BASIC DRUG IDENTIFICATION/CONFIRMATION BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY/NITROGEN PHOSPHORUS DETECTION

14.1 POLICY

This test method may be used to identify and/or confirm the presence of select basic drugs in biological samples. Quantitative results obtained through the use of this method will only be reported within the 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, and appropriately documented in the batch file.

14.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification, confirmation and/or quantitation of select basic drugs 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 compounds.

14.3 PRINCIPLE

The targeted compounds and internal standard, metycaine, 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 termed extracts, are injected into a gas chromatograph (GC) equipped with a nitrogen phosphorus detector (NPD), where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, the mass-to-charge ratios of each compound and related fragments are measured. Multiple-point, internal standard calibration is used to generate a calibration curve (NPD and MS). The concentration of any target compound identified in a sample is determined from its calibration curve.

14.4 SPECIMENS

14.4.1 The specimen volume is 1 mL.

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

14.4.3 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.

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

14.5 REAGENTS, MATERIALS AND EQUIPMENT

14.5.1 REAGENTS

14.5.1.1 Acetonitrile

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14.5.1.2 Ammonium carbonate (saturated)

14.5.1.3 Ammonium hydroxide (NH_4OH, concentrated)

14.5.1.4 Boric acid (H_3BO_3)

14.5.1.5 n-butyl chloride

14.5.1.6 Certified blank blood

14.5.1.7 Chloroform

14.5.1.8 Deionized water (DI H_2O)

14.5.1.9 Ethyl acetate

14.5.1.10 Hydrochloric acid (HCl, concentrated)

14.5.1.11 3N Hydrochloric acid

Add 125 mL concentrated hydrochloric acid to 300mL DI H_2O in a glass flask. Dilute to 500 mL with DI H_2O. Store in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

14.5.1.12 Methanol

14.5.1.13 Potassium chloride (KCl)

14.5.1.14 Sodium borate decahydrate ($Na_2B_4O_7 \bullet 10H_2O$)

14.5.1.15 0.13M sodium borate solution (saturated)

In a glass flask, dissolve 4.9 g $Na_2B_4O_7 \bullet 10H_2O$ in approximately 75 mL DI H_2O. Dilute to 100mL 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 six months. Volumes may be adjusted provided proportions remain the same.

14.5.1.16 Sodium carbonate (Na_2CO_3)

14.5.2 MATERIALS

14.5.2.1 Autosampler vials, inserts and caps

14.5.2.2 Disposable 16 x 100mm tubes

14.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

14.5.2.4 Disposable pipette tips

14.5.2.5 Disposable safety closures for 16mm tubes

14.5.2.6 Disposable transfer pipettes

14.5.2.7 GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 μm film thickness, or equivalent)

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14.5.2.8 Laboratory glassware (graduated cylinders, flasks)

14.5.2.9 Volumetric glassware (flasks)

14.5.3 EQUIPMENT

14.5.3.1 Agilent GC (6890 or equivalent) equipped with an NPD detector

14.5.3.2 Agilent MS (5973 or equivalent)

14.5.3.3 Calibrated, adjustable air-displacement pipettes

14.5.3.4 Centrifuge

14.5.3.5 pH Meter and/or indicating pH paper

14.5.3.6 Rotary mixer

14.5.3.7 Vacuum aspirator

14.5.3.8 Vortex mixer

14.6 STANDARDS, CALIBRATORS AND CONTROLS

14.6.1 STANDARDS

14.6.1.1 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.

14.6.1.2 Stock standards are purchased from an approved reference material supplier and include the following:

<u>Group A</u>

a. Bupropion: 1.0 mg/mL
b. Citalopram: 1.0 mg/mL
c. Dextromethorphan: 1.0 mg/mL
d. Diphenhydramine: 1.0 mg/mL
e. Tramadol: 1.0 mg/mL
f. Venlafaxine: 1.0 mg/mL

<u>Group B</u>

a. Alprazolam: 1.0 mg/mL
b. Amitriptyline: 1.0 mg/mL
c. Chlorpheniramine: 1.0 mg/mL
d. Chlorpromazine: 1.0 mg/mL
e. Clonidine: 1.0 mg/mL
f. Codeine: 1.0 mg/mL
g. Cyclobenzaprine: 1.0 mg/mL
h. Doxepin: 1.0 mg/mL
i. Fentanyl: 1.0 mg/mL
j. Fluoxetine: 1.0 mg/mL
k. Hydrocodone: 1.0 mg/mL
l. Lidocaine: 1.0 mg/mL
m. MDA: 1.0 mg/mL

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n. MDMA: 1.0 mg/mL
o. Meperidine: 1.0 mg/mL
p. Methamphetamine: 1.0 mg/mL
q. Nordiazepam: 1.0 mg/mL
r. Nortriptyline: 1.0 mg/mL
s. Oxycodone: 1.0 mg/mL
t. Propoxyphene: 1.0 mg/mL
u. Sertraline: 1.0 mg/mL
v. Trazodone: 1.0 mg/mL
w. Verapamil: 1.0 mg/mL
x. Zolpidem: 1.0 mg/mL

NOTE: Metycaine internal standard is purchased as a solid reference material and weighed at time of working standard preparation.

14.6.1.3 Working standard – Group A (10 ng/µL)

a. Using a calibrated pipette, measure 1 mL each of stock standards into a 100 mL class-A volumetric flask.
b. Add methanol or acetonitrile to the flask to the designated volume.
c. The final concentration of the Group A working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

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14.6.1.4 Working standard – Group B (10 ng/µL)

a. Using a calibrated pipette, measure 0.5 mL each of stock standards (1.0 mL for amitriptyline, nortriptyline, fluoxetine and trazodone) into a 50 mL class-A volumetric flask.
b. Add methanol or acetonitrile to the flask to the designated volume.
c. The final concentration of the Group B working standard is 10 ng/µL (20 ng/µL amitriptyline, nortriptyline, fluoxetine and trazodone). The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

14.6.1.5 Working standard – Miscellaneous basic drugs

a. For the identification and/or quantitation of basic drugs other than those in Group A or B, working standards may be prepared as above in 14.6.1.3 or 14.6.1.4. Working standards may be prepared from stock standards obtained from an approved supplier or, if necessary, from in-house prepared stock solutions.
b. The concentrations of the prepared working standards may be adjusted, provided that final calibrator and control concentrations are documented in the batch file.
c. Lot numbers of the prepared working standards must be documented in the batch file.

14.6.1.6 Working internal standard (10 ng/µL)

a. Dissolve 10 mg metycaine in 10 mL methanol. Dilute to 1 L in ethyl acetate.
b. The final concentration of the working internal standard is 10 ng/μL. The working internal standard is stored at room temperature in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.
c. For miscellaneous basic drug quantitation, alternative internal standards may be used at the Forensic Scientist's discretion, including deuterated compounds.

14.6.2 CALIBRATORS

14.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 14.7 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.

14.6.3 CONTROLS

14.6.3.1 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.)

14.6.3.2 Positive Controls

a. At least two positive whole blood controls are tested with every quantitative batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added at time of extraction.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards should be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot or supplier must be used, the working control standard should be prepared by someone other than the person that prepared the working standard.
d. The control working standard (10 ng/μL) is prepared as described in 14.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 14.7 SAMPLE PREPARATION. For miscellaneous basic drug quantitation, control target concentrations are at the Forensic Scientist's discretion. Alternatively, quality control personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

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14.7 SAMPLE PREPARATION

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

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

14.7.3 Add 1 mL of certified blank whole blood into each of the Group A and B calibrator tubes, and the positive and negative control tubes.

14.7.4 Prepare a 1:10 dilution of the Group A working standard. (1 ng/µL)

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

14.7.5 Using the Group A working standard and the prepared dilution, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 (100 ng/mL)	100	1 ng/µL
Calibrator 2 (250 ng/mL)	25	10 ng/µL
Calibrator 3 (500 ng/mL)	50	10 ng/µL
Calibrator 4 (1000 ng/mL)	100	10 ng/µL

NOTE: For screening only, Group A calibrator 3 may be used for retention time and mass spectrum match for compound identification. For quantitation of miscellaneous basic drugs, target calibrator levels are at the discretion of the Forensic Scientist.

14.7.6 Add 50µL of the Group B working standard to its designated tube.

14.7.7 Using the Group A control working standard, spike the positive controls according to the following table.

Control Description	Volume (µL) Added	Working Control Standard
Low Control (200 ng/mL)	20	10 ng/µL
High Control (800 ng/mL)	80	10 ng/µL

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14.7.8 Sample 1 mL of each case sample into its respective tube.

14.7.9 Add 50 µL of the working internal standard solution to each tube and briefly vortex mix. Final concentration of the internal standard is 500 ng/mL.

14.7.10 Add 3 mL n-butyl chloride to each tube.

14.7.11 Cap the tubes and place on a rotary mixer for 20 minutes.

14.7.12 Centrifuge the tubes for 10 minutes at 3500rpm.

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

14.7.14 Add 200 µL 3N HCl to each tube.

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

14.7.16 Centrifuge the tubes for 5 minutes at 2000-3500 rpm.

14.7.17 Aspirate the organic layer to chemical waste.

14.7.18 Add 100µL saturated ammonium carbonate to each tube.

14.7.19 Add 100µL concentrated ammonium hydroxide to each tube and vortex mix.

14.7.20 Add 150µL chloroform to each tube and vortex mix for at least 30 seconds.

14.7.21 Cap tubes and centrifuge 5 minutes at 2000-3500 rpm.

14.7.22 Transfer the bottom (chloroform) layer to two labeled autosampler vials with inserts and cap.

14.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation 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 BASIC for each line. For miscellaneous basic drug quantitation, instrumental parameters may be adjusted at the discretion of the Forensic Scientist (e.g., selected ion monitoring (SIM), temperature program).

14.9 DATA ANALYSIS

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

14.9.2 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. The Group B calibrator is used to generate an internal standard, single-point, linear regression with a 1/a weighting factor.

14.9.3 Whenever possible, quantitation should be based on NPD data; however, quantitation based on MSD data is allowable if the reason is documented and approved. Alternatively, any compound may be quantified using a SIM method, as appropriate.

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

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

14.10 CRITERIA FOR BATCH ACCEPTANCE

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

14.10.1 Calibrators and calibration curves

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

14.10.1.2 Retention times shall be within ±2% of those in calibrator 3. These are inclusive ranges.

NOTE: If performing SIM quantitation of miscellaneous basic drugs, retention times shall be within ±2% and ion ratios must be within ±20% of those in the updating calibrator.

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

14.10.1.4 The calibration curves for those compounds in the quantitative standard shall have a correlation coefficient ≥0.99.

14.10.2 Controls

14.10.2.1 The negative control(s) shall not identify any target compound above its limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions.

14.10.2.2 Positive controls (quantitative)

a. Chromatographic peaks for target compounds and internal standard shall appear symmetrical.

b. Retention times for target compounds and internal standard shall be within ±2% of those in calibrator 3. These are inclusive ranges.

NOTE: If performing SIM quantitation of miscellaneous basic drugs, retention times shall be within ±2% and ion ratios must be within ±20% of those in the updating calibrator.

c. Quantitative results for all compounds 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 one Group A positive control must meet these criteria for all compounds for the batch to be acceptable for reporting of Group A compounds.

e. For miscellaneous basic compound quantitation, positive spiked controls must meet criteria in 14.10.2.2.a-c above for that compound to be reported from the batch. Failure of one or both spiked controls for one compound does not affect reporting of other compounds.

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14.11 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.

14.11.1 Quantitative acceptance

14.11.1.1 Any chromatographic peak for Group A compounds shall appear symmetrical.

14.11.1.2 The retention times for any reportable compounds are ±2% of those in calibrator 3. These are inclusive ranges.

NOTE: If performing SIM quantitation of miscellaneous basic drugs, retention times shall be within ±2% and ion ratios must be within ±20% of those in the updating calibrator.

14.11.1.3 The quantitative results for each identified compound must be within the dynamic range of the test method.

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

14.11.2 Qualitative acceptance

14.11.2.1 Any chromatographic peak for compounds identified shall appear symmetrical.

14.11.2.2 The retention times for any identified compounds are ±2% of those in Group A calibrator 3 or the Group B calibrator. These are inclusive ranges.

14.12 REPORTING

14.12.1 Quantitative reporting

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

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

14.12.1.3 Converted results are truncated to no more than two significant figures for reporting.

a. For example: tramadol is measured as 425.69 ng/mL.

b. The unit conversion step truncates the result to 425 ng/mL and then represents the result as 0.425 mg/L.

c. The result is truncated to 0.42 mg/L (two significant figures) and reported.

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

14.12.2 Qualitative reporting

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14.12.2.1 To appropriately identify and report a compound as present in a case sample, the following must be demonstrated:

a. The retention times for any identified compounds are ±2% of those in calibrator 3 for Group A compounds. For Group B compounds, retention times are ±2% of those in the Group B calibrator. These are inclusive ranges.

b. A mass spectral match of the detected compound to that in an approved mass spectral library or comparison to a mass spectrum in a calibrator or control. When using a library match, spectrum agreement should be 75 or greater wherever possible, taking into consideration the appearance and abundance of ions specific to that compound (an extracted ion match may be necessary). A reference spectrum for the compound found in a published article, research paper or other reference material may be acceptable if an electronic library match is not feasible, provided the source is documented.

c. Chromatography must meet criteria for acceptance found in 14.11.1.1 and 14.11.2.1.

14.12.2.2 Any compounds initially identified using this method must be confirmed using this or another test method on a separate sampling before reporting.

14.13 METHOD PERFORMANCE

14.13.1 Group A Compound Quantitation

14.13.1.1 Lower limit of quantification: 0.1 mg/L

14.13.1.2 Dynamic range: 0.1 mg/L – 1.0 mg/L

14.13.1.3 Upper limit of quantification: 1.0 mg/L

14.13.2 For miscellaneous basic compound quantitation, results are reported within the dynamic range of the batch.

14.14 TRACEABILITY

14.14.1 Traceability of the reference materials is provided through the certificate of analysis provided by the approved reference material supplier.

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APPENDIX A
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Pulsed Splitless
Inlet Liner	4mm gooseneck w/glass wool plug
Temperature	250˚C
Pulse Pressure	45.0 psi
Pulse Time	1.00 min
Purge Flow	3.0 mL/min
Purge Time	0.00 min
Autosampler	
Gas Type	Helium
Injection Volume	3.0 µL
Solvent Wash A	Ethyl acetate
Solvent Wash B	Ethyl acetate
Pre-injection Wash	4
Post-injection Wash	4
Sample Pumps	2

Oven/Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.5 mL/min
Initial Temperature	90˚C
Initial Time	1.00 min
Ramp Rate	15.00˚C/min
Final Temperature	180˚C
Final Time	0.00 min
Ramp Rate	10.00˚C/min
Final Temperature	300˚C
Final Time	10.00 min
Front Detector/NPD	
Temperature	320˚C
H_2 Flow	3.0 mL/min
Air Flow	50.0 mL/min
N_2 Flow (Makeup)	15.0 mL/min

MASS SPECTROMETER

Solvent Delay	3.00 min	MS Quad Temperature	150°C
EM Offset	200	MS Source Temperature	230°C
Mode	Scan	Scan Range	40-550
Transfer Line Temperature	28[illegible]˚C		

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LIST OF CHANGES

Revision Date	Description	Page Number
10/03/12	Method approved by Washington State Toxicologist. See DRA dated 09/28/12. Method released for use in evidentiary testing on 10/03/12.	All
04/22/13	References to "Group B positive control " throughout were changed to "Group B calibrator." Spiked positive controls were introduced in place of prepared, pooled, whole blood controls. See detailed changes in DRA dated 04/12/13.	3-10
10/28/13	Removed ketamine and methadone from Group A Working Standard and the list of compounds in 14.6.1.2. Added chlorpromazine to list of compounds in Group B Working Standard in 14.6.1.2. See detailed changes in DRA dated 10/17/13.	3-4
3/16/15	Amended wording for deviation approval by a member of TLD Management in 14.1. Reagent and sample preparation sections modified to reflect use of 0.13M sodium borate solution instead of pH9 phosphate buffer. See DRA dated 3/11/15.	1, 2, 6

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