CONFIRMATION OF TRAZODONE BY LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

30.1 POLICY

This test method may be used to confirm the presence of trazodone (TRZ), with trazodone-d_6 (TRZ-d_6) internal standard, in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, or a Supervisor, and appropriately documented in the batch file.

30.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of trazodone present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compound.

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30.3 PRINCIPLE

The targeted compound and internal standard are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, the specimens, now formed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile phase and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion-monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point internal standard calibration is used to generate a calibration curve. The concentration of any target compound identified in a sample is determined from its calibration curve.

30.4 SPECIMENS

30.4.1 The specimen volume is 0.2 mL.

30.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

NOTE: Matrix-matching of calibrators and controls is not required for quantitation of trazodone in tissue homogenate samples, as determined through evaluation of alternative matrix (liver homogenate) during method validation.

30.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

30.4.4 Analysis of larger specimen volumes must be approved and documented.

30.5 REAGENTS, MATERIALS AND EQUIPMENT

30.5.1 REAGENTS

30.5.1.1 Acetonitrile

30.5.1.2 Certified blank blood

30.5.1.3 Deionized water (DI H_2O)

30.5.1.4 Ethyl acetate

30.5.1.5 Extraction solvent

Add 20 mL ethyl acetate to a glass flask. Add 20 mL hexanes and mix. For use on date of preparation only. Adjustments to final volume are permitted as long as proportions are maintained.

30.5.1.6 Formic acid (concentrated)

30.5.1.7 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.

30.5.1.8 Hexanes

30.5.1.9 Methanol

30.5.1.10 Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)

30.5.1.11 0.1M 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 buffer in glass bottle at room temperature for up to 6 months. Adjustments to final volume are permitted as long as proportions are maintained.

30.5.2 MATERIALS

30.5.2.1 Autosampler vials, inserts and caps

30.5.2.2 Disposable 16 x 100mm tubes with closures

30.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

30.5.2.4 Disposable pipette tips

30.5.2.5 HPLC column (Agilent Zorbax Eclipse Plus C18; 4.6 mm x 75 mm ID, d_p=3.5 μm, or equivalent)

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30.5.2.6 Laboratory glassware (graduated cylinders, flasks)

30.5.2.7 Solvent filters (0.45 μm pore size; reduced cellulose, other)

30.5.2.8 Volumetric glassware (flasks)

30.5.3 EQUIPMENT

30.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

30.5.3.2 Agilent MS with API-ES source (SL model or equivalent)

30.5.3.3 Calibrated, adjustable piston pipettes

30.5.3.4 Centrifuge

30.5.3.5 Evaporator (Biotage, formerly Zymark, TurboVap)

30.5.3.6 Rotary mixer

30.5.3.7 Heated mechanical stir plate

30.5.3.8 Solvent filtration apparatus

30.5.3.9 Vortex mixer

30.6 STANDARDS, CALIBRATORS AND CONTROLS

30.6.1 STANDARDS

30.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards which in turn are used to produce calibrators, positive controls and the working internal standard.

30.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a. Trazodone: 1.0 mg/mL
b. Trazodone-d_6: 0.1 mg/mL

30.6.1.3 Working standard (20 ng/μL)

a. Using calibrated pipettes, measure 500 µL of TRZ stock into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 20 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted provided that proportions remain constant.

30.6.1.4 Working internal standard (1 ng/μL)

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a. Using calibrated pipettes, measure 250 µL of TRZ-d_6 into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to designated volume.
c. The final concentration of the working internal standard is 1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted provided that proportions remain constant.

30.6.2 CALIBRATORS

30.6.2.1 Calibrators are prepared in certified blank whole blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 30.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

30.6.3 CONTROLS

30.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

30.6.3.2 Positive Controls

a. Three positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
d. The control working standard (20 ng/µL) is prepared as described in 30.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 30.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house or externally sourced positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix (Refer to NOTE in 30.4.2).

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30.7 SAMPLE PREPARATION

30.7.1 Label a clean 16 x 100mm tube for each member of the test batch (i.e. calibrator, control, case sample).

30.7.2 Place 1 mL of sodium borate solution into each tube.

30.7.3 Add 0.2 mL of certified blank whole blood into each of the six calibrator tubes, the three positive control tubes and the negative control tube(s).

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

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

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

a. Using calibrated pipettes, combine 0.1 mL of the 2 ng/μL working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

30.7.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator Description	Volume (μL) Added	Working Standard
Calibrator 1 (50 ng/mL)	50	0.2 ng/ μL
Calibrator 2 (100 ng/mL)	100	0.2 ng/ μL
Calibrator 3 (250 ng/mL)	25	2 ng/ μL
Calibrator 4 (500 ng/mL)	50	2 ng/ μL
Calibrator 5 (1000 ng/mL)	100	2 ng/ μL
Calibrator 6 (2000 ng/mL)	20	20 ng/ μL

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

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

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

a. Using calibrated pipettes, combine 0.1 mL of the 2 ng/μL control working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

30.7.9 Using the control working standard and the prepared dilutions, spike the positive controls according to the following table.

Control Description	Volume (μL) Added	Control Working Standard
Control 1 (150 ng/mL)	150	0.2 ng/μL
Control 2 (750 ng/mL)	75	2 ng/μL
Control 3 (1600 ng/mL)	160	2 ng/μL

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30.7.10 If in-house positive controls are being used, transfer 0.2 mL of each into their labeled tubes.

30.7.11 Sample 0.2 mL of each case sample into its respective tube.

30.7.12 Add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.

30.7.13 Cap the tubes and briefly vortex mix.

30.7.14 Add 2 mL extraction solvent to each tube.

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

30.7.16 Centrifuge the tubes for 10 minutes at 3500 rpm.

30.7.17 Transfer the organic layer to clean, labeled 10 mL centrifuge or screw cap tubes.

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

30.7.19 Reconstitute the extracts with addition of 100 uL of methanol to each tube. Briefly vortex mix the tubes. If, necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

30.7.20 Transfer the extracts to labeled autosampler vials and cap.

30.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation or Open Lab to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is TRAZODONE.M for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g. using method RINSE.M), or this may be done manually.

30.9 DATA ANALYSIS

30.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.

30.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

30.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

30.9.4 Technical review of the batch is conducted according to the criteria listed below.

30.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

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30.10.1 Calibrators and calibration curves

30.10.1.1 Chromatographic peaks for TRZ and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

30.10.1.2 Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

30.10.1.3 Quantitative results for TRZ in each calibrator shall be within ±20% of their target values with the exception of calibrator 1, which shall be within ±25% of its target. These are inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.

30.10.1.4 The calibration curve for TRZ shall have a correlation coefficient ≥0.99.

30.10.2 Controls

30.10.2.1 The negative control(s) shall not identify TRZ above its limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

30.10.2.2 Positive controls

a. Chromatographic peaks for TRZ and internal standard shall appear symmetrical.
b. Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for TRZ in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
d. At least two positive controls must meet these criteria for TRZ for the batch to be accepted.

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30.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

30.11.1 Any chromatographic peaks for TRZ and internal standard shall appear symmetrical.

30.11.2 The retention time for TRZ is ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

30.11.3 The quantitative result for TRZ must be within the dynamic range of the test method.

30.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

NOTE: If venlafaxine has been identified in a case sample, 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. Methocarbamol was also shown to interfere with m/z 148, 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.

30.12 REPORTING

30.12.1 Results are reported in units of milligrams per liter (mg/L).

30.12.2 The whole integer, truncated results are converted from ng/mL to mg/L.

30.12.3 Converted results are truncated to two significant figures for reporting.

a. For example: trazodone is measured as 209.32 ng/mL.

b. The unit conversion step truncates the result to 209 ng/mL and then represents the result as 0.209 mg/L.

c. The result is truncated to 0.20 mg/L (two significant figures) and reported.

30.12.4 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

30.13 METHOD PERFORMANCE

30.13.1 Limit of detection: 5.0 ng/mL

30.13.2 Lower limit of quantification: 50 ng/mL (0.05 mg/L)

30.13.3 Dynamic range: 50 - 2000 ng/mL

30.13.4 Upper limit of quantitation: 2000 ng/mL (2.0 mg/L)

30.14 TRACEABILITY

30.14.1 Traceability of the reference materials to SI units is provided through the certificates of analysis provided by the approved reference material supplier.

30.15 REFERENCES

30.15.1 Sarah Swenson and Dawn Sklerov, in-house method development.

30.15.2 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).

30.15.3 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).

30.15.4 Justin L. Poklis, Carl E. Wolf, Ashley Goldstein, M. Lauren Wolfe, Alphonse Poklis. Detection and Quantification of Tricyclic Antidepressants and Other

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Psychoactive Drugs in Urine by HPLC/MS/MS for Pain Management Compliance Testing. *J. Clin Lab Anal.* 26(4): 286-294 (July 2012).

30.15.5 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).

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APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.80 mL/min
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile
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	15 sec
Flush-port Solvent	Acetonitrile

MASS SPECTROMETER

Ion Mode	(+) SIM	Nebulizer Gas	Nitrogen
EM Gain	1.0	Nebulizer Pressure	30 psi
Peakwidth	0.08 min	Drying Gas	Nitrogen
		Drying Gas Flow	13 L/min
		Drying Gas Temperature	350 °C
		Capillary Voltage	4kV
Signals	Ion	Ion Ratios	
Trazodone	372, 176, 148	176/372, 148/372	
Trazodone-d_6	378, 182	182/378	

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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
3/16/16	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

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