001. ETHANOL IN BIOLOGICAL SPECIMENS BY DUAL HEADSPACE GAS CHROMATOGRAPHY (HS/GC)

1.1. PRINCIPLE:

Volatile compounds are analyzed in biological fluids by gas chromatography using N-propyl alcohol as the internal standard. Water containing an internal standard is added simultaneously with the biological sample as it is sampled using an automatic diluter. It is then sealed in a headspace sample vial prior to analysis. Volatile sample components are extracted from the non-volatile sample components by heating, pressurizing the vial and then sampling from the equilibrated gas phase above the sample phase. One milliliter of this gas phase mixture is injected onto a column, which splits into 2 different gas chromatographic columns. The volatile compounds are separated based on their respective molecular weights and polarities and detected with a flame ionization detector. The identification of ethanol and other volatile compounds is made by comparing the relative retention times of the unknown to the retention time of an internal standard. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of volatile compounds in the sample.

By using 2 different columns that cause the volatiles to separate in different but known ways, a more specific identification is possible. The possibility of an interfering or co-eluting peak is also considerably reduced since it is unlikely to elute on both columns at the same retention time.

Samples are screened using the Volatiles Screen analytical method on the HS/GC. Positive samples are then quantified using the Ethanol Quantitation analytical method on the HS/GC.

1.2. BACKGROUND AND PHARMOKINETIC INFORMATION:

Effects of these drugs can be found in the corresponding section of the Toxicology Training Program.

1.3. SPECIMEN HANDLING:

1.3.1. BLOOD: Use blood specimens collected in gray top evacuated tubes (containing 100 mg sodium fluoride and 20 mg potassium oxalate). Blood specimens collected in other containers may be analyzed. The optimum sample size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.

1.3.2. URINE: Use urine specimens collected in urine collection bottles. Urine specimens collected in other containers may be analyzed. The optimum specimen size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.

1.3.3. VITREOUS FLUID: Use vitreous fluid specimens collected in red top tubes. Vitreous fluid specimens collected in other containers may be analyzed. The optimum specimen size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.

1.3.4. Keep specimens at 2°C to 8°C until analyzed; bring the specimens to room temperature before analysis.

1.4. SAFETY PRECAUTIONS:

1.4.1. Wear safety glasses, laboratory coats, and gloves when handling reagents, samples, and controls.

1.4.2. Sample aliquoting procedures should be performed under a bio-safety hood (BSL2) using universal precaution for blood borne pathogen (BBP) at all times.

1.4.3. Processed headspace vials are both heated and pressurized; take caution when removing from the autosampler, as they may burst upon impact.

1.4.4. All chemical hazard and safety information can be found in their SDS following SOP 126_SafetyDataSheet.

1.5. REAGENTS AND SUPPLIES:

1.5.1. N-propyl alcohol (high purity)

1.5.2. Commercially prepared Ethanol Calibrators (0.01, 0.02, 0.05, 0.08, 0.20, 0.40, 0.50 g/dL)

1.5.3. Commercially prepared Whole Blood Reference Control (targeted at 0.05 g/dL and 0.20 g/dL per manufacturer)

1.5.4. Commercially prepared Serum Interference Control (targeted at 0.15 g/dL Ethanol, 0.08 g/dL Acetone, 0.08 g/dL Isopropanol, and 0.04 g/dL Methanol per manufacturer)

1.5.5. 2 L volumetric flask

1.5.6. 5 mL volumetric pipettes

1.5.7. Adjustable Pipetter 10-100 µL

1.5.8. Agilent 10 mL headspace vials or equivalent

1.5.9. 20 mm butyl septa

1.5.10. 20 mm aluminum seals

1.5.11. Headspace vial racks

1.5.12. Automatic diluter

1.5.13. Tube rocker

1.5.14. Homogenizer

1.6. INSTRUMENTATION:

1.6.1. Two sets of instrumentation are maintained for ethanol analysis, either can be used:

1.6.1.1. Agilent Technologies 8890 Gas Chromatograph with dual flame ionization detector and Agilent Technologies 7697A Headspace Sampler (Headspace 2)

1.6.1.2. Agilent Technologies 6890N Gas Chromatograph with dual flame ionization detector Agilent Technologies 7697A Headspace Sampler (Headspace 1)

1.6.2. Chemstation™ software

1.7. STANDARDS, CONTROLS, AND INTERNAL STANDARD PREPARATION:

Label all reagents with reagent name, lot/tracking number, preparation date, preparer's initials, and expiration date. Record the standard reagent information in LIMS.

1.7.1. Ethanol Working Standard

Prepared by manufacturer. After opening the ethanol standard ampoule, transfer the solution to its individual glass vial and cap with a screw cap. Standards are good for 2 weeks from the time they are opened. Store vials in refrigerator at 2°C to 8°C when not in use.

1.7.2. Internal Standard Preparation

1.7.2.1. N-propyl Alcohol Internal Standard (IS) Preparation:

Add 200 µL N-propyl Alcohol to 2 L ultrapure water and mix well. Add 250 mg of sodium fluoride to prevent mold growth. Label reagent bottle with internal standard name, lot number, analyst's initials, and tracking number. Internal standard solution is good for 1 year from the month of preparation and can be stored at room temperature.

1.7.3. Control Preparation

All controls must be prepared from a separate stock source than the standards. Separate vendors and/or lot numbers are sufficient to fill this requirement.

1.7.3.1. Whole Blood Reference Control:

Prepared by manufacturer. Upon receipt, store at -10°C to -20°C until use. After opening, store in the refrigerator at 2°C to 8°C when not in use; expiration is 21 days from the time it is opened.

1.7.3.2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol):

Prepare according to manufacturer instructions. Store in the refrigerator at 2°C to 8°C; expiration is 30 days from the time it is prepared.

1.8. PROCEDURE:

1.8.1. Calibration Procedure

1.8.1.1. Calibrators consist of seven levels: 0.01, 0.02, 0.05, 0.08, 0.20, 0.40, 0.50 g/dL.

1.8.1.2. Generate a calibration curve at the beginning of screen batch of analysis with calibrators in order of increasing concentration. A minimum of 5 calibrators is needed to construct a valid calibration curve for quantitative calculations.

Note: Ethanol quantitation run on the same day as the screen use the calibration curve of the corresponding screen for quantitation. Ethanol quantitation run on a different day must have a calibration curve run at the beginning of the batch.

1.8.1.3. Calibrate the gas chromatograph prior to use with calibrators in order of increasing concentration. The same calibration curve is used for the screen and quantitation.

1.8.1.4. Values must fall within ± 10% of the target values before new calibrator lots can be introduced. If a previously validated calibrator is not available, then a prepared control must be used to verify a newly prepared calibrator.

1.8.1.5. Record verification results in LIMS. Make a new preparation/verification note under "Batch Comment."

1.8.2. Quality Control Procedure

1.8.2.1. Quality controls samples consist of:

1.8.2.1.1. Low and High Whole Blood Reference Control is used to evaluate the accuracy of the ethanol result.

1.8.2.1.2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol) is used to evaluate the presence of methanol, acetone, and isopropanol.

1.8.2.1.3. An Internal Standard Blank Control, containing only the IS, is used to evaluate carry over and contaminations within the auto dilutor.

1.8.2.2. The controls are used for the screen and quantitation.

Note: The Serum Interference Control is not used in the Ethanol Quantitation.

1.8.2.3. New Whole Blood Reference Lot:

1.8.2.3.1. Before a new Low or High Whole Blood Reference Control lot can be introduced, it must be verified by running 7 replicates of the control. The target for the new Low or High control is set using the mean value of the 7 replicates.

1.8.2.3.2. Record Whole Blood Reference Control results in LIMS. Make a new preparation/verification note under "Batch Comment."

1.8.2.3.3. Values for the new Low or High control must fall within ± 10% of the target values.

1.8.2.4. New Serum Interference Control Lot:

1.8.2.4.1. Responses for each of the 4 peaks in the Serum Interference Control must be greater than the screen lower reporting limits before the new lot can be introduced.

1.8.2.4.2. Record Serum Interference Control results in LIMS. Make a new preparation/verification note under "Batch Comment."

1.8.3. Internal Standard Procedure

1.8.3.1. Internal Standards must be present in all samples within a batch.

1.8.3.2. New lots of Internal Standard must be validated prior to use in casework. Instrument response for the new lot of Internal Standard must be comparable to the one that is currently in use.

1.8.3.3. Chromatography for Internal Standard must meet acceptance criteria. (Peak shape and Internal Standard response must be consistent throughout the analytical batch).

1.8.3.4. Record the preparation and verification results in LIMS and on hardcopy using the relevant form. File the original form with verification batch. Make a new preparation/verification note under "Batch Comment."

1.8.4. Sample Preparation Procedure

1.8.4.1. Bring calibrators, controls, and samples to room temperature.

1.8.4.2. Mix blood samples for at least 3 minutes on a tube rocker. Clotted samples should be homogenized prior to sampling and this step should be indicated in the batch worksheet.

1.8.4.3. Use the automatic diluter to dispense 1.4 mL of internal standard mixture and 0.05 mL of calibrators, controls, and samples into a labeled headspace vial. Cap each headspace vial immediately after dispensing.

1.8.4.4. An internal standard blank is prepared by placing the internal standard mix into a sample vial.

1.8.4.5. Seal the headspace cap onto the specimen by crimping each vial.

1.8.4.6. Using the Chemstation™ software, enter the analytical sequence in the following order for screens:

Calibrators (in increasing order)

Internal Standard Blank

Serum Reference Control

Low Whole Blood Control

High Whole Blood Control

≤ 20 screen injections

Low Whole Blood Control

High Whole Blood Control

≤ 20 screen injections

Low Whole Blood Control

High Whole Blood Control, etc.

Note: Any changes to the sequence template must be approved by a supervisor prior to analysis.

1.8.4.7. Load the vials onto the autosampler tray according to the analytical sequence and analyze the samples with the Chemstation™ Ethanol program. Typical HS/GC conditions are included in the Instrument Log binder.

1.8.4.8. After the batch has been authorized by a technical review, prepare new aliquots of the whole blood control and the samples positive for ethanol as described in steps 1-5. Samples positive for methanol, acetone, and isopropanol will be quantitated according to the Volatiles Quantitation SOP.

1.8.4.9. The sequence order for ethanol quantitation continues on the batch in the following order:

Low Whole Blood Control

High Whole Blood Control

≤ 20 quantitation injections

Low Whole Blood Control

High Whole Blood Control

≤ 20 quantitation injections

Low Whole Blood Control

High Whole Blood Control, etc.

Note: Any changes to the sequence template must be approved by a supervisor prior to analysis.

1.8.4.10. Note: Ethanol quantitation run on a different day than the screen are to follow the analytical sequence for screens. (1.8.4.6)

1.8.4.11. Load the vials onto the autosampler tray according to the analytical sequence and analyze the samples with the Chemstation™ Ethanol program. Typical HS/GC conditions are included in the Instrument Log binder.

1.9. ACCEPTANCE CRITERIA AND CALCULATIONS:

1.9.1. Internal Standard: internal standard recovery must be consistent for all calibrators, controls, and samples.

1.9.2. Chromatographic Peak: Peak should be symmetrically shaped with no indication of coelution. Certain assays may include analytes which produce less symmetrical peaks than the theoretical ideal standard. In these instances, peak shape should be evaluated within the context of the current method validation, including historical and contemporaneous calibrators and controls. The baseline must be flat and stable and returns to same label, with data system integration accurately defining the peak. In the event, where manual integration is necessary due to improper integration performed by the data system, (i.e. retention time shift, tailing, baseline noise, asymmetry etc.), it must be justified and the reason for manual integration must be noted on the data analysis paperwork for review purposes. Consultation with supervisor or senior scientist should be sought when needed and exceptions must be noted on the batch worksheet prior to submission for review.

1.9.3. A calibration curve is derived by comparison between the ratios of the calibrator ethanol peak areas to their respective internal standard peak areas. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of compounds in the sample.

1.9.3.1. The Chemstation™ software calculates a "least squares" line.

1.9.3.2. The calibrator coefficient (r^2) must be ≥ 0.99.

1.9.3.3. A minimum of 5 calibrators is needed to construct the calibration curve.

1.9.3.4. No more than 2 calibration points may be removed. Calibration points removed from the curve must be noted under Batch Comment in LIMS. Calibration points may be removed if the calculated concentration for the targeted concentration is outside acceptance range, poor chromatography, signal to noise, and/or poor internal standard recovery. Calibration points may not be dropped solely to make controls pass.

1.9.3.5. If the highest or lowest calibration points are removed then the reporting limits must be adjusted accordingly.

1.9.4. The dual HS/GC generates 2 values for each injection because it uses 2 separate columns.

1.9.4.1. Internal Standard blank: The mean of the blank results must be less than the lower reporting limits for ethanol, methanol, acetone, and isopropanol.

1.9.4.2. Low and High Whole Blood Control: The mean of the 2 values for Low Whole Blood and the mean of the 2 values for High Whole Blood Control must be within ± 10% of the in-house target concentration for their particular lot numbers.

1.9.4.3. Serum Interference Control: The mean of the 2 values for Serum Interference Control must fall within ± 10% of the manufacturer target concentration for ethanol and responses must be greater than the lower reporting limits for methanol, acetone, and isopropanol.

1.9.4.4. Samples: The mean of the sample results is used to calculate the result as described below.

1.9.4.4.1. Screen results for Ethanol:

1.9.4.4.1.1. For any LE samples > LE lower reporting limit, the 2 values generated are truncated after the 3rd decimal as they are entered on LIMS. The 2 values generated from the Volatile Screen must fall within ± 10% of their mean.

1.9.4.4.1.2. For any ME samples > ME lower reporting limit, the 2 values generated are truncated after the 3rd decimal as they are entered on LIMS. The 2 values generated from the Volatile Screen must fall within ± 10% of their mean.

1.9.4.4.2. Screen results for Methanol, Acetone, and Isopropanol:

1.9.4.4.2.1. For all samples with responses for Methanol, Acetone, and Isopropanol that are greater than the corresponding screen lower reporting limits, report as "Positive".

1.9.4.4.2.2. These samples will be quantitated according to the Volatiles Quantitation SOP.

1.9.4.4.3. Quantitation results for Ethanol: The 2 values generated are truncated after the 3rd decimal as they are entered on LIMS. Each value generated from Ethanol Quantitation must fall within ± 10% of their mean.

1.9.4.4.4. The mean of the Volatile Screen result and the mean of the Ethanol Quantitation result must fall within 10% of each other. If these requirements are met, the lowest value of the 4 values generated is reported to the 2nd decimal place.

Note: The calculations for values generated from the instrument to be within specified limits are conducted in LIMS, but should be verified by the analysts

1.9.4.4.5. Any samples with a result greater than the highest calibrator may be re-analyzed using a smaller aliquot of the sample, so the result falls within the calibration concentration range. Alternatively, the sample result may be reported as having a concentration greater than the value of the highest calibrator. Dilutions should be prepared with the appropriate matrix and documented on the analytical results.

1.9.4.4.6. Add remark on batch documentation and analytical data if sample is unsuitable for testing due to matrix condition or is there is insufficient sample for analysis. The submitting agency is notified on the final report.

1.9.5. Both the instrument and LIMS calculations are verified annually as described in the Calculation Verification SOP.

1.9.6. Possible Carryover: Samples with analytical results observed above 0.79 g/dL (prior to multiplying with dilution factors) must be evaluated for possible carryover. To evaluate carryover, reinject a check standard, the sample in question, and another check standard as needed.

1.9.7. Any quantitated sample with a result greater than the highest calibrator may be reanalyzed using a smaller aliquot of the sample such that the result falls within the calibration concentration range. Dilutions should be prepared with the appropriate matrix and documented on the analytical results. Alternatively, any confirmation sample result may be reported as having a concentration greater than the value of the highest calibrator.

1.10. REPORTING RESULTS:

1.10.1. Calibration results are printed on hardcopy.

1.10.2. Blank control and Serum control results are printed and entered to the third decimal place on LIMS. No additional blank or serum controls are included for quantitation.

1.10.3. Low and High Whole Blood Control results:

1.10.3.1. For screens: results are printed and entered to the third decimal place on LIMS.

1.10.3.2. For quantitations: results are printed and entered to the third decimal place on LIMS.

1.10.4. Sample results:

1.10.4.1. For screens: results are printed and entered to the third decimal place on LIMS.

1.10.4.2. For quantitations: results are printed and entered to the third decimal place on LIMS.

1.10.4.3. Measurement uncertainty is calculated for each quantitative result by multiplying the ethanol result by the Expanded Measurement Uncertainty (%). The resulting uncertainty is truncated and reported to three decimal places, not to exceed two significant figures.

1.10.5. Reagent information and Batch QC information are printed on hardcopy and entered on LIMS.

1.10.6. Any deviations from this procedure must be recorded, in LIMS and the analytical documentation, and are subject to authorization by a qualified reviewer. Notes relating to batch QC information are included under "Criteria Exceptions". Other notes relating to the batch are included under "Batch Comment."

1.10.7. Any exceptions to the criteria stated within this procedure and all data affected by the exceptions must be clearly documented and authorized during technical review.

1.11. LIMITATIONS OF PROCEDURE:

1.11.1. For Ethanol:

1.11.1.1. The LE reporting limit = 0.01 g/dL.

1.11.1.2. The ME reporting limit = 0.02 g/dL.

1.11.1.3. The upper reporting limits for both LE and ME cases = 0.50 g/dL.

1.11.2. For Methanol, Acetone, and Isopropanol: the screen lower reporting limits are:

Analyte	Headspace 1 Response	Headspace 2 Response
Methanol	10	4
Isopropanol	50	15
Acetone	100	30

1.11.3. Carryover: No carryover observed up to 0.79 g/dL

1.12. PROCEDURE NOTES:

1.12.1. System Suitability Test (SST) must be conducted each day prior to analysis and must be evaluated prior to analysis of a batch containing case samples. The SST is a Serum Interference Control prepared for a previous batch.

1.12.2. Batch size is limited to 50 case samples whenever possible. Prior approval by Supervisor is required to conduct batches with >50 case samples. Batches with >20 case samples must be bracketed by another set of Low and High Controls every 20 samples. The batch sequence must also be verified by a peer prior to submitting it for review.

1.12.3. Instrument parameters

1.12.3.1. Headspace 1

Initial Temp	40°C
Run Time	2.50 min
Mode	Split
Split Ratio	1.99:1

Split Flow	47.9 mL/min

1.12.3.2. Headspace 2

Initial Temp	40°C
Run Time	2.50 min
Mode	Split
Split Ratio	1.5:1
Split Flow	33 mL/min

1.12.4. HSGC Column: Agilent J & W DB-ALC1 column 30 m length 0.32 mm diameter, or similar

1.12.5. HSGC Column: Agilent J & W DB-ALC2 column 30 m length 0.32 mm diameter, or similar

1.12.6. There is one sample transfer recorded for the screen and, when applicable, a separate sample transfer recorded for quantitation.

1.12.7. Any sample that overloads the detector may be diluted and reanalyzed.

1.12.8. Any sample with large extraneous peaks should be evaluated for the presence of other volatile compounds and/or interferences. For some common interferences, see 001A_Appendix 1.1 Relative Retention Times for Miscellaneous Volatile Compounds.

1.13. REFERENCES:

1.13.1. Agilent Chemstation help index, topic: Calibration curves.

1.13.2. Roger L. Firor and Chin-Kai Meng, "Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler".

1.13.3. Restek Corp.'s "A Technical Guide for Static Headspace Analysis Using GC".

1.13.4. Matthew T. Barnhill, Jr., Donald Herbert, and David J. Wells, Jr., "Comparison of Hospital Laboratory Serum Alcohol Levels Obtained by Enzymatic Method with Whole Blood Levels Forensically Determined by Gas Chromatography" Journal of Analytical Toxicology Vol. 31 2007

1.13.5. Butala, Steven J.M., PhD, "Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Direct Injection GC/FID: Addendum: Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Headspace GC/FID" Bureau of Environmental Chemistry, Utah Department of Health.

1.14. AUTHORIZATION

QA Manager Approval: Signature: Date: 12/28/2023

Lab Director Approval: Signature: Date: 12/28/23