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4.1.2 Protocol for GCMS-15-2, GCMS-14-2 and LS-TX-GCMS12

4.1.2.1 Analyte(s)

See toxicology scope of analysis in Toxicology Procedure Manual 1.14

4.1.2.2 Specimen Requirements

2 mL blood.

<2 mL may be used if sample quantity is limited, however, positive results shall be reported qualitatively.

4.1.2.3 General Description of Method

- An internal standard GC/MS confirmation and identification of acidic, neutral and basic drugs.
- Quantitation of selected acidic, neutral and basic drugs.
- Extraction of the analytes of interest from the biological matrix is accomplished by using a solid phase extraction (SPE) method that has been adapted from a United Chemical Technologies (UCT) method, formerly Worldwide Monitoring Corp.

4.1.2.4 Equipment and Reagents

- GC/MS equipped with a suitable column for separating the analytes of interest from other drugs and coextractives (i.e. 15 meter DB5 capillary column).
- UCT standard SPE vacuum tank, manifold, vacuum source, reagents, and SPE columns as specified in the UCT procedure manual code EMB200DAUZ050191.
- Internal standard(s): deuterated analogs of phencyclidine, diazepam, nordiazepam, butalbital, phenobarbital, alprazolam and/or others where appropriate.
- Authentic drug standards for calibration standards and positive controls (see below).
- The usual assortment of laboratory glassware, reaction vessels, pipettes, reagent grade chemicals, vortexers and shakers.

4.1.2.5 Sample Preparation and Extraction

A homogeneous blood sample is assured by gently rocking the specimen on the Labquake Shaker for at least 5 minutes. If the specimen is clotted, homogenizing glassware can be used to obtain a liquid sample. All sample handling will be performed in the biological safety cabinet using the universal biohazard handling techniques.

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4.1.2.5.1 Sample Preparation

4.1.2.5.1.1 Standard Calibrators

- Prepare a standard curve by pipetting 2 mL blank blood and 20 µL each of the appropriate ANB standard and cocaine standard into a clean, labeled 16 x 125 mm culture tube.
- See 4.1.2.11.1 below for analyte concentrations in standards.

4.1.2.5.1.2 Controls

- Prepare a negative control by pipetting 2 mL of blank blood into clean, labeled 16 x 125 mm culture tube.
- Prepare low, medium and high positive controls by pipetting 2 mL blank blood and 20 µL of the appropriate control(s) into clean, labeled 16 x 125 mm culture tubes.
- Prepare a UTAK ANB Blood 100 ng/mL Whole Blood Toxicology Control:
 - Remove from freezer and allow to thaw at room temperature with cap on prior to use.
 - Control should not be thawed in a water bath or by heating.
 - Swirl gently by hand for 3 - 4 minutes to ensure a homogeneous mixture.
 - Pipet 2 mL into a clean labeled 16 x 125 mm culture tube.
 - The thawed control is stable for 30 days when stored at 2 - 8° C.
- See 4.1.2.2.11.2 for analyte concentrations of low, medium, high and UTAK controls.

4.1.2.5.1.3 Unknowns

- Pipette 2 mL sample blood into a clean, labeled 16 x 125 mm culture tube. Less is allowable if sample quantity is limited. For low volume samples, add DI water to bring total sample volume to 2 mL.

4.1.2.5.1.4 Additional Sample Preparation for all Standards, Controls and Unknowns

- Add 1 mL of the internal standard solution to each standard, control and unknown. See 4.1.2.2.12.2 for internal standard analyte concentrations.
- Q.S. to 5 mL with DI water. Mix/vortex.
- Centrifuge for 10 minutes at ≥3000 rpm.
- Transfer supernatant into clean, labeled 16 x 125 mm culture tubes.
- Discard tubes containing pellets.
- Add 4 mL 100 mM phosphate buffer (pH 6.0); mix / vortex, pH should be 6.0 ± 0.5.
- Adjust with 100 mM monobasic or dibasic sodium phosphate as needed.

4.1.2.5.2 Condition Clean Screen Extraction Column

To appropriately labeled extraction columns placed on a manifold pass the following reagents individually through all columns under gravity or low vacuum (<3 in Hg):

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- 3 mL anhydrous methyl alcohol (CH₃OH), aspirate.
- 3 mL DI water, aspirate.
- 1 mL 100 mM phosphate buffer (pH 6.0), aspirate.

NOTE: Aspirate at ≤ 3 inches Hg to prevent sorbent drying.

4.1.2.5.3 Apply Sample

- Carefully, decant the sample mixture into its respective extraction column.
- Allow gravity to pull the sample through the column. (If vacuum assisted aspiration is used, use a flow rate of no more than 2 mL/min.)

4.1.2.5.4 Wash Column

NOTE: The vacuum pump on the extraction manifold should be turned on prior to washing the columns.

- 3 mL DI water, aspirate
- 1 mL 100 mM acetic acid, aspirate
- DRY COLUMN (≥ 10 INCHES Hg FOR 30 MIN)
- 500 µL hexane; aspirate

4.1.2.5.5 Elute Acidic and Neutral Drugs

NOTE: Acidic and Neutral Drug eluate may not need to be collected and analyzed in every case. Analysts should use screen results and other appropriate information to aid them in this determination.

Place clean, labeled 12 x 75 mm culture tubes into rack, place the rack inside the manifold tank ensuring each column will elute into its respective tube.

- 2 mL hexane/ethyl acetate (30/70); collect eluate using gravity or less than 2 mL/min vacuum.
- Use vacuum pump to remove the last of the eluate from column.
- Retain extraction columns.
- Evaporate eluate to dryness (≤ 40° C) in TurboVap.
- Reconstitute in an appropriate volume of ethyl acetate; use screening values and the following table as a guide.

Standard / Control	Suggested Solvent Volume (µL)
1	100
2	100
3	300

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4	300
5	400
6	400
Low	100
Medium	300
High	400
UTAK	100

- Transfer to clean, labeled autosampler vial. Ex: Microliter 12x32 crimp vial or equivalent.
- Cap with red rubber septum and metal crimp cap.
- Run on an appropriate GC/MS.

4.1.2.5.6 Wash Column

- 3 mL methanol, aspirate
- DRY COLUMN (≥ 10 INCHES Hg FOR 5 MIN)

4.1.2.5.7 Elute Basic Drugs

Place clean, labeled 12 x 75 mm culture tubes into rack, place the rack inside the manifold tank assuring each column will elute into its respective tube.

- 2 mL methylene chloride / isopropanol / ammonium hydroxide in the following appropriate ratios: (78/20/2), (39/10/1), (23.4/6/0.6), (19.5/5/0.5).
- **NOTE:** Prepare elution solvent daily. Add ammonium hydroxide to isopropanol then add the methylene chloride (pH >10).
- Collect eluate under gravity. Use vacuum pump to remove the last of the eluate from the column.

4.1.2.5.8 Evaporate Eluates

- Evaporate just to dryness ($\leq 40^\circ$ C) in TurboVap.
- Reconstitute in an appropriate volume of a 50:50 mixture of methyl alcohol and ethyl acetate; use screening values and the following table as a guide.

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Standard / Control	Suggested Solvent Volume (µL)
1	60
2	60
3	60
4	100
5	100
6	150
Low	60
Medium	100
High	150
UTAK	60

- Transfer to clean, labeled autosampler vial. Ex: Microliter 12x32mm conical crimp vial or equivalent.
- Cap with red rubber septum and metal crimp cap.
- Run on an appropriate GC/MS.

4.1.2.6 GC/MS Instrument Setup

- See the Toxicology ANB-Dedicated GC-MS (ISQ) Schedule for details regarding maintenance prior to batch analysis.

4.1.2.7 Data Analysis

4.1.2.7.1 Controls and Standards

- Each batch analysis should contain a negative, low, medium and high control. When available, an external control shall be run. Additionally, the following standards shall be analyzed;
 - Tier I
 - Tier II
- The positive controls and standards should contain all analytes of interest. Analytes of interest are defined as the analytes that are included in the scope of analysis.
- When multiple positive controls are run within a batch they must fall within +/-20% of the target concentration for an acceptable quantitative analysis. If one control does not meet the acceptance criteria, no action is necessary. If more than one control does not meet acceptance

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criteria all positive case samples in the batch should be repeated. Qualitative results may be reported when reanalysis is not possible or practical. Verification of control lot number and expiration date shall occur at the time calibrator/control packs are reviewed.

- The negative control should not contain enough of the analyte of interest to be confirmed.

4.1.2.7.2 Unknowns

- Casework samples shall be analyzed for a minimum of Tier I substances. Analytes detected during Tier I analysis, outside the scope of Tier I, may be reported by the casework scientist. The requirements in 4.1.2.9 apply.
- Casework samples shall be analyzed for Tier II substances when requested. Additionally, the casework scientist may initiate a full or partial Tier II analysis based upon observations within the data.
- The casework scientists may report analytes of interest that are outside the scope of Tier I and Tier II. The requirements in 4.1.2.9 apply.

4.1.2.7.3 Blanks

- Each casework pair of samples, acidic/neutral and basic, should have a minimum of one solvent blank run between them. Additional blanks may be run if necessary.
- The blank should not contain enough analyte of interest to be confirmed. If it does, this indicates carryover and the subsequent casework sample will need to be reinjected.

4.1.2.8 Standard Calibration Curve

- Calibration models, internal standards and peak detection algorithms have been validated for each quantified analyte as follows:

AN Fraction	Curve Type	Weighting	Origin	Quant Ion	I.S./Ion	Algorithm
butalbital	quadratic	Inverse (1/x)	Ignore	124	D5-butalbital/173	ICIS
carisoprodol	quadratic	Inverse (1/x)	Ignore	55	D5-butalbital/173	ICIS
meprobamate	quadratic	Inverse (1/x)	Ignore	144	D5-butalbital/173	ICIS
phenobarbital	linear	Inverse (1/x)	Ignore	204	D5-phenobarbital/209	ICIS

BSC Fraction	Curve Type	Weighting	Origin	Quant Ion	I.S./Ion	Algorithm
alprazolam	quadratic	Inverse (1/x)	Ignore	308	D5-alprazolam/313	ICIS

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diazepam	quadratic	Inverse (1/x)	Ignore	256	D5-diazepam/261	ICIS
methadone	quadratic	Inverse (1/x)	Ignore	223	D5-diazepam/261	ICIS
nordiazepam	quadratic	Inverse (1/x)	Ignore	242	D5-nordiazepam/247	ICIS
tramadol	quadratic	Inverse (1/x)	Ignore	263	D5-diazepam/261	ICIS
zolpidem	quadratic	Inverse (1/x)	Ignore	235	D5-diazepam/261	ICIS

- Calibration model information in the processing method or Quan Browser shall not be changed unless validated.
- A standard curve should be generated with $r^2 \geq 0.95$ for each analyte. If the r^2 value is within this range for some analytes, but not others, the supervisor shall determine how the data shall be interpreted. Verification of calibrator lot number and expiration date shall occur at the time calibrator/control packs are reviewed.
- Evaluate the curve by examining the value of the calibrators. Values of $\pm 20\%$ from the target calibrator concentration are acceptable except for standard 1 which allows $\pm 30\%$.
- At least 4 calibrators must be included in the calibration curve. No more than 2 calibrators may be removed from the calibration curve.
- If the lowest or highest calibrators are removed from the curve, the change in the working range may require a repeat analysis of case specimens that lie outside the new range. Alternately, the cases may be reported out as " $<$ (new lowest calibrator)" or " $>$ (new highest calibrator)" at the discretion of the unit supervisor.

4.1.2.9 Chromatographic and Mass Spectral Quality Control

- Chromatographic Quality
 - Chromatographic quality for Toxicology SIM data is defined as a reasonably symmetrical shaped peak consistent with those observed in calibrators and positive controls and is able to be differentiated from a negative control.
 - Chromatographic quality for Toxicology Full Scan data is defined as a resolved peak, not always symmetrical in nature, for three m/zs consistent with the analyte of interest and the TIC. The observable peak should be able to be differentiated from a negative control.
 - Flexibility is given to the experienced analyst to prevent misidentification and under-identification.
- Retention Time
 - Whenever possible, the retention time of positive analytes shall match a known reference standard run with each batch of unknowns. If a known reference standard is unavailable, a relative retention time based upon deuterated internal standards should be used. It is recognized that retention times may "shift", slightly, from that of the known reference standard. Flexibility is given to the experienced analyst to prevent misidentification and under-identification.
- Mass spectrum and ion ratios
 - Whenever possible, the mass spectrum and ion ratios of an identified analyte shall match a known reference standard. If a known reference standard is unavailable, a library match using an approved library is acceptable. It is recognized that ion ratios may change, slightly, from that of the known reference standard based upon factors such as

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analyte concentration, co-eluting substances and background noise. Flexibility is given to the experienced analyst to prevent misidentification and under-identification.

- Library Matches
 - If a known reference standard is unavailable, a library match from an accepted library may be used to aid in identification of an analyte. Approved libraries are:
 - DD2010
 - SWGDRG
 - nistdemo
 - mainlib
 - caymanspectrallibrary
 - Any MSP in-house library in which the name of the analyte, lot number and manufacturer have been recorded

Note: Chromatographic Quality and Mass Spectral Quality shall also be evaluated for all calibrators and controls. Calibrators and controls are not required to meet the criteria of showing chromatographic peaks for three m/zs (three m/zs should be observed in the mass spectrum however). All other criteria, a reasonably symmetrical peak, able to be differentiated from a negative control, retention time and mass spectral data shall be assessed. By completing the calibrate/control request within Forensic Advantage, the reviewer is stating that all applicable requirements have been met.

4.1.2.10 Reporting the Results

- Quantitative results will be reported in ng/mL.
- Quantitative results will be truncated (not rounded) and reported to the nearest whole number.
- If an analyte is present at a concentration < LOQ and ≥ LOD, it will be reported as "< (LOQ)".
- If an analyte is present at a concentration < LOD, it will be reported as "not detected".
- If an analyte is present above the ULOQ, it will be reported as ">(ULOQ)"
- If a sample was analyzed using less than 2 mL of sample, positive results will be reported out qualitatively.
- Qualitative positive results will be reported as "Detected (Not Quantified)".
- See also 4.6 Drug Reporting Guidelines for Forensic Advantage.

4.1.2.11 Drug Concentrations in Standards and Controls

4.1.2.11.1 Standards

Concentrations after dilution into 2 mL blank blood (ng/mL):

Analyte	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Alprazolam	10	25	50	100	250	500
Butalbital	200	500	1000	2500	5000	10000
Carisoprodol	200	500	1000	2500	5000	10000

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Diazepam	10	50	100	250	500	1000
Meprobamate	200	500	1000	2500	5000	10000
Methadone	10	50	100	250	500	1000
Nordiazepam	10	50	100	250	500	1000
Phenobarbital	10	50	100	250	500	1000
Tramadol	10	50	100	250	500	1000
Zolpidem	10	25	50	100	250	500

4.1.2.11.2 ANB Controls

Concentrations after dilution into 2 mL blank blood (ng/mL):

Analyte	Low	Medium	High	UTAK*
Alprazolam	20	200	400	100
Butalbital	400	4000	8000	1000
Carisoprodol	400	4000	8000	1000
Diazepam	20	400	800	100
Meprobamate	400	4000	8000	1000
Methadone	20	400	800	100
Nordiazepam	20	400	800	100
Phenobarbital	20	400	800	100
Tramadol	20	400	800	100
Zolpidem	20	200	400	100

*The UTAK control does not require dilution.