1 = response of the quantifying product ion
9.8.1.2. 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% (minimum window 7.2 seconds) 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 3).

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 to 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.

11. Validation of Method

Parameter	Result (TSQ04)	
Bias (Interday)	U-47700	**Blood:** Low -4.59% High -2.28%
		Liver: Low -3.85% High -0.72%
	U-49900	**Blood:** Low -0.75% High 5.33%
		Liver: Low -1.51% High 2.00%
	U-51754	**Blood:** Low -13.91% High -2.60%
		Liver: *Low -22.13% High -14.32%

Precision (Interday)	**U-47700**	**Blood:** Low 2.87% High 4.53%
		Liver: Low 4.74% High 3.53%
	U-49900	**Blood:** Low 2.19% High 4.91%
		Liver: Low 7.67% High 6.08%
	U-51754	**Blood:** Low 5.05% High 5.88%
		Liver: Low 7.15% High 3.99%
Calibration model	**U-47700** - 1/x Weighting	
	U-49900 - 1/x Weighting	
	U-51754 - 1/x Weighting	
Carryover	Tested to 2X high calibrator (1000ng/mL) with no detectable carryover.	
Interference Studies	No interfering signal observed from Internal Standard, Non-target analytes, or matrix.	
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	Minimal matrix effect observed for all analytes (less than 10% difference between target analytes and IS).	
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	Blood: U-47700 - Experimentally derived -1.99 ng/mL **Administratively set to 2.0 ng/mL**	
	Blood: U-49900 - Experimentally derived - 1.45 ng/mL **Administratively set to 2.0 ng/mL**	
	Blood: U-51754 - Experimentally derived - 1.83 ng/mL **Administratively set to 2.0 ng/mL**	
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**Blood: 5.0 ng/mL** (All analytes)	
	Liver: **5.0 ng/mL** - (on column) (All analytes)	

Dilution Integrity	Not determined as specimens not typically diluted. Will determine on case-by-case basis.
Processed Sample Stability (re-analyze after 11 days)	Specimens determined to be stable for up to 11 days (Re-capped and stored refrigerated).
Recovery	**U-47700** - Blood: 76% Liver: 78% **U-49900** - Blood: 90% Liver: 85% **U-51754** - Blood: 74% Liver: 70%

*** U-51754 control was evaluated using a mix of donor livers with results more consistent with the blood levels. A matrix effect with the laboratory's blank bovine liver matrix seems to cause lower than expected QC levels. Further evaluation is required prior to quantitative analysis of U-51754 in liver.**

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.

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 mg/L.

12.4.2. Analyte concentrations entered into Toxlog shall not exceed 3 decimal places (e.g. a result of 7.4 ng/mL shall be reported as 0.007mg/L)

12.5. U-51754 vs U-48800 - While U-51754 and U-48800 have the same molecular weight and fragment very similarly on the Mass Spectrometer, (identical parent and transition ions) the two compounds can be differentiated by this method. Use of the U-48800 reference specimen will help identify the differences on a batch to batch basis.

12.5.1. U-48800 elutes approximately 0.08 min. earlier than U-51754.

12.5.2. The 298m/z vs 218m/z ion ratios differ enough between the two compounds that the ±20% acceptance criteria will prevent the incorrect compound from being identified **in most cases** - vigilance is still required.

12.5.3. If identified, U-48800 shall be reported qualitatively.

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.