Section Three
Blood Toxicology

3.6 Qualitative Liquid-Liquid Extraction Methods for GC/MSD Confirmation

3.6.1 Liquid-Liquid Extraction Procedure for the Recovery of Neutral and Basic Drugs from Blood

3.6.1.1 BACKGROUND

This method is a general blood extraction procedure for a variety of commonly encountered neutral and basic drugs along with their metabolites. The method is based upon the principle of liquid/liquid extraction. Positive controls are spiked for a resulting concentration of 200ng/mL or 500ng/mL of drugs of interest. The sample pH is adjusted with a pH 9.2 borate buffer and extracted with n-butyl chloride. An optional back extraction procedure removes most frequently encountered interfering substances. Two internal standards are used to monitor extraction efficiency and chromatographic performance. Gas chromatography in conjunction with full scan mass spectrometry is used to confirm the presence of basic and neutral analytes of interest.

3.6.1.2 SCOPE

This method is a general blood extraction procedure for a variety of commonly encountered neutral and basic drugs, along with their metabolites. This method has also been shown capable of extracting some acidic drugs (e.g. butalbital) if sufficient quantity is present in the sample. This method does not efficiently extract some basic compounds, such as morphine and hydromorphone, due to pKa/pH considerations. The method allows for the analyst to use either methanol or ethyl acetate as a reconstitution solvent. Some benzodiazepines are more efficiently extracted using ethyl acetate than methanol as a reconstitution solvent. In addition, samples reconstituted in ethyl acetate can also be derivatized to increase sensitivity and detection of some compounds. Some drugs are more efficiently extracted using methanol as a reconstitution solvent. It is at the analyst's discretion to determine which solvent to use.

3.6.1.3 EQUIPMENT AND SUPPLIES

3.6.1.3.1 Tube Rocker
3.6.1.3.2 Vortex Mixer
3.6.1.3.3 Evaporative concentrator equipped with nitrogen tank.
3.6.1.3.4 Laboratory centrifuge capable of 3400rpm
3.6.1.3.5 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
3.6.1.3.6 16X100mm round bottom glass screw-top tubes
3.6.1.3.7 Screw Cap for 16mm O.D. tubes
3.6.1.3.8 GC/MS Automated Liquid Sampler (ALS) vials
3.6.1.3.9 GC/MS Vial Microinsert

3.6.1.3.10 Gas Chromatograph equipped with a Mass Selective Detector
3.6.1.3.11 5%-Diphenyl-95%-Dimethyl-siloxane copolymer capillary GC column, 12.5 to 30M.

3.6.1.4 REAGENTS

Refer to Manual section 5.12 for solution preparation instructions.

3.6.1.4.1 Methanol (ACS Grade)
3.6.1.4.2 n-Butyl chloride (ACS Grade)
3.6.1.4.3 Borate Buffer (pH 9.2)
3.6.1.4.4 Deionized/Distilled (DI) Water
3.6.1.4.5 1% Hydrochloric Acid in Methanol
3.6.1.4.6 100mM Hydrochloric Acid
3.6.1.4.7 Ethyl Acetate (ACS Grade)
3.6.1.4.8 Silylating Agent (select from):
BSTFA/1% TMCS or MSTFA

3.6.1.5 REFERENCE MATERIAL

3.6.1.5.1 Positive Control

Positive Control can be prepared with the working solution described below and/or obtained commercially.

3.6.1.5.1.1 **Positive Control Stock Solution**

Obtain 1mg/mL stock drug standard solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.6.1.5.1.2 **Positive Control Working Solution**

Add the designated volume of stock solution to 10mL methanol. A minimum of four compounds must be used.

Solution is stable for 6-months when stored at room temperature or 12-months when stored under refrigeration. Re-make solution if deterioration is noted.

Stock Solution (1.0mg/mL)	Volume (µL)
Amitriptyline	20
Caffeine	20
Codeine	20
Diphenhydramine	20
Lidocaine	20

Meperidine	20
Methadone	20
Nicotine	20
PCP	20
Trazodone	50
Methamphetamine	20

3.6.1.5.2 Internal Standard

3.6.1.5.2.1 **Stock Solutions**
1 mg/mL Benzphetamine
1mg/mL Papaverine

3.6.1.5.2.2 **Working Internal Standard Solution [10ng/µL]**
Add 100µL Benzphetamine and Papaverine stock solutions to 10mL volumetric ball flask. QS with methanol.
Solution is stable for three months when stored at room temperature.

3.6.1.5.3 Negative Control
Negative Whole Blood

3.6.1.5.4 Reference Standards

3.6.1.5.4.1 **Stock Standard Solution**
Obtain 1mg/mL stock drug standard solutions through Cerilliant, Grace, Sigma or other appropriate vendor.

3.6.1.5.4.2 **Working Drug Standard Solution**
Add the designated volume of stock solution to 10mL methanol.

3.6.1.6 PROCEDURE

3.6.1.6.1 Initial set-up
For each control and case sample, label two screw-top extraction tubes and one ALS vial with microinsert.

3.6.1.6.2 Positive Control Samples
The same lot of negative blood must be used for the preparation of both negative and positive spiked controls.

3.6.1.6.2.1 Prepare control sample by adding 200µL mixed working control solution to 2mL negative whole blood or pipette a 2mL sample of commercially-obtained whole blood positive control.

3.6.1.6.2.2 When the optional back extraction is used, prepare an additional positive control to parallel the back extraction process.

3.6.1.6.2.3 When some samples in a batch are going to be reconstituted with methanol and others with ethyl acetate, a separate positive control must be run for each reconstitution solvent.

3.6.1.6.3 Casework Samples

Transfer 2mL casework sample to screw top extraction tube.

3.6.1.6.4 Negative Control Sample

Transfer 2mL negative whole blood to extraction tube. If some samples are going to be run with ethyl acetate and some with methanol as a reconstitution solvent, a negative control must be prepared and run for each reconstitution solvent. If the optional back extraction is used, prepare an additional negative control to parallel the back extraction process.

3.6.1.6.5 Internal Standard

3.6.1.6.5.1 Add 20µL of internal standard mixture and vortex. If benzodiazepines are of interest, 3µL of Prazepam (1 mg/mL) may also be spiked into the sample.

NOTE: If the analyst has reason to suspect the sample may contain one or more of these internal standard compounds, it is permissible for the analyst to eliminate one of the internal standard compounds (e.g. papaverine) or replace one of the internal standard compounds with an appropriate alternative (prazepam may be used). Clear notation of the replacement, along with justification, must be included in the analysis notes. If the analyst prefers to use a different internal standard than those listed here, s/he must confer with the toxicology discipline leader in the selection of said internal standard.

3.6.1.6.5.2 Allow sample to stand 10 minutes.

3.6.1.6.6 Initial Extraction

3.6.1.6.6.1 Add 2mL borate buffer. Vortex.

3.6.1.6.6.2 Pipet 4mL n-butyl chloride into each tube, cap.

3.6.1.6.6.3 Place tube on rocker for 10 minutes.

3.6.1.6.6.4 Centrifuge for 10 minutes at 3400 rpm.

3.6.1.6.6.5 Transfer the n-butyl chloride layer (upper) to second screw-top tube.

3.6.1.6.6.6 Add 50uL 1% HCl in methanol.

3.6.1.6.6.7 Evaporate to dryness under N_2 at ≤37°C.
If no clean-up proceed to 3.6.1.6.8.

3.6.1.6.7 Optional Sample Clean-up

3.6.1.6.7.1 Reconstitute with 50uL 100mM HCl.

3.6.1.6.7.2 Add 1mL of n-Butyl Chloride. Vortex.

3.6.1.6.7.3 Rock for 5 minutes.

3.6.1.6.7.4 Centrifuge for 5 minutes at 3400 rpm.

3.6.1.6.7.5 Discard upper n-Butyl Chloride layer.

3.6.1.6.7.6 Add 2mL of pH 9.2 borate solution. Vortex

3.6.1.6.7.7 Add 4 mL of n-Butyl Chloride.

3.6.1.6.7.8 Rock for 5 minutes.

3.6.1.6.7.9 Centrifuge for 5 minutes at 3400 rpm.

3.6.1.6.7.10 Transfer upper n-Butyl Chloride layer into screw-top tube.

3.6.1.6.7.11 Evaporate to just dryness under N_2 at ≤45°C.

3.6.1.6.8 Reconstitution

3.6.1.6.8.1 Add 50uL Methanol or Ethyl Acetate to the residue, vortex.

3.6.1.6.8.2 Transfer extract to labeled ALS vial with microinsert.

3.6.1.6.9 Preparation for Analysis Run

3.6.1.6.9.1 Into Sequence log table, enter the case samples, blanks and controls.

3.6.1.6.9.2 Load samples, standards, blank and controls into the quadrant rack as noted in the sequence table.

3.6.1.6.10 Optional Derivatization (samples that are extracted with ethyl acetate may be derivatized at the analyst's discretion)

3.6.1.6.10.1 After ethyl acetate extraction samples have run on the GC-MSD, add 20uL silylating agent to the sample. In addition to the case samples, the extracted positive and negative controls must also be derivatized and run.

3.6.1.6.10.2 Heat at about 75 degrees Celsius for approximately 15 minutes.

3.6.1.6.10.3 Allow samples to cool; run on GC-MSD.

3.6.1.6.11 GC-MSD Acquisition Parameters

3.6.1.6.11.1 Refer to instrument METHOD printout for current acquisition parameters.

3.6.1.6.11.2 Current acquisition method must be stored centrally as a hard or electronic copy.

3.6.1.6.12 GC-MSD Qualitative Detection and Identification Criteria

3.6.1.6.12.1 For the identification of compounds not included in positive control, analyze appropriate non-extracted reference materials.

3.6.1.6.12.2 The presence of a drug compound is indicated if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and

there are no significant differences in the mass spectral data. NOTE: early eluting drugs, as well as drugs known to have similar retention times and mass spectral fragmentation patterns (e.g. phentermine and methamphetamine), may not differ from the retention time of the applicable reference material by more than ±0.1 minutes.

3.6.1.7 QUALITY ASSURANCE REQUIREMENTS

3.6.1.7.1 General

3.6.1.7.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

3.6.1.7.1.2 Refer to toxicology manual sections 5.2, 5.8, and 5.10 for quality assurance and reference material authentication requirements.

3.6.1.8 ANALYSIS DOCUMENTATION

3.6.1.8.1 Case results are to be recorded in the LIMS system.

3.6.1.8.2 A packet containing original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

3.6.1.8.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

3.6.1.9 REFERENCES

3.6.1.9.1 Procedure for Basic Drug Analysis, Courtesy of Jim Hutchison, Montana Department of Justice, Forensic Services Division, 2005.

3.6.1.9.2 Procedure for Back Extraction, Courtesy of Jim Hutchison, Montana Department of Justice, Forensic Services Division, 2006.

3.6.1.9.3 Strong Bases Extractions - Screening SOP, Courtesy of Dr. Graham Jones, Office of the Chief Medical Examiner, Edmonton, Canada, 2003.

3.6.1.9.4 Jones, G., *Postmortem Toxicology*. pp. 98-102, ***in:*** Clarke's Analysis of Drugs and Poisons, 3rd Edition, Moffat, A.C,

Osselton, M.D. and Widdop, B., eds., Pharmaceutical Press, 2004.

3.6.1.9.5 Hearn, W.L. and Walls, H.C., Strategies for Postmortem Toxicology Investigation. pp. 937-939. ***in:*** Drug Abuse Handbook, S.B. Karch, ed., CRC Press, Boca Raton, FL, 1998.

Revision History

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Blood Toxicology

3.6 Qualitative Liquid-Liquid Extraction Methods for GC/MSD Confirmation
3.6.1 Liquid-Liquid Extraction Procedure for the Recovery of Neutral and Basic Drugs from Blood.

Revision #	Issue Date	History/Comments
1	04-25-2002	Original Issue in SOP format
2	05-27-2003	Updated, Clarifications
3	11-21-2006	Addition of internal standard, extraction process restructured.
4	07-28-2008	Clarified that negative blood used to prepare positive control is the same lot as used for negative control.
5	10-29-2012	Removed requirement to run positive control in duplicate. Added option to reconstitute in ethyl acetate and derivatize.
6	03/13/2015	Made provision for cases that may contain internal standard compound(s). Added LIMS reporting requirement. Clarified retention time requirements for certain compounds. Clarified the method scope and relocated procedure summary to background section. Consolidated quality assurance paragraphs. Minor formatting and grammar changes. Added additional control requirement (negative) for optional back extraction. Added pipettors and centrifuge to supplies list. Replaced “Alltech” with “Grace” for RM vendor.