CONFIRMATION OF TRAZODONE BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

30.1 METHOD

This test method may be used to confirm the presence of trazodone in biological specimens. Trazodone (TRZ) and internal standard (TRZ-d_6) are isolated from biological matrices by liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source.

30.2 SPECIMENS

The specimen volume is 0.2 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in liver (tissue) homogenate or serum/plasma specimens (see 30.4.3.2).

30.3 REAGENTS, MATERIALS AND EQUIPMENT

30.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H_2O) is specified, laboratory general-use DI H_2O is used in reagent preparation. Organic solvents are reagent grade, unless otherwise specified.

- Acetonitrile (ACN), reagent grade and LC-MS grade
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade (or equivalent from a high-purity filtration system)
- Ethyl acetate (EtAC)
- Extraction solvent

  Add 20 mL ethyl acetate to a glass flask. Add 20 mL hexanes and mix. Use solvent on date of preparation only.
- Formic acid (concentrated)
- 0.1% Formic acid

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade DI H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the acid in a glass bottle at room temperature for up to one year.
- Hexanes

- Methanol (MeOH)
- Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)
- 0.13M Sodium borate solution (saturated)

  In a 100 mL volumetric flask, dissolve 4.9 g $Na_2B_4O_7 \cdot 10H_2O$ in approximately 75 mL DI H_2O. Dilute to 100 mL with DI H_2O and mix thoroughly (may require low heating). The weighed contents may not go completely into solution – this is normal. Store the solution in a glass bottle at room temperature for up to 6 months.

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

30.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- HPLC column (Agilent Zorbax Eclipse Plus C18; 4.6 mm x 75 mm ID, d_p=3.5 µm, or equivalent)
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

30.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS with API-ES source (SL/6130 model, or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heated stir plate, rotary mixer, solvent filtration apparatus, vortex mixer)

30.4 STANDARDS, CALIBRATORS AND CONTROLS

30.4.1 STANDARDS

- Working standard: 20 ng/µL
- Working control standard: 20 ng/µL
- Working internal standard (TRZ-d_6): 1 ng/µL

30.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 30.5 SAMPLE PREPARATION.

30.4.3 CONTROLS

30.4.3.1 At least one negative whole blood control and three positive whole blood controls are tested with every batch, prepared as described in 30.5.

30.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

30.4.3.3 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls.

30.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

30.5 SAMPLE PREPARATION

NOTE: Methocarbamol was shown to interfere with m/z 148 in method validation, co-eluting with the trazodone peak. An alternative method must be used for confirmation of trazodone if methocarbamol is also identified in the case sample.

NOTE: Organic solvents used in sample preparation are reagent grade.

30.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample)

30.5.2 Add 1 mL 0.13M sodium borate solution into each tube.

30.5.3 Using a calibrated pipette, add 0.2 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

30.5.4 Prepare a 1:10 dilution of the working standard. (2 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

30.5.5 Prepare a 1:100 dilution of the working standard. (0.2 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

30.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 50 ng/mL	50	0.2 ng/µL	1:100
Calibrator 2 – 100 ng/mL	100	0.2 ng/µL	1:100
Calibrator 3 - 250 ng/mL	25	2 ng/µL	1:10
Calibrator 4 - 500 ng/mL	50	2 ng/µL	1:10
Calibrator 5 - 1000 ng/mL	100	2 ng/µL	1:10
Calibrator 6 - 2000 ng/mL	20	20 ng/µL	WS

30.5.7 Prepare a 1:10 dilution of the control working standard. (2 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

30.5.8 Prepare a 1:100 dilution of the control working standard. (0.2 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

30.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions of the control working standard.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Control 1 – 150 ng/mL	150	0.2 ng/µL	1:100
Control 2 – 750 ng/mL	75	2 ng/µL	1:10
Control 3 - 1600 ng/mL	160	2 ng/µL	1:10

30.5.10 Using a calibrated pipette, sample 0.2 mL of each case sample into its respective tube.

30.5.11 Using a calibrated pipette or verified repeater-pipette, add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.

30.5.12 Briefly vortex mix.

30.5.13 Add 2 mL extraction solvent to each tube.

30.5.14 Cap the tubes and place on a rotary mixer for 5 minutes.

30.5.15 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

30.5.16 Transfer the organic layer to a clean, labeled centrifuge or screw-cap tube.

30.5.17 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

30.5.18 Reconstitute the extracts with the addition of 100 µL MeOH to each tube and briefly vortex mix. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

30.5.19 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

30.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – TRAZODONE (instrumental parameters in Appendix A)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4

  Result comparisons – truncated, whole integer values in units of ng/mL

NOTE: If venlafaxine has been identified in a specimen, trazodone qualifier ion (m/z 148) chromatography and integration should be carefully evaluated upon review, as venlafaxine will produce an m/z 148 peak at a retention time slightly in advance of the trazodone peak.

30.7 REPORTING

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), and truncated to two significant figures for reporting.

30.8 METHOD PERFORMANCE

- Limit of detection: 5 ng/mL (0.005 mg/L)
- Lower limit of quantification: 50 ng/mL (0.05 mg/L)
- Dynamic range: 50 – 2000 ng/mL (0.050 – 2.0 mg/L)
- Upper limit of quantitation: 2000 ng/mL (2.0 mg/L)

30.9 REFERENCES

- Sarah Swenson and Dawn Sklerov, in-house method development.
- Eva Choong, Serge Rudaz, Astrid Kottelat, Sophie Haldemann, Davy Guillarme, Jean-Luc Veuthey, Chin B. Eap. Quantification of 4 antidepressants and a metabolite by LC-MS for therapeutic drug monitoring. *Journal of Chromatography B* 879: 1544-1550 (2011).
- Bhavin N. Patel, Naveen Sharma, Mallika Sanyal, Pranav S. Shrivastav. High throughput and sensitive determination of trazodone and its primary metabolite, *m*-chlorophenylpiperazine, in human plasma by liquid chromatography – tandem mass spectrometry. *Journal of Chromatography B* 871: 44-54 (2008).

- Justin L. Poklis, Carl E. Wolf, Ashley Goldstein, M. Lauren Wolfe, Alphonse Poklis. Detection and Quantification of Tricyclic Antidepressants and Other Psychoactive Drugs in Urine by HPLC/MS/MS for Pain Management Compliance Testing. *J. Clin Lab Anal.* 26(4): 286-294 (July 2012).
- Tatsuo Shinozuka, Masaru Terada, Einosuke Tanaka. Solid phase extraction and analysis of 20 antidepressant drugs in human plasma by LC/MS with SSI method. *Forensic Science International* 162: 108-112 (2006).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.8 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN (LC-MS grade)
Initial composition	90% A, 10% B
0 – 2 min	% B increased to 30%
Hold time	5.0 min (30% B)
Re-equilibration	5.0 min
Column temp	40°C
Autosampler	
Injection volume	1.0 µL
Injection flush-port	Active
Flush-port time/volume	15 sec
Flush-port solvent	ACN (LC-MS grade)

MASS SPECTROMETER

Ion mode	(+) SIM	Nebulizer gas	Nitrogen
EM gain	1.	Nebulizer pressure	30 psi
Peak width	0.08 min	Drying gas	Nitrogen
		Drying gas flow	13 L/min
		Drying gas temp	350 °C
		Capillary voltage	4 kV

Compound	Ions	Ion Ratios
Trazodone	372, 176, 148	176/372, 148/372
Trazodone-d_6	378, 182	182/378

LIST OF CHANGES

Revision Date	Description	Page Number
08/20/14	Method approved by Washington State Toxicologist. See DRA dated 8/14/14. Method released for use in evidentiary testing as of 8/20/14.	All
03/16/16	Added wording for adjustment of prepared volumes in 30.5.1.7 and clarification to 30.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates in 30.6.1.3 and 30.6.1.4. Added option for use of a column rinse method to 30.8. Edited 30.12.3 to reflect that only two significant figures are used for reporting and added "Printed Copies are Uncontrolled" to footer. Other minor edits throughout.	All
05/08/17	Wording added to 30.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 30.7. Edited 30.10.2.2.d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-8
7/9/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 30.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 30.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Formatting and minor edits throughout.	All