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Section 2.3.8: Solid Phase Methods for GC/MSD Confirmation Extraction of Opiates Employing the United Chemical Technologies 200 mg CLEAN SCREEN DAU Extraction Column	Rev. 1	02/02	10/02
Section One: Screening of Blood and Urine for Drugs of Abuse: Background and SOP for Screening of whole Blood and Urine by Enzyme Immunoassay.	Rev. 1	04/02	01/03
Section 4.1.1: Analysis of Alcohol and Common Volatile Solvents Quantitative Analysis for Ethanol and Qualitative Anal for Other Volatiles by Duel Column Headspace GC	Rev. 1	05/02	01/03

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Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.9 Qualitative Confirmation of Gamma Hydroxybutyrate in Urine Samples and GHB Containing Products

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2.3.9.1 BACKGROUND

This method provides a solid phase extraction option for the extraction of Gamma-hydroxybutyrate/Gamma-Hydroxybutyric Acid (GHB). GHB occurs naturally in minute quantities as a result of the metabolism of the inhibitory neurotransmitter, GABA.[6,8] The drug GHB is a potent tranquilizer that was previously used as an anesthetic and as a treatment for major depressive illnesses, alcohol withdrawal, and narcolepsy.[1-8] Legitimate use is limited due the side effects associated with the drug. GHB can produce visual disturbances, nausea, vomiting, drowsiness, dizziness, severe respiratory depression, unconsciousness and involuntary muscle spasms.[1,5,6] Overdoses can require emergency medical treatment including intensive care due to the respiratory depression, bradycardia and coma.[5,6,8]

The use of GHB has been increasing since the 1980s and currently is popular among body builders, teenagers and on the club/dance scene. Body builders use the drug due its alleged role as a growth hormone releasing agent to enhance muscle growth.[5,6,8] GHB has not been proven to possess any anabolic effects.[6] GHB has gained notoriety as a date rape drug due to its ability to produce short-term amnesia and decreased inhibitions.[1-8] The FDA banned the use of GHB in 1990 except for FDA approved physical supervised protocols.[6,8]

A dose of GHB consists of a capful that is usually approximately one teaspoon. This results in a dose anywhere from 2.5 to 4.0 grams of GHB. The taste of GHB has been described as salty or soapy, the odor is said to be mothball-like.[5,6] Due to the short half-life of GHB (0.3 to 1.0 hours[5], 27 ± 5 minutes[7]) the person will re-administer every 45 minutes to 1 hour. The onset of effects is 15 to 60 minutes. The effects of the drug will be detectable during a DRE exam for 4 to 6 hours. GHB is classified as a central nervous system depressant. The observed effects include horizontal and vertical nystagmus, lack of convergence, body tremors, and slowed breathing. The person will also exhibit a lowered pulse, blood pressure, and body temperature. In addition, the muscle tone will exhibit flaccidity and the person may be in a trance-like state, the pupils will exhibit a lack of reaction to light.[5] Lower doses will promote an agitated, combative state however their pulse and other vitals will be depressed.[5,6]

Combining GHB with alcohol plus a stimulant or marihuana allows the user to remain conscious during use. This allows them to experience the euphoric "buzz" that is the desired effect of its abuse. The desired effect is a state of relaxation and tranquility, a pleasant drowsiness, mild euphoria, hallucinations and a release of inhibitions. Combining GHB with alcohol or other central nervous system depressant will provide an additive depressant effect.[5]

GHB is detectable in blood for up to eight hours and in urine for up to 12 hours [5,6,7]. Peak plasma levels are obtained in 20 to 45 minutes.[7] Peak urine GHB concentrations on the order of 1100 µg/mL are observed within the first four hours after a 100 mg/kg oral dose.[3,7]

GHB is manufactured by reacting butyrolactone with sodium hydroxide in an aqueous solution in the presence of a low molecular weight alcohol (methanol, ethanol).[5,6] The average yield of GHB is 70%. If the yield of the manufacturing process is low, there will be significant amounts of the lactone present in the product. Abuse of this compound will continue due to its relatively simple synthesis and the availability and low cost of starting materials.[5] Users can ingest Gamma butyral lactone (GBL), a degreaser and floor stripper, and it is converted in vivo into GHB. GBL is therefore diverted from legitimate sources to either be taken directly or converted to GHB.

This compound has been referred to by a variety of names as listed in the table below.

Street Names	Marketing Names
"G"	Revitalize
"G" caps	Rejuvenate
Liquid X	Renewtrient
Soap	Revivarant
Easy Lay	Blue Nitro
	Thunder Nectar
	Rest-Eze
	Energy Drink

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There are thousands of documented GHB overdoses with numerous deaths. The danger in GHB use stems from its steep dose-response curve. A small increase in dose can create a dramatic difference in adverse effects. This makes the potential of overdosing with GHB very high. This is compounded by the fact that GHB effects users so differently. A dose that one individual uses could adversely effect another, thus word of mouth is a poor determiner of how much of the substance to use.

2.3.9.2 PRINCIPLE

GHB is extracted first into acetone, which is dried and reconstituted with phosphate buffer (pH 6.0). This sample extract is applied to a pretreated/conditioned SPE column. This method requires no heating to create the GHB-TMS derivative. The SPE elutant is evaporated and a di-

TMS derivative of GHB is prepared. The resulting derivative can be analyzed by either full scan or SIM GC/MSD in EI mode.

2.3.9.3 EQUIPMENT

2.3.9.3.1 Evaporative Concentrator (Zymark Turbo-Vap or equivalent).

2.3.9.3.2 Laboratory Centrifuge

2.3.9.3.3 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating GHB and its analogs in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl)

2.3.9.4 SUPPLIES

2.3.9.4.1 <u>Glassware</u>
Tapered tip 16X144 centrifuge tubes (Fisher catalog 05-538-41C or equivalent)
Snap caps (Fisher 05-538-41N or equivalent)
GC/MS vials (HP 5182-0865 or equivalent)
GC/MS vial microinserts (HP 5183-2088 or equivalent)
2.3.1.11.3.2 1.5mL snap cap centrifuge tubes (Fisher # or equivalent).

2.3.9.4.2 CLEAN SCREEN® GHB SPE columns (United Chemical Technologies, Inc., #ZSGHB020 or equivalent)

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2.3.9.5 REAGENTS

2.3.9.5.1 Hexane (Fisher #H303 or equivalent)

2.3.9.5.2 Sodium phosphate monobasic (Fisher #S369 or equivalent)

2.3.9.5.3 Sodium phosphate dibasic (Fisher #S374 or equivalent)

2.3.9.5.4 Dimethylformamide (Fisher #D119 or equivalent)

2.3.9.5.5 Ammonium hydroxide (Fisher #A669 or equivalent)

2.3.9.5.6 Methanol (Fisher #A454 or equivalent)

2.3.9.5.7 Ethyl Acetate (Ansys #203 or Fisher #E145-1 or equivalent)

2.3.9.5.8 <u>Silylating Agent (select from)</u>
BSTFA/1% TMCS (Pierce #38842ZZ or equivalent)
MSFTA (Pierce #48910 or equivalent)
BSTFA with 1%TMCS (Pierce #38831 or equivalent)

2.3.9.6 STANDARDS AND SOLUTIONS

Refer to section 2.6 for buffer solution preparation.

2.3.9.6.1 GHB Stock Solution
1.0mg/mL (Radian International #G-001 or equivalent)

2.3.9.6.2 0.1M Phosphate Buffer

2.3.9.6.3 99:1 $CH_3OH:NH_4OH$
Place ~80mL of methanol in a 100mL volumetric flask. Add 1mL of ammonium hydroxide, QS to 100mL.
Prepare fresh daily.

2.3.9.7 PROCEDURE

2.3.9.7.1 Initial set-up

2.3.9.7.1.1 Label GHB SPE extraction columns as follows:

- *GHB-NC* (Negative Control)
- *GHB-PC* (Positive Control)
- Laboratory numbers of samples without prefix.

2.3.9.7.1.2 Label Tapered-end centrifuge tubes and GC/MS vials as follows:

- *GHB-NC* (Negative Control)
- *GHB-PC* (Positive Control)
- Laboratory numbers of samples without prefix.
- *GHB-NES* (Non-extracted GHB standard)

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2.3.9.7.2 Preparation of GHB Controls and Standards

2.3.9.7.2.1 Spiked Urine - GHB Positive Control [200µg/mL]
Add of GHB 1mg/mL stock to negative urine. Vortex.

2.3.9.7.2.2 Non-Extracted Standard [200µg]
Place 200uL of GHB stock into taped-end centrifuge tube.

2.3.9.7.3 Extraction procedure

2.3.9.7.3.1 To 200uL of specimen, calibrators, negative and positive controls.

2.3.9.7.3.2 Add 25uL GHB-D6 internal standard.

2.3.9.7.3.3 Add 1mL of acetone, vortex for 15 seconds.

2.3.9.7.3.4 Centrifuge tube at ≈3300rpm for 10 minutes.

2.3.9.7.3.5 Transfer solvent from tube into tapered-end centrifuge tube.

2.3.9.7.3.6 Evaporate solvent with nitrogen at 80°C in TurboVap apparatus.

2.3.9.7.3.7 Reconstitute the evaporated extracts with 200uL of 0.1 M phosphate buffer (pH 6.0). Vortex 15 seconds.

2.3.9.7.4 Column Conditioning

Prepare CLEAN SCREEN® GHB SPE column as follows:

2.3.9.7.4.1 Apply 3mL of MeOH; aspirate at ≤ 3 inches of Hg.

2.3.9.7.4.2 Apply 3mL of DI H2O; aspirate ≤ 3 inches of Hg.

2.3.9.7.4.3 Apply 3 mL of 0.1M Phosphate Buffer (pH 6.0), aspirate ≤ 3 inches of Hg.

2.3.9.7.5 Sample Application

Add sample to prepared column with air displacement pipet (MLA, Eppendorf). Aspirate at 1 inch Hg.

2.3.9.7.6 Collection of Extract

Place tapered bottom centrifuge tube into collection rack. Add 1mL of MeOH/NH_4OH (99:1) to original sample centrifuge tube (from step 6.3.5), vortex.
Decant onto column and collect extract.

2.3.9.7.7 Concentration of Extract

Place tubes from vacuum manifold into TurboVap apparatus. Evaporate solvent with nitrogen at 70°C.

2.3.9.7.8 Derivatization Procedure

2.3.9.7.8.1 Add 100µL of Ethyl acetate and 100µL of BSTFA with 1% TCMS to evaporated extracted samples, spiked standards and non-extracted standard.

2.3.9.7.8.2 Transfer derivative to GC/MS ALS vial for analysis.

2.3.9.7.8.3 Inject 1 µL into GC/MS.

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2.3.9.7.9 Gas Chromatography/Mass Spectrometry (GC/MS) Parameters

2.3.9.7.9.1 Oven program, Injector and Interface Temperatures:
Refer to Method print-out that follows SOP.

2.3.9.7.9.2 Sample should be analyzed in full scan acquisition. Refer to attached GC/MSD method print-out for current parameters.

2.3.9.7.10 Detection and Identification Criteria
The qualitative presence of GHB can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

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2.3.9.8 REFERENCES

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12.3.9.8.3 Stephens, B. and Baselt, R.C. *Driving Under the Influence of GHB?* J Anal Tox, 1994, 18:357-358.

12.3.9.8.4 ElSohly, M.A. and Salamone, S.J. *Prevalence of Drugs used in Cases of Alleged sexual Assault* J Anal Tox, 1999, 23:141-146.

12.3.9.8.5 Chase, D.A., *Gamma Hydroxy Butyrate,* "*GHB*", Presentation at IACP DRE Conference, Minnesota, 1999.

12.3.9.8.6 Good, P.J., *Selected Abuse Substances,* Presentation at IACP DRE Conference, Portland, Oregon, 1998.

12.3.9.8.7 Determination of Gamma-Hydroxybutyric Acid by GC/MS, Dade County Medical Examiner's Toxicology Lab SOP.

12.3.9.8.8 Microgram, Volume XXXI, No. 3, March 1998.

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Section Two
2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation
2.4.1 General Extraction of Urine Samples for Qualitative Confirmation of Basic and Neutral or Acidic Drugs

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2.4.1.1 BACKGROUND

These extraction procedures are extensions of the TOXI-LAB® TOXI-A and TOXI-B thin layer chromatography (TLC) drug detection systems. The samples are extracted as with the TLC system, however, instead of concentrating the extract onto a disc, the solvent extract is concentrated and placed into an automated liquid sampler (ALS) vial for analysis by a gas chromatograph equipped with a mass selective detector (GC/MSD).

2.4.1.2 PRINCIPLE

This procedure describes the extraction of drug compounds from urine. Depending upon the pK_a of a drug compound, either Toxi-A or Toxi-B tubes are used. Basic compounds are extracted with a Toxi-A tube. Addition of urine to the Toxi-A tubes results in the urine becoming alkaline (pH=9) into 1,2-Dichloroethane, dichloromethane, heptane and isopropanol. Acidic compounds are isolated from an acidified solution (pH=4.5) into methylene chloride and heptane with zinc chloride to facilitate the extraction process. The extraction is achieved with an Ansys Toxi-B extraction tube. Either resulting extract is analyzed by full scan GC/MS in EI mode.

2.4.1.3 EQUIPMENT AND SUPPLIES

2.4.1.3.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.1.3.2 Electric Warmer with Omega-12 extraction solvent concentrator (Ansys 118/153)

2.4.1.3.3 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.1.3.4 Disposable Aluminum Concentration Cups (Ansys 152)

2.4.1.3.5 Glassware
GC/MS vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.4.1.3.6 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973) and a HP-5MS Ultra low bleed (5%-Diphenyl-95%-Dimethylsiloxane copolymer) capillary column (25M).

2.4.1.4 REAGENTS

2.4.1.4.1 ANSYS TOXI-TUBES A and B (Ansys 109A-100/ 109B-100)

2.4.1.5 CONTROLS

2.4.1.5.1 Toxi-Control No. 19 – Morphine, amphetamine, imipramine, methadone, propoxyphene, phenobarbital, secobarbital and benzoylecgonine (Ansys 191AB).

2.4.1.5.2 Toxi-Control No. 2 – Amphetamine, methamphetamine, nicotine and cotinine. (Ansys 170B).

2.4.1.6 STANDARDS

2.4.1.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

2.4.1.7 PROCEDURE

2.4.1.7.1 Initial set-up
Label TOXI-TUBES A or B, and GC/MS vials with microinserts, with negative control, TC-19 and or TC-2 and appropriate laboratory numbers.

2.4.1.7.2 Extraction Procedure Toxi-A Extraction (Basic or Neutral Compounds)
Transfer 5 mL of urine specimen, negative urine or appropriate Toxi-Control to a TOXI-TUBE A (pH=9).
Rock TOXI-TUBE A for 15 minutes.

2.4.1.7.3 Centrifuge tube at 2500 rpm for 15 minutes.

2.4.1.7.4 Transfer solvent from tube into concentration cup in Omega-12 extraction solvent concentrator. Allow cups to warm prior to the addition of extract.

2.4.1.7.5 Evaporate solvent to approximately 200µL on electric warmer.

2.4.1.7.6 Transfer solvent to labeled GC/MS ALS vial with microinsert.

2.4.1.7.7 Extraction Procedure Toxi-B Extraction (Acidic Compounds)

2.4.1.7.8 Transfer 4.5 mL of urine specimen, negative urine or Toxi-Control 19, to a TOXI-TUBE B (pH=4.5).

2.4.1.7.9 Rock TOXI-TUBE B for 15 minutes.

2.4.1.7.10 Centrifuge tube at 2500 rpm for 15 minutes.

2.4.1.7.11 Transfer solvent from tube into concentration cup in Omega-12 extraction solvent concentrator.

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2.4.1.7.12 Evaporate solvent to approximately 200μL on electric warmer.

2.4.1.7.13 Transfer solvent to labeled GC/MS ALS vial with microinsert.

2.4.1.7.4 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

2.4.1.7.4.1 Inject 1 μL into GC/MS using the ALS.

2.4.1.7.4.2 Analyze sample extract in full scan acquisition. Refer to attached GC/MSD method printout for current analysis parameters.

2.4.1.7.5 Detection and Identification Criteria

2.4.1.7.5.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

2.4.1.7.5.2 Acceptable retention time window is ±5%.

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Toxicology Section

Section Two
Urine Toxicology

2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation

2.4.2.1 Qualitative Confirmation of Gamma-Hydroxybutyrate (GHB) in Urine Samples and GHB Containing Products

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2.4.2.1.1 BACKGROUND

This method provides two qualitative analysis options for the liquid-liquid extraction of samples suspected of containing γ-Hydroxybutyrate/γ-Hydroxybutyric Acid (GHB). GHB occurs naturally in minute quantities as a result of the metabolism of the inhibitory neurotransmitter, GABA.[6,8] The drug GHB is a potent tranquilizer that was previously used as an anesthetic and as a treatment for major depressive illnesses, alcohol withdrawal, and narcolepsy.[1-8] Legitimate use is limited due the side effects associated with the drug. GHB can produce visual disturbances, nausea, vomiting, drowsiness, dizziness, severe respiratory depression, unconsciousness and involuntary muscle spasms.[1,5,6] Overdoses can require emergency medical treatment including intensive care due to the respiratory depression, bradycardia and coma.[5,6,8]

The use of GHB has been increasing since the 1980s and currently is popular among body builders, teenagers and on the club/dance scene. Body builders use the drug due its alleged role as a growth hormone releasing agent to enhance muscle growth.[5,6,8] GHB has not been proven to possess any anabolic effects.[6] GHB has gained notoriety as a date rape drug due to its ability to produce short-term amnesia and decreased inhibitions.[1-8] The FDA banned the use of GHB in 1990 except for FDA approved physician supervised protocols.[6,8]

A dose of GHB consists of a capful that is usually approximately one teaspoon. This results in a dose anywhere from 2.5 to 4.0 grams of GHB. The taste of GHB has been described as salty or soapy, the odor is said to be mothball-like.[5,6] Due to the short half-life of GHB (0.3 to 1.0 hours[5], 27 ± 5 minutes[7]) the person will re-administer every 45 minutes to 1 hour. The onset of effects is 15 to 60 minutes. The effects of the drug will be detectable during a DRE exam for 4 to 6 hours. GHB is classified as a central nervous system depressant. The observed effects include horizontal and vertical nystagmus, lack of convergence, body tremors, and slowed breathing. The person will also exhibit a lowered pulse, blood pressure, and body temperature. In addition, the muscle tone will exhibit flaccidity and the person may be in a trance-like state, the pupils will exhibit a lack of reaction to light.[5] Lower doses will promote an agitated, combative state however their pulse and other vitals will be depressed.[5,6]

Combining GHB with alcohol plus a stimulant or marihuana allows the user to remain conscious during use. This allows them to experience the euphoric "buzz" that is the desired effect of its abuse. The desired effect is a state of relaxation and tranquility, a pleasant drowsiness, mild euphoria, hallucinations and a release of inhibitions. Combining GHB with alcohol or other central nervous system depressant will provide an additive depressant effect.[5]

GHB is detectable in blood for up to eight hours and in urine for up to 12 hours [5,6,7]. Peak plasma levels are obtained in 20 to 45 minutes.[7] Peak urine GHB concentrations on the order of 1100 μg/mL are observed within the first four hours after a 100 mg/kg oral dose.[3,7]

GHB is manufactured by reacting butyrolactone with sodium hydroxide in an aqueous solution in the presence of a low molecular weight alcohol (methanol, ethanol).[5,6] The average yield of GHB is 70%. If the yield of the manufacturing process is low, there will be significant amounts of the lactone present in the product. Abuse of this compound will continue due to its relatively simple synthesis and the availability and low cost of starting materials.[5] Users can ingest Gamma butyral lactone (GBL), a degreaser and floor stripper, and it is converted in vivo into GHB. GBL is therefore diverted from legitimate sources to either be taken directly or converted to GHB.

This compound has been referred to by a variety of names as listed in the table below.

Street Names	Marketing Names
"G"	Revitalize
"G" caps	Rejuvenate
Liquid X	Renewtrient
Soap	Revivarant
Easy Lay	Blue Nitro
	Thunder Nectar
	Rest-Eze
	Energy Drink

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There are thousands of documented GHB overdoses with numerous deaths. The danger in GHB use stems from its steep dose-response curve. A small increase in dose can create a dramatic difference in adverse effects. This makes the potential of overdosing with GHB very high. This is compounded by the fact that GHB effects users so differently. A dose that one individual uses could adversely effect another, thus word of mouth is a poor determiner of how much of the substance to use.

2.4.2.1.2 PRINCIPLE - EXTRACTION OPTION ONE

GHB is isolated from an acidified solution into methylene chloride and heptane with zinc chloride to facilitate the extraction process. The extraction is achieved with an Ansys Toxi-B extraction tube. The

extraction is followed by the creation of a di-TMS derivative of GHB. The derivative is analyzed by full scan GC/MS in EI mode. This method may not provide adequate sensitivity for weaker concentrations of GHB.

2.4.2.1.3 EQUIPMENT EXTRACTION OPTION ONE

2.4.2.1.3.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.2.1.3.2 Evaporative Concentrator (Zymark Turbo-Vap or equivalent)

2.4.2.1.3.3 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.2.1.3.4 Glassware

2.4.2.1.3.4.1 Tapered tip 16X144 centrifuge tubes (Fisher catalog 05-538-41C or equivalent)

2.4.2.1.3.4.2 Snap caps (Fisher 05-538-41N or equivalent)

2.4.2.1.3.4.3 GC/MS vials (HP 5182-0865 or equivalent)

2.4.2.1.3.4.4 GC/MS vial microinserts (HP 5183-2088 or equivalent)

2.4.2.1.3.5 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating GHB and its analogs in toxicological specimens. (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl)

2.4.2.1.4 REAGENTS EXTRACTION OPTION ONE

2.4.2.1.4.1 ANSYS TOXI-TUBES B (109B-100)

2.4.2.1.4.2 <u>Silylating Agent (select from)</u>
MSFTA (Pierce #48910 or equivalent)
BSTFA with 1%TMCS (Pierce #38831 or equivalent)

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2.4.2.1.5 STANDARDS EXTRACTION OPTION ONE

2.4.2.1.5.1 Stock Standard Solution
1.0mg/mL (Radian International #G-001 or equivalent).

2.4.2.1.6 PROCEDURE EXTRACTION OPTION ONE

2.4.2.1.6.1 <u>Initial set-up</u>

2.4.2.1.6.1.1 Label TOXI-TUBES B as follows:

- *GHB-NC* (Negative Control)
- *GHB-PC* (Positive Control)
- Laboratory numbers of samples without prefix.

2.4.2.1.6.1.2 Label Tapered-end centrifuge tubes and GC/MS vials as follows:

- *GHB-NC* (Negative Control)
- *GHB-PC* (Positive Control)
- Laboratory numbers of samples without prefix.
- *GHB-NES* (Non-extracted GHB standard)

2.4.2.1.6.2 Preparation of Controls

2.4.2.1.6.2.1 **Spiked Urine**
GHB Positive Control [200µg/mL]
Add 900uL of GHB 1mg/mL stock to 3600uL negative urine. Vortex.

2.4.2.1.6.2.2 **Non-Extracted Standard [200µg]**
Place 200uL of GHB stock into taped-end centrifuge tube.

2.4.2.1.6.3 Extraction procedure

2.4.2.1.6.3.1 Extract 4.5 mL of specimen, negative or spiked urine in TOXI-TUBE B (acidic extraction @pH=4.5).

2.4.2.1.6.3.2 Rock TOXI-TUBE for 15 minutes.

2.4.2.1.6.3.3 Centrifuge tube at 2500 rpm for 15 minutes.

2.4.2.1.6.3.4 Transfer solvent from tube into tapered-end centrifuge tube.

2.4.2.1.6.3.5 Evaporate solvent to approximately 50µL with nitrogen at 40°C in TurboVap apparatus.

2.4.2.1.6.4 Derivatization Procedure

2.4.2.1.6.4.1 Add 40µL silylating agent to evaporated extracted samples, spiked standards and non-extracted standard. Cap tube with snap cap.

2.4.2.1.6.4.2 Vortex tube.

2.4.2.1.6.4.3 Place tube in 60°C sandbath for 15 minutes.

2.4.2.1.6.4.4 Remove tube from sandbath. Allow sample to cool. Transfer derivative to labeled GC/MS ALS vial for analysis.

2.4.2.1.6.4.5 Inject 1 µL into GC/MS.

2.4.2.1.6.5 Gas Chromatography/Mass Spectrometry (GC/MS) Parameters

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2.4.2.1.6.5.1 Refer to following method for oven program, and injector and interface temperatures.

2.4.2.1.6.5.2 Sample should be analyzed in full scan acquisition. Refer to attached GC/MSD method printout for current parameters to be employed for analysis.

2.4.2.1.6.6 Detection and Identification Criteria

2.4.2.1.6.6.1 The presence of GHB can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

2.4.2.1.6.6.2 Acceptable retention time window is ± 2%.

2.4.2.1.7 PRINCIPLE - EXTRACTION OPTION TWO

GHB is isolated from an acidified solution into ethyl acetate. The extraction is followed by the derivatization of GHB with BSTFA/1% TMCS and 60µL acetonitrile. The derivative is analyzed by SIM and/or full scan GC/MS in EI mode.

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2.4.2.1.8 EQUIPMENT EXTRACTION OPTION TWO

2.4.2.1.8.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.2.1.8.2 Evaporative Concentrator (Zymark Turbo-Vap or equivalent)

2.4.2.1.8.3 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.2.1.8.4 Glassware

2.4.2.1.8.4.1 Screw-top 16x100mm centrifuge tubes (Fisher #14-959 or equivalent)

2.4.2.1.8.4.2 Screw caps (Fisher 14-930-15E or equivalent)

2.4.2.1.8.4.3 Tapered tip 16X144 centrifuge tubes (Fisher #05-538-41C or equivalent)

2.4.2.1.8.4.4 Snap caps (Fisher #05-538-41N or equivalent)

2.4.2.1.8.4.5 GC/MS vials (HP 5182-0865 or equivalent)

2.4.2.1.8.4.6 GC/MS vial microinserts (HP 5183-2088 or equivalent)

2.4.2.1.8.5 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar

capillary column with a phase composition capable of efficiently separating GHB and its analogs in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl)

2.4.2.1.9 REAGENTS EXTRACTION OPTION TWO

2.4.2.1.9.1 Concentrated Sulfuric Acid (Fisher #A300S-500 or equivalent) diluted to 0.1N.

2.4.2.1.9.2 Ethyl Acetate (Ansys #203 or Fisher #E145-1 or equivalent)

2.4.2.1.9.3 Acetonitrile (Fisher #A996-1 or equivalent)

2.4.2.1.9.4 BSTFA with 1%TMCS (Pierce #38831 or equivalent)

2.4.2.1.10 STANDARDS EXTRACTION OPTION TWO

2.4.2.1.10.1 GHB Stock Solution
1.0mg/mL (Radian International #G-001 or equivalent).

2.4.2.1.11 PROCEDURE EXTRACTION OPTION TWO

2.4.2.1.11.1 Preparation of Controls

2.4.2.1.11.1.1 100μg/mL and 200μg/mL Spiked Urine Positive Controls

100μg/mL: Add 100uL of GHB 1mg/mL stock to 950uL negative urine. Vortex.

200μg/mL : Add 200uL of GHB 1mg/mL stock to 800uL negative urine. Vortex.

2.4.2.1.11.1.2 Non-Extracted Standard [100μg] Place 100uL of GHB stock into taped-end centrifuge tube.

2.4.2.1.11.2 Extraction procedure

2.4.2.1.11.2.1 Place 1.0mL of specimen, negative or spiked urine in round bottom centrifuge tube.

2.4.2.1.11.2.2 Add 250uL of cold 0.1N H_2SO_4 and vortex.

2.4.2.1.11.2.3 Add 6mL ethyl acetate. Cap.

2.4.2.1.11.2.4 Rock tube for 15 minutes.

2.4.2.1.11.2.5 Centrifuge tube at 2500 rpm for 10 minutes.

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2.4.2.1.11.2.6 Transfer solvent from tube into tapered-end centrifuge tube.
2.4.2.1.11.2.7 Re-extract urine with an additional 6mL ethyl acetate. Cap.
2.4.2.1.11.2.8 Rock tube for 15 minutes.
2.4.2.1.11.2.9 Centrifuge tube at 2500 rpm for 15 minutes.
2.4.2.1.11.2.10 Transfer solvent from tube into tapered-end centrifuge tube.
2.4.2.1.11.2.11 Evaporate the combined solvent with nitrogen at ≤40°C in TurboVap apparatus.

2.4.2.1.11.3 Derivatization Procedure
2.4.2.1.11.3.1 Add 30µL BSTFA/1 % TMCS and 60µL acetonitrile to evaporated samples, spiked standards and non-extracted standard. Cap tube with snap cap.
2.4.2.1.11.3.2 Vortex tube.
2.4.2.1.11.3.3 Place tube in 70°C sandbath for 15 minutes.
2.4.2.1.11.3.4 Remove tube from sandbath and allow to cool. Transfer derivative to GC/MS ALS vial for analysis.
2.4.2.1.11.3.5 Inject 2 µL into GC/MS.

2.4.2.1.11.4 Gas Chromatography/Mass Spectrometry (GC/MS) Parameters
2.4.2.1.11.4.1 Refer to attached GC/MSD method printout for current parameters for analysis and quantitation.
2.4.2.1.11.4.2 Sample should be analyzed full scan acquisition mode.

2.4.2.1.11.5 Detection and Identification Criteria
The presence of GHB can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.
2.4.2.1.11.5.1 Chromatographic Criteria
The retention time of the analyte should fall within ±2% of the retention time exhibited by GHB standards.

2.4.2.1.11.5.2 Full Scan Acquisition

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Full scan data should be compared against within run GHB standards.

2.4.2.1.12 REFERENCES

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2.4.2.1.12.2 Ferrare, S.D., Tedeschi, L. Frison, G., et. al, *Therapeutic gamma-hydroxybutyric acid monitoring in plasma and urine by gas chromatography-mass spectrometry.* J Pharm. Biomed Anal, 1993, 11(6):483-487.

2.4.2.1.12.3 Stephens, B. and Baselt, R.C. *Driving Under the Influence of GHB?* J Anal Tox, 1994, 18:357-358.

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Toxicology Section

Section Two
Urine Toxicology

2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation

2.4.3 Qualitative Benzodiazepines and Ancillary Compounds in Urine

2.4.3.1 BACKGROUND

Benzodiazepines continue to be the most prescribed group of therapeutic agents. Approximately 20 benzodiazepines are approved for use in the US.[2] Benzodiazepines were first introduced in 1960s in the pursuit of the perfect sedative hypnotic agent and have replaced barbiturates as the major class of CNS-depressant drugs.[2] Chlordiazepoxide (Librium®) was originally in 1962 introduced followed by the introduction of Diazepam (Valium®) in 1968. There are four main classes of benzodiazepines, the 1,4-benzodiazepines, the triazolobenzodiazepines, the diazolobenzodiazepines, and the 7-nitrobenzodiazepines. Refer to the following chart for a compilation of benzodiazepines currently prescribed in the US or ones that are commonly encountered.

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1,4-Benzodiazepines	*Trade Name*	*Major Metabolite(s)*
Diazepam	Valium®	Nordiazepam, Oxazepam, Temazepam
Nordiazepam	---	Oxazepam
Oxazepam	Serax®	Glucuronide conjugate
Temazepam	Restoril®	Oxazepam
Clorazepate	Tranxene®	Nordiazepam, Oxazepam
Chlordiazepoxide	Librium®	Demoxepam, Nordiazepam, Oxazepam
Halazepam	Paxipam®	3-Hydroxy-Halazepam, Nordiazepam, Oxazepam
Quazepam	Dormalin®, Doral®	2-Oxoquazepam
Flurazepam	Dalmane®	Desalkylflurazepam
Lorazepam	Ativan®	3-Glucuronide
7-Nitrobenzodiazepines		
Clonazepam	Klonopin®	7-Aminoclonazepam
Flunitrazepam	Rohypnol® *Not Prescribed in US*	7-Aminoflunitrazepam
Triazolobenzodiazepines		
Alprazolam	Xanax®	α-Hydroxy-alprazolam, 4-Hydroxy-alprazolam
Triazolam	Halcion®	α-Hydroxy-triazolam
Estazolam	ProSom®	---
Diazolobenzodiazepine		
Midazolam	Versed (Parenteral)	α-Hydroxymidazolam

Benzodiazepines are used primarily as antiepileptics in the treatment of seizure disorders, as anxiolytics for the short-term relief of anxiety disorders, as sedative-hypnotics for the treatment of sleep, disorders and as muscle relaxants to relieve spasticity. The primary side effects that accompany their use include dose-related extensions of the intended actions. These include sedation and sleepiness/drowsiness. In addition, other undesired effects that will influence the outcome of field sobriety tests include ataxia, a blocked ability to coordinate movements, a staggering walk and poor balance, lethargy/apathy, indifferent or sluggish, metal confusion, disorientation, slurred speech and amnesia. Impairment of motor abilities, especially a person's ability to drive an automobile is common. This impairment is compounded by the drug-induced suppression of ones' ability to assess his or her own level of physical and mental impairment. Alcohol and other CNS depressants (e.g., barbiturates antidepressants, etc.) will increase CNS depressant effects, such as impairment of psychomotor function and sedation, in an addition manner.[4-6]

The benzodiazepines are lipid soluble and are absorbed well from the GI tract with good distribution to the brain. They are metabolized primarily in the liver. Their CNS active metabolites extend their duration of action. The benzodiazepines work by enhancing, facilitating or potentiating the action of the inhibitory neurotransmitter GABA. They serve to increase the frequency of GABA-mediated chloride ion channel opening.

Benzodiazepines are metabolized primarily in the liver via several different microsomal enzyme systems.[6] Many products of their metabolism are active. Since many of the active metabolites have been marketed as therapeutic agents, it is difficult to ascertain which drug was ingested solely upon the basis of the results of analysis. Current drug therapy will assist in determining the source of a particular compound. The detection of a particular agent is determined partly by whether its metabolism yields active metabolites. Excretion of the benzodiazepines is predominantly in the urine. Depending upon the particular benzodiazepine, the urine may contain parent compounds, N-dealkylation and oxidative (hydroxylation) metabolism products and/or glucuronide conjugates.

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2.4.3.2 SCOPE

This extraction method is a modification of the method developed by Valentine, et al., for the extraction of benzodiazepines from urine.[1] This procedure is to prepare benzodiazepines in urine for either qualitative or quantitative GC/MSD analysis. Two urine aliquots are subjected to a Glucuronidase hydrolysis followed by extraction with chloroform-isopropanol. Following evaporation, one extract is reconstituted with ethyl acetate while the other is derivatized with a silylating agent. Each of the resulting extracts is analyzed by GC/MSD.

2.4.3.3 EQUIPMENT AND SUPPLIES

2.4.3.3.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.4.3.2 Laboratory oven or waterbath capable of achieving 60°C (Fisher or equivalent)

2.4.3.3.3 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.3.3.4 Drybath (Fisher or equivalent)

2.4.3.3.5 Evaporative Concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.4.3.3.6 Glassware
16X100mm tubes (Fisher 14-959-35AA or equivalent)
Screw caps (Fisher 14-930-15E or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap caps (Fisher 05-538-41N or equivalent)
GC/MS ALS vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.4.3.3.7 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl).

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2.4.3.4 REAGENTS

Refer to manual section 2.6 for preparation instructions.

2.4.3.4.1 Glucuronidase (Sigma G-0876 or equivalent)

2.4.3.4.2 2M Acetate buffer, pH ≈4.8

2.4.3.4.3 50mM Sodium Bicarbonate, pH=11

2.4.3.4.4 Chloroform/Isopropanol 9:1

2.4.3.4.5 Ethyl Acetate (Ansys #203 or Fisher #E145-1 or equivalent)

2.4.3.4.6 Silylating Agents
MSFTA (Pierce #48910 or equivalent) or
BSTFA with 1%TMCS (Pierce #38831 or equivalent)

2.4.3.5 CONTROLS

2.4.3.5.1 Liquid Urine Control containing a minimum of Oxazepam or Nordiazepam (BioRad 443, Utak 88121 or equivalent)

2.4.3.5.2 Drug Mix (Alltech 601826 {Medazepam, Oxazepam, Lorazepam, Diazepam, Temazepam, and Bromazepam}or similar)

2.4.3.6 STANDARDS

Obtain as required.

Standards (in methanol)	*Potential Vendors*
Alprazolam	Cerilliant A-903, Alltech 01427
α-Hydroxyalprazolam	Cerilliant A-907, Alltech 01545
Bromazepam	Cerilliant B-903, Alltech 6013563
Chlordiazepoxide	Cerilliant C-022
Norchlordiazepoxide	Alltech 6013433
Clonazepam	Cerilliant C-907, Alltech 017943
7-Aminoclonazepam	Cerilliant A-916
Diazepam	Cerilliant D-907, Alltech 017213
Estazolam	Cerilliant E-901, Alltech 601560
Flurazepam	Cerilliant F-003, Alltech 017953
Flunitrazepam	Cerilliant F-907, Alltech 6015123
7-aminoflunitrazepam	Cerilliant A-911
Lorazepam	Cerilliant L-901, Alltech 013583
Medazepam	Alltech 013573
Midazolam	Cerilliant M-908
4-hydroxymidazolam	Cerilliant H-902
Nitrazepam	Cerilliant N-906, Alltech 017933
Nordiazepam	Cerilliant N-905, Alltech 013453
Oxazepam	Cerilliant O-902, Alltech 013703
Temazepam	Cerilliant T-907, Alltech 013833
Triazolam	Cerilliant T-910, Alltech 014283
α-Hydroxytriazolam	Cerilliant T-911
Oxazepam Glucuronide	Alltech 01541

Refer to an opiate SOP for opiate standards.

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2.4.3.7 PROCEDURE

2.4.3.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Oxazepam, temazepam, nordiazepam and lorazepam. Add 10µL of stock solution to labeled tapered bottom centrifuge tube. Derivatize as described in 2.4.3.7.6.

2.4.3.7.2 Initial set-up

Label extraction tubes, tapered-bottom derivatization tubes and GC/MS vials with microinserts as follows for both the underivatized/ethyl acetate (EA) and derivatized extractions (TMS) for the negative control (NC), positive control (PC) and appropriate laboratory numbers without prefix.

2.4.3.7.3 Sample Preparation

- Transfer 6-mL of urine specimen, negative urine or positive control to extraction tube.
- If a drug mix is used as an additional control, add drug mix solution to 6-mL of negative urine. For a 0.1mg/mL solution, use 100µL.

2.4.3.7.4 Sample Hydrolysis

- To each extraction tube add:
  - 200µL 2M acetate buffer
  - 100µL β-Glucuronidase
- Cap and vortex *gently* to mix.
- Place in 60°C laboratory oven or waterbath for two hours.
- Allow samples to cool before proceeding with solvent extraction.

2.4.3.7.5 Extraction

- To each tube add:
  - 2mL 50mM sodium bicarbonate to each tube.
  - 4mL of chloroform/isopropanol {9:1}.
- Rock for 15 minutes.
- Centrifuge at 3500 rpm for 15 minutes.
- Transfer lower organic phase from tube into labeled tapered bottom tube.
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at 37°C.

2.4.3.7.6 Derivatization

- To one set of tapered-bottom tubes add:
  - 20µL ethyl acetate
  - 30µL of MSTFA.
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 15 minutes in 75°C dry bath.
- Remove from heat and allow to cool.
- Transfer derivative to labeled GC/MS ALS vial with microinsert.

2.4.3.7.7 Reconstitution with Ethyl Acetate

- To remaining set of tapered bottom tubes, add:
  - 50µL ethyl acetate.
  - Vortex.
  - Transfer extract to labeled GC/MS ALS vial with microinsert.

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2.4.3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.4.3.8.1 Analysis Parameters

2.4.3.8.1.1 Inject 1 μL into GC/MS using the ALS.

2.4.3.8.1.2 Analyze sample extract in full scan acquisition.

2.4.3.8.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.4.3.8.2 Detection and Identification Criteria

2.4.3.8.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.4.3.9 APPLICATION OF METHOD TO OTHER ANALYTES

2.4.3.9.1 This method is applicable to other compounds, which require an enzymatic hydrolysis to liberate the compound of interest. Both the ethyl acetate extraction and the TMS derivative can be applied toward the identification of these compounds.

2.4.3.9.2 This method has proven useful in the identification of opiate class compounds such as codeine, morphine, 6-monacetylmorphine and hydrocodone.

2.4.3.9.3 Appropriate standards should be prepared as required.

2.4.3.10 REFERENCES

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Section Two
Urine Toxicology

2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation
2.4.4 Qualitative 11-nor-9-THC-Δ^9-COOH (Carboxy-THC)

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2.4.4.1 BACKGROUND

Cannabis sativa use dates back to 2700 B.C.[2,5] Marijuana refer to a mixture of the leaves and flowering tops.[3] The smoke from burning cannabis includes 61 different cannabionoids.[2,6] The major active ingredient in marijuana is delta–9-tetrahydrocannabinol (Δ^9-THC). The Δ^9-THC content varies from 2 to 10% with an average of four to five percent. The quality of marijuana is reported to have improved over the last 20 years due to superior cultivation practices. The medicinal effects of marihuana include antinausea, muscle relaxing, anticonvulsant and reduction of intraocular pressure.[6] Cannabis therefore has found use as an antiemetic to deal with the nausea associated with anticancer chemotherapy and for relief for those suffering from glaucoma. The debate continues on the legalization of the drug.

Several factors come into play when considering the behavioral effects of (Δ^9)-THC. These include the route of administration (smoked or ingested), the THC concentration of the plant (dose), the experience of the user, the user's vulnerability to psychoactive effects, and the setting of the use.[5,6] The desirable effects of marihuana include an increased sense of well-being, mild euphoria, relaxation and a mild sedative-hypnotic effect.[5,6] Its clinical effects are similar to those of alcohol and the antianxiety agents.[5] The side-effects of marihuana use include impairment of cognitive functions, alteration of the user's perception of time and distance, reaction time, learning and short-term memory.[2,5,6] Marijuana has been shown to interfere with a person's ability or willingness to concentrate. Cannabis causes temporal disintegration such that the individual loses the ability to store information in the short term and is easily distracted.[2] Impairment from use is thought to last from 4 to 8-hours with more recent studies reporting 3 to 6 hours. Dr. Huestis reported that most behavioral and physiological effects return to baseline within three to six hours after use with residual effects in specific behaviors for up to 24 hours.

Impairment of coordination and tracking behavior has been reported to persist several hours beyond the perception of the high.[6] Due to the variable period of impairment the relating of urine carboxy-THC to the time of use, and thus impairment, requires the development of the

scenario surrounding the stop for DUI. The presence of carboxy-THC in urine only indicates exposure to MH at some previous indeterminate time.

The physiological effects may include an increase in heart rate and blood pressure, conjunctival suffusion, vasodilation, dry mouth and throat and a decrease in respiratory rate. The individual may also experience increased hunger (munchies).

Δ^9-THC is rapidly metabolized to the inactive metabolite, carboxy-THC.[1,4,5,6] In urine, this major metabolite, carboxy-THC is pursued due to Δ^9-THC only being present in minute quantities.[6] Carboxy-THC in urine has been conjugated with glucuronic acid to improve excretion. The detection time of carboxy-THC in urine following marijuana use varies dependent upon various pharmacological factors such as the dose obtained, the route of administration and the rates of metabolism and excretion.[1] Δ^9-THC is deposited in body fat due to it's high lipid solubility. It is slowly released from this storage depot over time.[1] The amount of Δ^9-THC stored in fat is a function of the amount, frequency and potency of drug exposure. The detection time can therefore vary from days to months.

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2.4.4.2 SCOPE

This method is to qualitatively confirm the presence of a major metabolite of marijuana, carboxy-THC, in urine specimens. Samples are subjected to an alkaline hydrolysis to liberate carboxy-THC from its glucuronide conjugate. Hydrolyzed samples are then made acidic with a phosphate buffer and extracted with hexane/ethyl acetate 87:13. Following centrifugation the extract is removed and dried under nitrogen. The dried extract is silylated to form a TMS derivative. The derivative is analyzed on a GC/MSD in SIM mode.

2.4.4.3 EQUIPMENT AND SUPPLIES

2.4.4.3.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.4.3.2 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.4.3.3 Waterbath (Fisher or equivalent)

2.4.4.3.4 Drybath (Fisher or equivalent)

2.4.4.3.5 Evaporative Concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.4.4.3.5 Whatman® pH Indicator Paper Strips (Fisher 09-876-17 or equivalent)

2.4.4.3.6 Glassware

16X100mm tubes (Fisher 14-959-35AA or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap caps (Fisher 05-538-41N or equivalent)
GC/MS ALS vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.4.4.3.7 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%diphenyl).

2.4.4.4 REAGENTS

Refer to manual section 2.6 for solution preparation instructions. Purity of chemicals should be certified ACS or equivalent.

2.4.4.4.1 1.0 N KOH

2.4.4.4.2 Saturated Potassium Phosphate Monobasic pH ≈1.8

2.4.4.4.3 87:13 Hexane with Ethyl Acetate (w/v)

2.4.4.4.4 Ethyl acetate

2.4.4.4.5 Silylating Agent (select from)

BSTFA/1% TMCS (Pierce #38842ZZ or equivalent)

MSFTA (Pierce #48910 or equivalent)

BSTFA with 1%TMCS (Pierce #38831 or equivalent)

2.4.4.5 STANDARDS

2.4.4.5.1 Stock Standard Solution

2.4.4.5.1.1 100µg/mL (+) 11-nor-9-carboxy-Δ^9-THC (Radian T-006 or equivalent).

2.4.4.5.2 Working Standard Solution (1800ng/mL)

2.4.4.5.2.1 Add 900µL Stock Solution to 49.1mL Methanol. Solution is stable for six months when stored at 4°C.

2.4.4.6 CONTROLS

2.4.4.6.1 Liquid Urine Control containing Carboxy-THC (Utak 66814, 66825, 98816 or equivalent)

2.4.4.6.2 60ng/mL Carboxy-THC Positive Control Urine

Add 100µL of working standard solution to 3mL of negative urine and mix.

2.4.4.6.3 Negative Control Urine (FS Personnel).

2.4.3.7 PROCEDURE

2.4.4.7.1 Initial set-up

Label extraction tubes, tapered bottom derivatization tubes and GC/MS vials with microinserts as follows for the negative control (NC), positive control (PC), Liquid Control and appropriate laboratory numbers without prefix.

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2.4.4.7.2 Sample Preparation

Transfer 3 mL of urine specimen, negative urine, positive control and liquid control to extraction tube.

2.4.4.7.3 Sample Hydrolysis

Add 0.5mL of 1.0N KOH to each extraction tube.
Vortex *gently* to mix.
Check resulting pH with pH indicator strip.
pH must be ≥ 12. If <12 add an additional 0.5mL of KOH.
Place in 40°C waterbath for 15 minutes.
Allow samples to cool before proceeding with solvent extraction.

2.4.4.7.4 Extraction

Original pH @ ≥ 12

- Add 1.5mL of phosphate buffer.
- Add 3mL of Hexane/Ethyl Acetate (87:13)
- Rock for 10 minutes.

Original pH @ <12

- Add 3.0mL of phosphate buffer.
- Add 4mL of Hexane/Ethyl Acetate (87:13)
- Rock for 15 minutes.

2.4.4.7.5 Centrifuge tubes at 3500 rpm for 10 minutes.

2.4.4.7.6 Transfer upper organic phase from tube into labeled tapered bottom tube.

2.4.4.7.7 Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at 37°C.

2.4.4.7.8 Derivatization

- To dried extract in tapered bottom tubes, add 50μL ethyl acetate and 50μL silylating reagent (BSTFA or MSTFA).
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 15 minutes in 95°C dry bath. Remove from heat and allow to cool.
- Transfer derivative to labeled GC/MS ALS vial with microinsert.

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2.4.3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.4.4.8.1 Inject 1μL TMS derivative into GC/MS using the ALS.

2.4.4.8.2 Analyze sample extract in SIM (selected ion monitoring) utilizing the ions 371, 473 and 488. Refer to attached GC/MSD method printout for current analysis parameters.

2.4.4.8.2.1 Detection and Identification Criteria
The presence of a drug compound can be established if there are no significant differences in the retention time and selected ion ratios are ±20%.

- Acceptable retention time window is +/-5%.

2.4.4.9 REFERENCES

2.4.4.9.1 Huestis, M.A., Mitchell, J.M. and Cone, E.J. *Detection Times of Marijuana Metabolites in Urine by Immunoassay and GC-MS* J. Anal. Tox. 19:443-449, 1995.

2.4.4.9.2 Huestis, M. *Marijuana*. pp. 246-264. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 1999.

2.4.4.9.3 *Cannabis, in: Clark's* Isolation and Identification of Drugs pp. 423-425, Moffat, A.C. ed., Pharmaceutical Press:London, 1986.

2.4.4.9.4 Drug Evaluation and Classification Training Manual, U.S. Dept. of Transportation, 1993.

2.4.4.9.5 Julien, R.M. Marijuana*: A Unique Sedative-Euphoriant-Psychedelic Drug. in:* A Primer of Drug Action. pp. 319-349, W.H. Freeman and Company:NewYork, 1998.

2.4.4.9.6 O'Brien, C.P. *Drug Addiction and Drug Abuse.* pp. 572-573. *in:* Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Hardman, J.G. ed., McGraw-Hill, 1996.

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.1 Amphetamine and Methamphetamine Extraction Employing the Ansys® Diagnostics Spec·Plus™ DAU Column

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2.3.1.1 BACKGROUND

Amphetamine and methamphetamine are sympathomimetic drugs that mimic the actions of naturally occurring stimulatory neurotransmitters. Although still prescribed for the treatment of attention deficit disorder (ADD), narcolepsy, and obesity, these compounds have a high potential for abuse. Methamphetamine is produced clandestinely often through the reduction of ephedrine/pseudoephedrine. Psychological side effects may include agitation, nervousness, restlessness, and paranoia. Physiological effects may include mydriasis, insomnia, increased blood pressure and heart rate. The manifestation of adverse affects is dependent on the time since drug administration.

2.3.1.2 PRINCIPLE

This procedure outlines the use of the ANSYS® Diagnostics, Inc SPEC·PLUS™ 3ml DAU column for the extraction of amphetamine and methamphetamine and amine compounds, from urine. ANSYS Technologies' SPEC™ Solid Phase Extraction products are manufactured with polypropylene plastic and bonded-silica impregnated on a glass fiber disc. The DAU column utilizes a copolymer sorbent which combines a strong cation exchange phase with a non-polar phase (reversed phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the urine sample. The copolymer binds the analyte primarily with ionic interactions with the anionic sorbent and to a lesser extent, by hydrophobic interactions. The cation exchange component (anionic sorbent) of the phase is effective for recovering amines which are present in the urine sample in a cationic form.

For the extraction of amphetamine, methamphetamine and other phenethylamines of interest, the urine is adjusted with a phosphate buffer and applied to a pre-conditioned SPE column. This pH adjustment maximizes the ionic character of the analyte and the sorbent to take full advantage of the cation exchange mechanism. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The column is subsequently washed with an

aqueous solvent, to selectively remove matrix components and interfering substances from the column. Next, the column is to dried to remove traces of solvent. When the column is dry, the analytes of interest are recovered from the column by disrupting the ionic bonds with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.1.3 EQUIPMENT AND SUPPLIES

2.3.1.3.1 SPEC·PLUS™ 3ml DAU column (Ansys 532-DAU)

2.3.1.3.2 Drybath (Fisher or equivalent)

2.3.1.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank;

2.3.1.3.4 Vacuum Manifold/pump

2.3.1.3.5 Glassware

16X100 Test Tubes (Fisher 14-961-29 or equivalent)

16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)

Snap Caps (Fisher 05-538-41N or equivalent)

GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)

GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.3.1.3.6 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.1.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.1.4.1 1.0 M Potassium hydroxide

2.3.1.4.2 0.1M Phosphate Buffer, pH 6.0

2.3.1.4.3 0.1M Acetic acid

2.3.1.4.4 1% Acidic Methanol

2.3.1.4.5 Isooctane (Fisher O-299-1 or equivalent)

2.3.1.4.6 Methanol (Fisher A412-4 or equivalent)

2.3.1.4.7 Ethyl Acetate (Fisher E145-4 or equivalent)

2.3.1.4.8 Ammonium Hydroxide (Fisher A669-500 or equivalent)

2.3.1.4.9 Elution Solvent

80ml ethyl acetate, 20 ml methanol, 2ml of NH_4OH ***Prepare fresh.***

2.3.1.4.10 1M Potassium phosphate dibasic (K_2HPO_4)

2.3.1.4.11 Derivatizing Agents - Select from the following:

Heptafluorobutyric Acid Anhydride (HFAA) (Pierce 63164 or equivalent)

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Pentafluoropropionic Acid Anhydride (PFAA) (Pierce 65193 or equivalent)

2.3.1.5 CONTROL

2.3.1.5.1 Toxi-Control No. 2, UTAK 98814, or equivalent control which contains both Amphetamine and Methamphetamine in the appropriate concentrations.

2.3.1.6 STANDARDS

2.3.1.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standard (mg/mL)	Potential Vendors
Methamphetamine	Cerilliant M-009, Alltech 010013
Amphetamine	Cerilliant A-007, Alltech 010023
MDMA	Cerilliant M-013, Alltech 014093
MDA	Cerilliant M-012, Alltech 014603
Phenylpropanolamine	Cerilliant P-038, Alltech 6017803
Phentermine	Cerilliant P-023, Alltech 017833
Ephedrine	Cerilliant E-024, Alltech 017403
Pseudoephedrine	Cerilliant P-035, Alltech 6013213
PMA	Cerilliant P-050

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2.3.1.7 PROCEDURE

2.3.1.7.1 Initial set-up

Label the test tubes and GC/MS vials with microinserts.

- Negative Control
- Positive Control
- Appropriate Laboratory Numbers

2.3.1.7.2 Manual Extraction Procedure Utilizing the SPEC·PLUS™ •3ml DAU column

2.3.1.7.2.1 Transfer 1mL of urine specimen, negative urine or appropriate control to the properly labeled test tube.

2.3.1.7.2.2 Add 500μL of 0.1M phosphate buffer, pH 6.0, and vortex.

2.3.1.7.2.3 Insert labeled SPEC·PLUS™ 3mL DAU column into vacuum manifold.

2.3.1.7.2.4 Add 200μL of methanol to column and aspirate at approximately 5 in. Hg (17 kPa) for approximately 1 minute.

2.3.1.7.2.5 Pour prepared sample into column and aspirate at approximately 5 in. Hg (17 kPa).

2.3.1.7.2.6 Add 500µL of 0.1M acetic acid and aspirate at approximately 5 in. Hg (17 kPa).

2.3.1.7.2.7 Increase vacuum to 10-20 in. Hg (34-68 kPa) and dry the extraction disc for a minimum of 1 minute.

2.3.1.7.2.8 Add 500µL of Methanol to the column and aspirate at approximately 5 in. Hg (17 kPa).

2.3.1.7.2.9 Increase the vacuum to 10-20 in Hg (34-68 kPa) and dry the disc for a minimum of 1 minute.

2.3.1.7.2.10 Open vacuum manifold, wipe collection tips, and insert collection holding rack containing the 16X144mm tapered tip centrifuge tubes.

2.3.1.7.2.11 Add 800µL of elution solvent to column and aspirate slowly, < 3 in. Hg (10kPa).

2.3.1.7.2.12 Increase vacuum to 5 in. Hg (17 kPa) to assist final amount of elution solvent through the disc.

2.3.1.7.2.13 Remove the tapered tip centrifuge tubes containing the collected samples from rack.

2.3.1.7.2.14 Add 50µL of 1% acidic methanol and vortex.

2.3.1.7.2.15 Evaporate to dryness under a gentle stream of nitrogen at approximately 35°C.

2.3.1.7.2.16 In the hood add 50µL of HFAA or PFAA, cap, and vortex.

2.3.1.7.2.17 Heat for 20 minutes at 70°C.

2.3.1.7.2.18 Cool to room temperature.

2.3.1.7.2.19 Add 1 mL of Isooctane and 1mL of 1M K_2HPO_4.

2.3.1.7.2.20 Cap and vortex.

2.3.1.7.2.21 Incubate at ~60°C for 15 minutes.

2.3.1.7.2.22 Cool.

2.3.1.7.2.23 Vortex

2.3.1.7.2.24 Centrifuge at 100rpm for 5 minutes to separate the layers

2.3.1.7.2.25 Transfer the isooctane (top) layer to an appropriately labeled ALS vial.

2.3.1.7.3 Automated Extraction Procedure Utilizing SPEC·PLUS™ - 3ml DAU column.

2.3.1.7.3.1 Refer to the following attached methods/printouts.

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2.3.1.7.4 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

2.3.1.7.4.1 Inject 1 µL into GC/MS using the ALS.

2.3.1.7.4.2 Analyze sample extract in full scan acquisition. Refer to attached GC/MSD method printout for current analysis parameters.

2.3.1.7.5 Detection and Identification Criteria

2.3.1.7.5.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus that of an authenticated standard.

2.3.1.7.5.2 Acceptable retention time window is ±5%.

2.3.1.8 REFERENCES

2.3.1.8.1 Automated SPEC® · Solid Phase Extraction Protocols for Drugs of Abuse Using the RapidTrace™ SPE Workstation, ANSYS Diagnostics, 1997.

2.3.1.8.2 SPEC·PLUS™·3ML·DAU Drugs of Abuse in Urine Extraction Applications, ANSYS Diagnostics, 1999.

2.3.1.8.3 Instructions for Urine of SPEC·Solid Phase Extraction Columns, SPEC·PLUS™ Solid Phase Extraction Columns with Filter, ANSYS Diagnostics, 1997.

2.3.1.8.4 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation
2.3.2 Extraction of Amphetamines Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

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2.3.2.1 BACKGROUND

Amphetamine and methamphetamine are sympathomimetic drugs that mimic the actions of naturally occurring stimulatory neurotransmitters. Although still prescribed for the treatment of attention deficit disorder (ADD), narcolepsy, and obesity, these compounds have a high potential for abuse. Methamphetamine is produced clandestinely often through the reduction of ephedrine/pseudoephedrine. Psychological side effects may include agitation, nervousness, restlessness, and paranoia. Physiological effects may include mydriasis, insomnia, increased blood pressure and heart rate. The manifestation of adverse affects is dependent on the time since drug administration.

2.3.2.2 PRINCIPLE

This procedure outlines the use of the UCT 200mg CLEAN SCREEN® DAU column for the extraction of Amphetamines from urine. The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the urine sample.

The cation exchanger will allow the anionic sorbent (-) to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption. The nonpolar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[2] The cation exchanger component of the phase is effective for amines which are present in the urine sample in a cationic form bonding ionically to the sorbent.[2]

For the extraction of amphetamines; the urine is adjusted to pH 6 with a phosphate buffer to maximize the ionic character of the analyte, and loaded onto a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the amine functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water

and a weak aqueous buffer, to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. When the column is dry the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.2.3 EQUIPMENT AND SUPPLIES

2.3.2.3.1 200 mg CLEAN SCREEN® extraction column (ZSDAU020 or ZCDAU020 or equivalent)
2.3.2.3.2 Drybath (Fisher or equivalent)
2.3.2.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.
2.3.2.3.4 Vacuum Manifold/pump
2.3.2.3.5 pH paper (Fisher 09-876-17 or equivalent)
2.3.2.3.6 Glassware
16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sample (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)
2.3.2.3.7 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.2.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.2.4.1 Methanol (Fisher A412-4 or equivalent)
2.3.2.4.2 Methylene Chloride (Fisher D37-4 or equivalent)
2.3.2.4.3 Dimethylformamide (DMF) (Fisher D119-500 or equivalent
2.3.2.4.4 Ethyl Acetate (Fisher E145-4 or equivalent)
2.3.2.4.5 Isopropanol (Fisher A416-1 or equivalent)
2.3.2.4.6 Ammonium Hydroxide (Fisher A669-500 or equivalent)

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2.3.2.4.7 100mM Phosphate Buffer (pH 6.0)
2.3.2.4.8 100mM Acetic Acid
2.3.2.4.9 Elution Solvent
Mix 78mL Methylene Chloride, 20mL Isopropanol and 2mL Ammonium Hydroxide. ***Make fresh.***
2.3.2.4.10 Derivatizing Agents - Select from the following:
Heptafluorobutyric Acid Anhydride (HFAA) (Pierce 63164 or equivalent)
Pentafluoropropionic Acid Anhydride (PFAA) (Pierce 65193 or equivalent)

2.3.2.5 CONTROL

2.3.2.5.1 Toxi-Control No. 2, UTAK 98814, or an equivalent control which contains both Amphetamine and Methamphetamine in the appropriate concentrations.

2.3.2.6 STANDARDS

2.3.2.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standard (1 mg/mL)	Potential Vendors
Methamphetamine	Cerilliant M-009, Alltech 010013
Amphetamine	Cerilliant A-007, Alltech 010023
MDMA	Cerilliant M-013, Alltech 014093
MDA	Cerilliant M-012, Alltech 014603
Phenylpropanolamine	Cerilliant P-038, Alltech 6017803
Phentermine	Cerilliant P-023, Alltech 017833
Ephedrine	Cerilliant E-024, Alltech 017403
Pseudoephedrine	Cerilliant P-035, Alltech 6013213
PMA	Cerilliant P-050

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2.3.2.7 PROCEDURE

2.3.2.7.1 Initial set-up
Label test tubes and GC/MSD vials with microinserts.

- Negative Control
- Positive Control
- Appropriate Laboratory Numbers

2.3.2.7.2 Extraction Procedure Utilizing the 200 mg CLEAN SCREEN® Extraction Column

2.3.2.7.2.1 Transfer 5mL of urine specimen, negative urine or appropriate Toxi-Control to the appropriate labeled test tube.

2.3.2.7.2.2 Add 2mL 100mM phosphate buffer and Vortex. Verify that pH is 6.0 ± 0.5, adjust

pH with 100mM monobasic or dibasic sodium phosphate, as necessary.

2.3.2.7.2.3 Insert labeled CLEAN SCREEN® extraction column into vacuum manifold.

2.3.2.7.2.4 Add 3mL of methanol to column and aspirate at ≤3 in. Hg.

2.3.2.7.2.5 Add 3mL of DI H_2O to column and aspirate at ≤3 in. Hg.

2.3.2.7.2.6 Add 1mL of 100mM phosphate buffer (pH 6.0) to column and aspirate at ≤3 in. Hg.

2.3.2.7.2.7 Pour sample onto column and aspirate at ≤3 in. Hg.

2.3.2.7.2.8 Wash column with 3mL DI H_2O and aspirate at ≤3 in. Hg.

2.3.2.7.2.9 Wash column with 1mL 100mM acetic acid and aspirate at ≤3 in. Hg.

2.3.2.7.2.10 Wash column with 3mL methanol and aspirate at ≤3 in. Hg.

2.3.2.7.2.11 Dry column for ≥ 5minutes at ≥10 inches Hg.

2.3.2.7.2.12 Open vacuum manifold, wipe collection tips, and insert collection holding rack containing labeled 16X144mm tapered tip centrifuge tubes.

2.3.2.7.2.13 Add 3mL of elution solvent to column and aspirate slowly, < 3 in. Hg.

2.3.2.7.2.14 Add 30µL of DMF to eluate.

2.3.2.7.2.15 Evaporate eluate to ~30µL at ≤40°C under a gentle stream of nitrogen.

2.3.2.7.2.16 Add 50µL of PFFA or HFFA, cap, and vortex.

2.3.2.7.2.17 Heat for 20 minutes at 70°C.

2.3.2.7.2.18 Evaporate to dryness at ≤40°C.

2.3.2.7.2.19 Reconstitute with 100µL ethyl acetate.

2.3.2.7.2.20 Transfer to the appropriately labeled ALS vial.

2.3.2.7.3 <u>Automated Extraction Procedure Utilizing 200 mg CLEAN SCREEN® Extraction Column</u>

2.3.2.7.3.1 Refer to the following attached methods/printouts.

2.3.2.7.4 <u>Gas Chromatography/Mass Spectrometry (GC/MS) Analysis</u>

2.3.2.7.4.1 Inject 1 µL into GC/MS using the ALS.

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2.3.2.7.4.2 Analyze sample extract in full scan acquisition. Refer to attached GC/MSD method printout for current analysis parameters.

2.3.2.7.5 Detection and Identification Criteria

2.3.2.7.5.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus that of an authenticated standard.

2.3.2.7.5.2 Acceptable retention time window is ±5%.

2.3.2.8 REFERENCES

2.3.2.8.1 UCT CLEAN SCREEN® Extraction Columns Application Manual

2.3.1.8.2 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation
2.3.3 Extraction of Benzodiazepines Employing the Ansys® Diagnostics Spec·Plus™ DAU Column

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2.3.3.1 BACKGROUND

Refer to manual section 2.4.3.

2.3.3.2 PRINCIPLE

This procedure outlines the use of the ANSYS® Diagnostics, Inc SPEC·PLUS™ 3ml SPE column for the extraction of benzodiazepines from urine. ANSYS Technologies' SPEC™ Solid Phase Extraction products are manufactured with polypropylene plastic and bonded-silica impregnated on a glass fiber disc. For benzodiazepines, a non-polar phase (reversed phase) retention mechanism is utilized. to interact effectively, with analytes of interest and minimally with interfering substances in the urine sample. The non-polar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[4]

Benzodiazepines form glucuronide conjugates to facilitate their excretion. An enzymatic hydrolysis is required to free them from the glucuronide sugar moiety. For the extraction of benzodiazepines, the urine is adjusted to pH 10.8 with a phosphate buffer, to maximize the hydrophobic/non-polar character of the analyte and the sorbent, and applied to a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The column is subsequently washed with an aqueous solvent, to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of solvent. When the column is dry, the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.3.3 EQUIPMENT AND SUPPLIES

2.3.3.3.1 SPEC·PLUS™ - 3mL DAU SPE column (Ansys 532-DAU or equivalent)
2.3.3.3.2 Drybath (Fisher or equivalent)
2.3.3.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.
2.3.3.3.4 Vacuum Manifold/pump

2.3.3.3.5 Glassware
16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)
pH paper (Fisher 09-876-17 or equivalent)

2.3.3.3.6 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.3.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.3.4.1 Isooctane (2,2,4 – trimethylpentane) (Fisher O-299-1 or equivalent)

2.3.3.4.2 1.0M Acetate Buffer, pH 3.8

2.3.3.4.3 1.5 M Phosphate Buffer, pH 10.8

2.3.1.4.4 Ethyl Acetate (Fisher E145-4 or equivalent)

2.3.1.4.5 Ammonium Hydroxide (Fisher A669-500 or equivalent)

2.3.3.4.6 Elution Solvent
To 98mL ethyl acetate add 2ml NH_4OH, mix.
Make Fresh.

2.3.3.4.7 Deionized/distilled water

2.3.3.4.8 β-Glucuronidase (Patella vulgata) Options
- Prepare from Patella vulgata Type L-II powder (Sigma G8132 or equivalent)
- Prepared Helix pomatia Type H-2 Solution (Sigma G0876 or equivalent)

2.3.3.4.9 Silylation Reagent Options
- MTBSTFA / 1% t-BDMCS (Pierce 48925 or equivalent)
- MSFTA (Pierce 48910 or equivalent)
- BSTFA (Pierce 38830 or equivalent)

2.3.3.5 CONTROL

2.3.3.5.1 Liquid Urine Control containing a minimum of Oxazepam or Nordiazepam (BioRad 443, Utak 88121 or equivalent)

2.3.3.5.2 Drug Mix (Alltech 601826 {Medazepam, Oxazepam, Lorazepam, Diazepam, Temazepam, and Bromazepam}or similar)

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2.3.3.5.3 Oxazepam Glucuronide (Alltech 01541 or equivalent.

2.3.3.6 STANDARDS

2.3.3.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards in Solution	*Potential Vendors*
Alprazolam	Cerilliant A-903, Alltech 01427
α-Hydroxyalprazolam	Cerilliant A-907, Alltech 01545
Bromazepam	Cerilliant B-903, Alltech 6013563
Chlordiazepoxide	Cerilliant C-022
Norchlordiazepoxide	Alltech 6013433
Clonazepam	Cerilliant C-907, Alltech 017943
7-Aminoclonazepam	Cerilliant A-916
Diazepam	Cerilliant D-907, Alltech 017213
Estazolam	Cerilliant E-901, Alltech 601560
Flurazepam	Cerilliant F-003, Alltech 017953
Flunitrazepam	Cerilliant F-907, Alltech 6015123
7-aminoflunitrazepam	Cerilliant A-911
Lorazepam	Cerilliant L-901, Alltech 013583
Medazepam	Alltech 013573
Midazolam	Cerilliant M-908
4-hydroxymidazolam	Cerilliant H-902
Nitrazepam	Cerilliant N-906, Alltech 017933
Nordiazepam	Cerilliant N-905, Alltech 013453
Oxazepam	Cerilliant O-902, Alltech 013703
Temazepam	Cerilliant T-907, Alltech 013833
Triazolam	Cerilliant T-910, Alltech 014283
α-Hydroxytriazolam	Cerilliant T-911

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2.3.3.7 PROCEDURE

2.3.3.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Oxazepam, temazepam, nordiazepam and lorazepam. Add 10μL of stock solution to labeled tapered bottom centrifuge tube.

2.3.3.7.2 Initial set-up

Label SPE·PLUS™·3ml·DAU extraction column, test tubes, tapered-bottom derivatization tubes and GC/MS vials with microinserts for the negative control (NC), positive control (PC), Oxazepam Glucuronide control, Standards, and appropriate laboratory numbers.

2.3.3.7.3 Manual Extraction Procedure Utilizing the SPEC·PLUS™ •3ml DAU column

2.3.3.7.3.1 Sample Preparation

Transfer 1.0mL of urine specimen, negative urine or positive control to labeled extraction test tube.

2.3.3.7.3.2 Sample Hydrolysis

For each extraction tube:

- Add 200μL 1.0M acetate buffer, pH 3.8
  - Vortex.
  - Verify that the resulting pH is approximately 4.
  - If necessary adjust pH.
- Add 200μL β-Glucuronidase.
  - Cap and vortex *gently* to mix.
- Place in 37°C laboratory oven or waterbath for three hours.
- Allow samples to cool before proceeding with solvent extraction.

2.3.3.7.3.3 Extraction

For each extraction tube:

- Add 1.5mL of phosphate buffer (pH 10.8)
  - Vortex.
  - Resulting pH should be approximately 10.
  - If necessary, adjust pH.
- Centrifuge at 3500 rpm for ≥5 minutes.
- Insert labeled SPE·PLUS™·3ml·DAU column in the vacuum manifold.
- Add 200μL of methanol to the column. Wait for 1 minute.
- Decant sample into column and aspirate at approximately 3-5 in. Hg (10-17kPa)
- Add 1mL of deionized water to column and aspirate at approximately 3-5 in. Hg (10-17kPa)
- Increase vacuum to 10-20 in. Hg (34-68kPa) and dry extraction disc for approximately 5 minutes.
- Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.

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- Add 800μL of elution solvent and apply ***gentle*** vacuum of <3 in. Hg (10 kPa) to aspirate the sample into the collection tube
- Increase vacuum to approximately 5 in. Hg (17 kPa) to assist the final amount of elution solvent through the disc.
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at 60°C.

2.3.3.7.3.4 Derivatization

- In hood, add 100μL silylating agent.
  - Cap tubes with snap caps.
  - Vortex.
- Heat tube in 90°C dry bath for 30 minutes.
- Remove from dry bath and allow to cool.
- Add 100μL of isooctane.
  - Vortex.
- Transfer derivative to labeled GC/MSD ALS vial with microinsert.

2.3.3.7.4 Automated Extraction Procedure Utilizing SPEC·PLUS™ - 3ml DAU column.

2.3.1.7.3.1 Refer to the following attached methods/printouts.

2.3.3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

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2.3.3.8.1 Analysis Parameters

2.3.3.8.1.1 Inject 1 μL into GC/MSD using the ALS.

2.3.3.8.1.2 Analyze sample extract in full scan acquisition.

2.3.3.8.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.3.3.8.2 Detection and Identification Criteria

2.3.3.8.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.3.3.9 REFERENCES

2.3.1.9.1 Automated SPEC® · Solid Phase Extraction Protocols for Drugs of Abuse Using the RapidTrace™ SPE Workstation, ANSYS Diagnostics, 1997.

2.3.1.9.2 SPEC·PLUS™·3ML·DAU Drugs of Abuse in Urine Extraction Applications, ANSYS Diagnostics, 1999.

2.3.1.10.3 Instructions for Urine of SPEC·Solid Phase Extraction Columns, SPEC·PLUS™ Solid Phase Extraction Columns with Filter, ANSYS Diagnostics, 1997.

2.3.1.10.4 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.4 Extraction of Benzodiazepines Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

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2.3.4.1 BACKGROUND

Refer to manual section 2.4.3.

2.3.4.2 PRINCIPLE

This procedure outlines the use of the 200mg UCT CLEAN SCREEN® DAU Column for the extraction of Benzodiazepines from urine The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the urine sample.

The retention mechanisms for the benzodiazepines are hydrophobic interactions and polar adsorption. The nonpolar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[2] Benzodiazepines form glucuronide conjugates to facilitate their excretion. An enzymatic hydrolysis is therefore required to free them from the glucuronide sugar moiety. For the extraction of benzodiazepines, the hydrolyzed urine is loaded onto a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by hydrophobic interaction of the functional groups present on both the analyte and the sorbent. The column is subsequently washed to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of aqueous and organic solvents. When the column is dry, the analytes of interest are recovered from the column with a basic organic solvent. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.4.3 EQUIPMENT AND SUPPLIES

2.3.4.3.1 200 mg CLEAN SCREEN® Extraction Column (ZSDAU020 or ZCDAU020 or equivalent)

2.3.4.3.2 Drybath (Fisher or equivalent)

2.3.4.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.3.4.3.4 Vacuum Manifold/pump

2.3.4.3.5 Glassware

16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.3.4.3.6 pH paper (Fisher 09-876-17 or equivalent)

2.3.4.3.7 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.4.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.4.4.1 ethanol (Fisher A412-4 or equivalent)
2.3.4.4.2 Ethyl Acetate (Fisher E145-4 or equivalent)
2.3.4.4.3 Hexane (Fisher H-292-1 or equivalent)
2.3.4.4.4 Deionized/distilled water
2.3.4.4.5 100 mM Phosphate buffer, pH 6.0
2.3.4.4.6 20 % Acetonitrile in 0.1 M phosphate buffer, pH 6.0
2.3.4.4.7 β-Glucuronidase (Patella vulgata)
2.3.4.4.8 Silylation Reagent Options

- MSFTA (Pierce 48910 or equivalent)
- MSFTA + 1% TMCS (Pierce 48915 or equivalent)
- BSTFA (Pierce 38830 or equivalent)
- BSTFA + 1% TMCS (Pierce 38831 or equivalent)

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2.3.4.5 CONTROL

2.3.4.5.1 Liquid Urine Control containing a minimum of Oxazepam or Nordiazepam (BioRad 443, Utak 88121 or equivalent)

2.3.4.5.2 Drug Mix (Alltech 601826 {Medazepam, Oxazepam, Lorazepam, Diazepam, Temazepam, and Bromazepam}or similar)

2.3.4.5.3 Oxazepam Glucuronide (Alltech 01541 or equivalent)

2.3.4.6 STANDARDS

2.3.4.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards in Solution	*Potential Vendors*
Alprazolam	Cerilliant A-903, Alltech 01427
α-Hydroxyalprazolam	Cerilliant A-907, Alltech 01545
Bromazepam	Cerilliant B-903, Alltech 6013563
Chlordiazepoxide	Cerilliant C-022
Norchlordiazepoxide	Alltech 6013433
Clonazepam	Cerilliant C-907, Alltech 017943
7-Aminoclonazepam	Cerilliant A-916
Diazepam	Cerilliant D-907, Alltech 017213
Estazolam	Cerilliant E-901, Alltech 601560
Flurazepam	Cerilliant F-003, Alltech 017953
Flunitrazepam	Cerilliant F-907, Alltech 6015123
7-aminoflunitrazepam	Cerilliant A-911
Lorazepam	Cerilliant L-901, Alltech 013583
Medazepam	Alltech 013573
Midazolam	Cerilliant M-908
4-hydroxymidazolam	Cerilliant H-902
Nitrazepam	Cerilliant N-906, Alltech 017933
Nordiazepam	Cerilliant N-905, Alltech 013453
Oxazepam	Cerilliant O-902, Alltech 013703
Temazepam	Cerilliant T-907, Alltech 013833
Triazolam	Cerilliant T-910, Alltech 014283
α-Hydroxytriazolam	Cerilliant T-911

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2.3.4.7 PROCEDURE

2.3.4.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Oxazepam, Temazepam, Nordiazepam and Lorazepam. Add 10μL of stock solution to labeled tapered bottom centrifuge tube.

2.3.4.7.2 Initial set-up

Label 200mg CLEAN SCREEN® Extraction Column, test tubes, tapered-bottom derivatization tubes and GC/MSD vials with microinserts for the negative control (NC), positive control (PC), Oxazepam Glucuronide control, Standards, and appropriate laboratory numbers without prefix.

2.3.4.7.3 Sample Preparation

- Transfer 5.0ml of urine specimen, negative urine or positive control to extraction tube.

2.3.4.7.4 Sample Hydrolysis

- To each extraction tube add:

- 2 ml β-Glucuronidase solution (pH 5.0)
- Cap and vortex *gently* to mix.
- Place in 65°C laboratory oven or waterbath for three hours.
- Centrifuge for 10 minutes at 2000 rpm and discard pellet.
- Allow samples to cool.

2.3.4.7.5 Extraction

- Insert labeled 200mg CLEAN SCREEN® DAU column in the vacuum manifold.
- Add 3mL of methanol to the column and aspirate at ≤3 in. Hg (<10 kPa). .
- Add 3mL of deionized water to the column and aspirate at ≤3 in. Hg (<10 kPa). .
- 1mL of 100mM phosphate buffer (pH 6.0) and aspirate at ≤ 3 in. Hg.
- Decant sample into column and aspirate at <3 in. Hg.
- Wash column with 2mL of deionized water and aspirate at ≤ 3 in. Hg.
- Wash column with 2mL 20% acetonitrile in 0.1M phosphate buffer (pH 6.0) and aspirate and aspirate at ≤ 3 in. Hg.
- Increase vacuum to ≥10 in. Hg (≥34 kPa) and dry extraction disc for approximately 5 minutes.
- Wash column with 2mL hexane and aspirate and aspirate at ≤ 3 in. Hg.
- Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.
- Add 3mL ethyl acetate to elute the sample from the column at < 3 in. Hg (<10 kPa).
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at ≤ 40°C.

2.3.4.7.6 Derivatization

In fume hood:

- Add 50 μL ethyl acetate.
- Add 50 μL silylating agent.
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 20 minutes in 70°C dry bath.
- Remove from heat and allow to cool.

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- Transfer derivative to labeled GC/MS ALS vial with microinsert.

2.3.4.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.3.4.8.1 Analysis Parameters

2.3.4.8.1.1 Inject 1 µL into GC/MS using the ALS.

2.3.4.8.1.2 Analyze sample extract in full scan acquisition.

2.3.4.8.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.3.4.8.2 Detection and Identification Criteria

2.3.4.8.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.3.4.9 REFERENCES

2.3.3.9.1 UCT CLEAN SCREEN® Extraction Columns Application Manual

2.3.3.9.2 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

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Forensic Services
Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.5 Extraction of Benzoylecgonine Employing the SPEC·PLUS™·DAU Extraction Column.

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2.3.5.1 BACKGROUND

Cocaine is a naturally occurring alkaloid derived from leaves of the South American shrub, Erythroxylon coca. Cocaine is also can be produced synthetically. Cocaine is one of the most potent stimulants to the central nervous system due to its mechanism of action, which involves blocking reuptake of stimulatory neurotransmitters. Cocaine is used licitly as a local anesthetic in ophthalmology. The positive effects of cocaine include an increased mental awareness and alertness, a sense of clarity and feelings of elation. The fictional detective Sherlock Holmes used cocaine for its transcendently stimulating and mind clarifying properties to the displeasure of Doctor Watson. As with all drugs, the effects of cocaine depend on the dosage, the form in which it is taken and the route of administration. Other significant factors include the setting or circumstances in which the drug is used and the expectations of the user. Side effects can include pupillary dilation, restlessness, dizziness, dyskinesia, tremor, dysphoria, and paranoia. Additional major side effects of cocaine use are a consequence of discontinued use. If the user does not readminister the drug, they may experience increased anxiety, agitation, restlessness and the disturbance of normal sleep patterns, which leads to fatigue. Due to these effects following cocaine use, an individual's ability to operate a motor vehicle is impaired both during and following cocaine use.

Routes of administration of cocaine include snorting, injection and smoking. The metabolism of cocaine and its metabolites involves hydrolysis, transesterification and n-demethylation. Cocaine metabolites detectable in urine include benzoylecgonine, ecgonine methyl ester, norcocaine and various arylhydroxy- and arylhydroxymethoxy-metabolites. The duration of the action of cocaine is limited by its rate of metabolism since its major metabolites are inactive. One of the active metabolites, cocaethylene is produced via transesterification when cocaine and ethanol are ingested concurrently.

2.3.5.2 PRINCIPLE

This procedure outlines the use of the SPEC·PLUS™ DAU SPE column for the extraction of Benzoylecgonine from urine. ANSYS Technologies' SPEC™ Solid Phase Extraction products are manufactured with polypropylene plastic and bonded-silica impregnated on a glass fiber disc. The DAU column utilizes a sorbent which combines a strong cation exchanger with a non-polar phase (reversed phase) to interact effectively, physically and chemically, with benzoylecgonine and minimally with interfering substances in the urine sample. The cation exchanger component of the phase is effective for compounds which are present in the urine in a cationic form. The sample pretreatment with 0.1M HCl ensures that the nitrogen group on the ecgonine portion of the cocaine molecule (pKa 8.6) bonds ionically to the sorbent. For the extraction of benzoylecgonine the urine is adjusted with a phosphate buffer to maximize the ionic character of the analyte. The sample is then applied to a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The column is subsequently washed with the aqueous solvent, to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of solvent. When the column is dry, the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

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2.3.5.3 EQUIPMENT AND SUPPLIES

2.3.5.3.1 SPEC·PLUS™ 3ml DAU column (Ansys 532-DAU or equivalent)

2.3.5.3.2 Drybath (Fisher or equivalent)

2.3.5.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.3.5.3.4 Vacuum Manifold/pump

2.3.5.3.5 <u>Glassware</u>

16X100 Test Tubes (Fisher 14-961-29 or equivalent)

16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)

Snap Caps (Fisher 05-538-41N or equivalent)

GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)

GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.3.5.3.6 pH paper (Fisher 09-876-17 or equivalent)

2.3.5.3.7 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g.

100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.5.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.5.4.1 Methanol (Fisher A412-4 or equivalent)
2.3.5.4.2 Ethyl Acetate (Fisher E145-4 or equivalent)
2.3.5.4.3 50% Methanol/water
2.3.5.4.4 0.1M Hydrochloric Acid
2.3.5.4.5 Elution Solvent
Mix 80mL ethyl acetate with 20mL methanol.
2.3.5.4.6 Silylating Agent (select from)

- MSTFA/1% TMCS (Pierce#48915 or equivalent)
- MSTFA (Pierce#48910 or equivalent)
- BSTFA/1% TMCS (Pierce#38831 or equivalent)
- BSTFA (Pierce#38830 or equivalent)

2.3.5.5 CONTROL

2.3.5.5.1 UTAK 66812-C or an equivalent control which contains benzoylecgonine in the appropriate concentrations.

2.3.5.6 STANDARDS

2.3.5.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standard (in methanol)	***Potential Vendors***
Benzoylecgonine	Cerilliant B-004, Alltech 018203

2.3.5.7 PROCEDURE

2.3.5.7.1 Initial set-up
Label the test tubes and GC/MS vials with microinserts.

- Negative Urine Control
- Positive Urine Control
- Appropriate Laboratory Numbers

2.3.5.7.2 Extraction Procedure Utilizing SPEC·PLUS™·DAU Extraction Column

2.3.5.7.2.1 Transfer 1mL of urine specimen, Negative Control or Positive Control to an appropriate labeled test tube.
2.3.5.7.2.2 Add 1mL 0.1M HCl and Vortex.
2.3.5.7.2.3 Insert labeled SPEC·PLUS™·DAU extraction column into vacuum manifold
2.3.5.7.2.4 Add 200µL of methanol to column and wait for one minute.

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2.3.5.7.2.5 Pour sample onto column and aspirate at 3-5 in. Hg (10-17 kPa)

2.3.5.7.2.6 Add 500µL of 0.1M HCl to column and aspirate at 3-5 in. Hg (10-17 kPa).

2.3.5.7.2.7 Add 500µL of 50% methanol/water to column and aspirate at 3-5 in. Hg (10-17 kPa).

2.3.5.7.2.8 Increase vacuum to 10-20 in. Hg. (34-68 kPa) and dry extraction disc for ≥5 minutes.

2.3.5.7.2.9 Open vacuum manifold, wipe collection tips, and insert collection holding rack containing the 16X144mm tapered tip centrifuge tubes.

2.3.5.7.2.10 Add 800µL of elution solvent to column and aspirate slowly at < 3 in. Hg (10kPa).

2.3.5.7.2.11 Increase vacuum to 5 in. Hg (17 kPa) to assist final amount of elution solvent through the disc.

2.3.5.7.2.12 Remove collection vials with elutes from rack.

2.3.5.7.2.13 Evaporate solvent to dryness under a gentle stream of nitrogen at approximately 60°C.

2.3.5.7.2.14 Add 25µL of ethyl acetate.

2.3.5.7.2.15 In the hood add 25µL of silylating agent.

2.3.5.7.2.16 Cap.

2.3.5.7.2.17 Vortex.

2.3.5.7.2.18 Heat for 15 minutes at 90°C.

2.3.5.7.2.19 Cool to room temperature

2.3.5.7.2.20 Transfer to the appropriately labeled ALS vial.

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2.3.5.7.3 <u>Automated Extraction Procedure Utilizing SPEC·PLUS™·DAU Extraction Column</u>

2.3.5.7.3.1 Refer to the following attached methods/printouts.

2.3.5.7.4 <u>Gas Chromatography/Mass Spectrometry (GC/MSD) Analysis</u>

2.3.5.7.4.1 Inject 1µL into GC/MSD using the ALS.

2.3.5.7.4.2 Analyze sample extract in full scan acquisition. Refer to attached GC/MSD method printout for current analysis parameters.

2.3.5.7.5 <u>Detection and Identification Criteria</u>

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.6 Extraction of Benzoylecgonine Employing United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column.

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2.3.6.1 BACKGROUND

Cocaine is a naturally occurring alkaloid derived from leaves of the South American shrub, Erythroxylon coca. Cocaine is also can be produced synthetically. Cocaine is one of the most potent stimulants to the central nervous system due to its mechanism of action, which involves blocking reuptake of stimulatory neurotransmitters. Cocaine is used licitly as a local anesthetic in ophthalmology. The positive effects of cocaine include an increased mental awareness and alertness, a sense of clarity and feelings of elation. The fictional detective Sherlock Holmes used cocaine for its transcendently stimulating and mind clarifying properties to the displeasure of Doctor Watson. As with all drugs, the effects of cocaine depend on the dosage, the form in which it is taken and the route of administration. Other significant factors include the setting or circumstances in which the drug is used and the expectations of the user. Side effects can include pupillary dilation, restlessness, dizziness, dyskinesia, tremor, dysphoria, and paranoia. Additional major side effects of cocaine use are a consequence of discontinued use. If the user does not readminister the drug, they may experience increased anxiety, agitation, restlessness and the disturbance of normal sleep patterns, which leads to fatigue. Due to these effects following cocaine use, an individual's ability to operate a motor vehicle is impaired both during and following cocaine use.

Routes of administration include snorting, injection and smoking. The metabolism of cocaine and its metabolites involves hydrolysis, transesterification and n-demethylation. Cocaine metabolites detectable in urine include benzoylecgonine, ecgonine methyl ester, norcocaine and various arylhydroxy- and arylhydroxymethoxy- metabolites. The duration of the action of cocaine is limited by its rate of metabolism since its major metabolites are inactive. One of the active metabolites, cocaethylene is produced via transesterification when cocaine and ethanol are ingested concurrently.

2.3.6.2 PRINCIPLE

This procedure outlines the use of the 200mg CLEAN SCREEN® DAU SPE column for the extraction of cocaine and benzoylecgonine from urine. The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the urine sample.

The cation exchanger will allow the anionic sorbent (-) to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption. The nonpolar aspect of the column serves to extract nonpolar compounds from a polar sample matrix.[2] The cation exchanger component of the phase is effective for compounds which are present in the urine sample in a cationic form bonding ionically to the sorbent.

For the extraction of cocaine and benzoylecgonine, the urine is adjusted to pH 6 with a phosphate buffer to maximize the ionic character of the analyte, and loaded onto a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the amine functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water and a weak aqueous buffer, to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. When the column is dry the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

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2.3.6.3 EQUIPMENT AND SUPPLIES

2.3.6.3.1 200 mg CLEAN SCREEN® Extraction Column (ZSDAU020 OR ZCDAU020 or equivalent)

2.3.6.3.2 Drybath (Fisher or equivalent)

2.3.6.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.3.6.3.4 Vacuum Manifold/pump

2.3.6.3.5 Glassware

16X100 Test Tubes (Fisher 14-961-29 or equivalent)

16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)

Snap Caps (Fisher 05-538-41N or equivalent)

GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)

GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.3.6.3.6 pH paper (Fisher 09-876-17 or equivalent)

2.3.6.3.7 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.6.3 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.6.4.1 Methylene Chloride (Fisher D37-4 or equivalent)

2.3.6.4.2 Isopropanol (Fisher A416-1 or equivalent)

2.3.6.4.3 Ammonium Hydroxide (Fisher A669-500 or equivalent)

2.3.6.4.4 Methanol (Fisher A412-4 or equivalent)

2.3.2.4.5 Ethyl Acetate (Fisher E145-4 or equivalent)

2.3.6.4.6 Deionized/distilled (DI) water

2.3.6.4.7 100mM Phosphate buffer pH 6.0

2.3.6.4.8 100mM HCl

2.3.6.4.9 Elution Solvent

Mix 70mL methylene chloride, 20mL isopropyl alcohol, and 2mL ammonia hydroxide.

2.3.6.4.10 Silylating Agent (select from)

- MSTFA/1% TMCS (Pierce#48915 or equivalent)
- MSTFA (Pierce#48910 or equivalent)
- BSTFA/1% TMCS (Pierce#38831 or equivalent)
- BSTFA (Pierce#38830 or equivalent)

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2.3.6.5 CONTROL

2.3.6.5.1 UTAK 66812-C or an equivalent control which contains benzoylecgonine in the appropriate concentrations.

2.3.6.6 STANDARDS

2.3.6.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards (in solution)	*Potential Vendors*
Benzoylecgonine	Cerilliant B-004, Alltech 018203
Cocaine	Cerilliant C-008, Alltech 018003
Ecgonine methyl ester	Cerilliant E-001, Alltech 014553
Norcocaine	Cerilliant N-005, Alltech 6015353
Cocaethylene	Cerilliant C-010, Alltech 6015363

2.3.6.7 PROCEDURE

2.3.6.7.1 Initial set-up

Label the test tubes and GC/MS vials with microinserts.

- Negative Control
- Positive Control
- Appropriate Laboratory Numbers

2.3.6.7.2 Extraction Procedure Utilizing 200mg CLEAN SCREEN® DAU Column

2.3.6.7.2.1 Transfer 5mL urine specimen, Negative Control or Positive Control to an appropriate labeled test tube.

2.3.6.7.2.2 Add 2mL 100mM phosphate buffer and Vortex. pH should be 6.0 ± 0.5. Adjust pH as necessary with 100mM monobasic or dibasic sodium phosphate.

2.3.6.7.2.3 Insert labeled CLEAN SCREEN® extraction column into vacuum manifold.

2.3.6.7.2.4 Add 3mL of methanol to column and aspirate at ≤3 in. Hg to prevent sorbent drying.

2.3.6.7.2.5 Add 3mL of DI H_2O to column and aspirate and aspirate at ≤3 in. Hg.

2.3.6.7.2.6 Add 1mL 100mM phosphate buffer (pH 6.0) to column and aspirate at ≤3 in. Hg.

2.3.6.7.2.7 Load sample onto column at 1 to 2 mL/minute.

2.3.6.7.2.8 Wash column with 2mL DI H_2O and aspirate at ≤3 in. Hg.

2.3.6.7.2.9 Wash column with 2mL of 100mM hydrochloric acid and aspirate at ≤3 in. Hg.

2.3.6.7.2.10 Wash column with 3mL of methanol and aspirate at ≤3 in. Hg.

2.3.6.7.2.11 Dry column by aspirating at ≥ 10 in. Hg for ≥ 5 minutes.

2.3.6.7.2.12 Open vacuum manifold, wipe collection tips, and insert collection holding rack containing the 16X144mm tapered tip centrifuge tubes.

2.3.6.7.2.13 Add 3mL of elution solvent to column and aspirate slowly, < 3 in. Hg (10kPa).

2.3.6.7.2.14 Remove collection vials with elutes from rack.

2.3.6.7.2.15 Evaporate to dryness under a gentle stream of nitrogen at ≤ 40°C.

2.3.6.7.2.16 Add 50µL ethyl acetate.

2.3.6.7.2.17 In fume hood, add 50µL silylating agent.

2.3.6.7.2.18 Cap.

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2.3.6.7.2.19 Vortex.
2.3.6.7.2.20 Heat for 20 minutes 70°C dry bath.
2.3.6.7.2.21 Remove from dry bath and cool to room temperature.
2.3.6.7.2.22 Transfer to the appropriately labeled ALS vial.

2.3.6.7.3 Automated Extraction Procedure Utilizing 200mg CLEAN SCREEN® extraction column.
2.3.6.7.3.1 Refer to the following attached methods/printouts.

2.3.6.7.4 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis
2.3.6.7.4.1 Inject 1 μL into GC/MS using the ALS.

2.3.6.7.4.2 Analyze sample extract in full scan acquisition. Refer to attached GC/MSD method printout for current analysis parameters.

2.3.6.7.5 Detection and Identification Criteria
2.3.6.7.5.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus that of an authenticated standard.
2.3.6.7.5.2 Acceptable retention time window is ±5%.

2.3.6.8 REFERENCES

2.3.6.8.1 UCT CLEAN SCREEN® Extraction Columns Application Manual.

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2.3.5.7.5.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus that of an authenticated standard.

2.3.5.7.5.2 Acceptable retention time window is ±5%.

2.3.5.8 REFERENCES

2.3.5.8.1 Automated SPEC® · Solid Phase Extraction Protocols for Drugs of Abuse Using the RapidTrace™ SPE Workstation, ANSYS, 1997.

2.3.5.8.2 SPEC·PLUS™·3ML·DAU Drugs of Abuse in Urine Extraction Applications, Ansys, 1999

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Toxicology Section

Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.7 Extraction of Codeine and Morphine Employing the SPEC·PLUS™·DAU Extraction Column.

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2.3.7.1 BACKGROUND

Morphine and codeine are natural derivatives of the opium poppy, *Papaver somiferum*. Opium contains several alkaloids including morphine, codeine and papaverine. Codeine is the phenolic methyl ether of morphine while heroin is a diacetyl derivative. Classified as opiates, codeine and morphine are used therapeutically primarily as analgesics and antitussives. While both morphine and codeine have potential for abuse, the illicit drug, heroin continues to be the second most widely abused drug in the United States (DAWN 2000). The effect these opiates exhibit is dependent upon their interactions with specific receptor sites within the central nervous system (CNS). In addition to analgesia and cough suppression, effects of opiate use include euphoria, respiratory depression, sedation, reduced GI motility/constipation, hypothermia, dysphoria, miosis, bradycardia, nausea, and physical tolerance and dependence.

Compound	Trade Name	Receptor/Action	Metabolites
Codeine	Tylenol 3®	μ agonist, δ agonist	morphine, norcodeine
Morphine	MSIR Roxanol MS Contin	μ agonist, κ agonist, δ agonist	minor quantities normorphine
Heroin	---------	μ agonist, κ agonist, δ agonist	6-monoacetylmorphine, morphine, small quantities of codeine (addicts)

2.3.7.2 PRINCIPLE

This procedure outlines the use of the ANSYS® Diagnostics, Inc SPEC·PLUS™ 3ml SPE column for the extraction of codeine and morphine from urine. ANSYS Technologies' SPEC™ Solid Phase Extraction products are manufactured with polypropylene plastic and bonded-silica impregnated on a glass fiber disc. The DAU column utilizes a sorbent which combines a strong cation exchanger with a non-polar phase (reversed phase) to interact effectively, physically and chemically, with benzoylecgonine and minimally with interfering substances in the urine sample. The cation exchanger component of the phase is effective for

compounds which are present in the urine in a cationic form. Codeine and morphine form glucuronide conjugates to facilitate their excretion. Prior to extraction, an enzymatic hydrolysis is required to free them from the glucuronide sugar moiety. For the extraction of codeine and morphine, the urine is adjusted with a low pH acetate buffer to maximize the ionic character of the analytes and the sorbent. The sample is then applied to a pre-conditioned SPE column. The conditioning creates an environment which allows for optimal interaction between the sorbent and the analytes of interest. The column is subsequently washed with the aqueous solvent, to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of solvent. When the column is dry, the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

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2.3.7.3 EQUIPMENT AND SUPPLIES

2.3.7.3.1 SPEC·PLUS™·DAU extraction column. (Ansys 532-DAU or equivalent)
2.3.7.3.2 Drybath (Fisher or equivalent)
2.3.7.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.
2.3.7.3.4 Vacuum Manifold/pump
2.3.7.3.5 Glassware
16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)
2.3.7.3.6 pH paper (Fisher 09-876-17 or equivalent)
2.3.7.3.7 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

2.3.7.3 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.7.4.1 Methanol (Fisher A412-4 or equivalent)
2.3.7.4.2 Ethyl Acetate (Fisher E145-4 or equivalent)
2.3.7.4.3 Ammonium Hydroxide (Fisher A669-500 or equivalent)
2.3.7.4.4 1.0M Acetate Buffer (pH 3.8)
2.3.7.4.5 0.1M Acetic Acid
2.3.7.4.6 1.5M Acetic Acid

2.3.7.4.7 1.5M Phosphate Buffer (pH 10.8)

2.3.7.4.8 Elution solvent

Mix 80mL of ethyl acetate, 20mL methanol and 2 mL ammonium hydroxide.

2.3.7.4.9 β-Glucuronidase Options

- Prepare from Patella vulgata Type L-II powder (Sigma G8132 or equivalent)
- Prepared Helix pomatia Type H-2 Solution (Sigma G0876 or equivalent)

2.3.7.4.10 Silylation Reagent Options

- MSFTA (Pierce 48910 or equivalent)
- BSTFA (Pierce 38830 or equivalent)

2.3.7.5 CONTROL

2.3.7.5.1 Liquid Urine Control containing a minimum of Morphine and/or Codeine (BioRad 478, Utak 66812-C or equivalent)

2.3.7.5.2 Drug Mix (Alltech 601827 {Codeine, Morphine, Hydromorphone, Oxycodone, Nalorphine and Diacetylmorphine }or similar)

2.3.7.5.3 Morphine-3β-D-glucuronide (Alltech [1mg/ml] M-031, [100μg/ml] M-018, or equivalent)

2.3.7.6 STANDARDS

2.3.7.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards (in solution)	***Potential Vendors***
Codeine	Cerilliant C006, Alltech 018013
Dihydrocodeine	Cerilliant D-019, Alltech 017773
Fentanyl	Cerilliant F-013, Alltech 013993
Heroin	Cerilliant H-038, Alltech 013653
Hydrocodone	Cerilliant H-003
Hydromorphone	Cerilliant H-004, Alltech 013553
Methadone	Cerilliant M-007, Alltech 018023
Morphine	Cerilliant M-005, Alltech 018033
Oxycodone	Cerilliant O-008, Alltech 013543
Oxymorphone	Cerilliant O-004, Alltech 013983

2.3.7.7 PROCEDURE

2.3.7.7.1 Standard Preparation

Prepare the following non-extracted standards.

TMS derivative: Morphine, Codeine.

Add 10μL of stock solution to labeled tapered bottom centrifuge tube.

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2.3.7.7.2 Initial set-up

Label SPEC·PLUS™·DAU extraction column, test tubes, tapered-bottom derivatization tubes and GC/MS vials with microinserts as follows for derivatized extractions (TMS) for the negative control (NC), positive control (PC), Morphine-3β-D-glucuronide control, Standards, and appropriate laboratory numbers without prefix.

2.3.7.7.3 Sample Preparation

- Transfer 1.0mL urine specimen, negative urine or positive control to extraction tube.
- Add 200μL of 1.0M acetate buffer (pH 3.8).
- Vortex.
- pH should be approximately 4, adjust if necessary using 0.1M acetic acid or KOH.

2.3.7.7.4 Sample Hydrolysis

- To each extraction tube add:
  - 200 μl β-Glucuronidase solution
- Cap and vortex *gently* to mix.
- Place in 60°C laboratory oven or waterbath for two hours.
- Allow samples to cool
- Add 1.0mL of phosphate buffer (pH 10.8)
- The resulting pH should be approximately 9.
- Adjust pH as necessary.
- Centrifuge for 5 minutes at 3000-3500 rpm

2.3.7.7.5 Extraction

- Insert labeled SPEC·PLUS™·DAU extraction column in the vacuum manifold.
- Add 200μL of methanol to the column and wait for one minute.
- Decant sample into column and aspirate at 3-5 in. Hg (10-17kPa).
- Wash column with the following and aspirate aspirate at 3-5 in. Hg (10-17kPa)
  - 500μL of deionized water.
  - 500μL 0.1 M acid.
  - 500μL methanol
- Increase vacuum to 10-20 in. Hg (34-68 kPa) and dry extraction disc for ≥ 5 minutes.
- Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.

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- Add 800µL elution solvent to the column and aspirate at < 3 in. Hg (<10 kPa) to aspirate solvent through disc into collection vial.
- Increase vacuum to 5in. Hg (17kPa) to assist final amount of elution solvent through disc.
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at approximately 60°C.

2.3.7.7.6 Derivatization

- In fume hood, add 50µL of silylating agent and 50µL ethyl acetate.
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 20 minutes in 60°C dry bath.
- Remove from heat and allow to cool.
- Transfer derivative to labeled GC/MSD ALS vial with microinsert.

2.3.7.7.7 Automated Extraction Procedure Utilizing SPEC·PLUS™ - 3ml DAU column.

2.3.8.7.7.1 Refer to the following attached method/printouts.

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2.3.7.7 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.3.7.7.1 Analysis Parameters

2.3.8.7.1.1 Inject 1 µL into GC/MSD using the ALS.

2.3.8.7.1.2 Analyze sample extract in full scan acquisition.

2.3.8.7.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.3.7.7.2 Detection and Identification Criteria

2.3.8.7.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.3.7.8 REFERENCES

2.3.7.8.1 Automated SPEC® · Solid Phase Extraction Protocols for Drugs of Abuse Using the RapidTrace™ SPE Workstation, ANSYS, 1997.

2.3.7.8.2 SPEC·PLUS™·3ML·DAU Drugs of Abuse in Urine Extraction Applications, Ansys, 1999.

2.3.7.8.3 Baselt RC, Disposition of Toxic Drugs and Chemicals in Man 5th ed., Chemical Toxicology Institute, 2000.

2.3.7.8.4 Hutchison TA & Shahan DR (Eds): DRUGDEX® System. MICROMEDEX, Inc., Greenwood Village, Colorado (Edition expires [12/01]).

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Section Two
Urine Toxicology

2.3 Solid Phase Extraction (SPE) Methods for GC/MSD Confirmation

2.3.8 Extraction of Opiates Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Extraction Column.

2.3.8.1 BACKGROUND

Morphine and codeine are natural derivatives of the opium poppy, *Papaver somiferum*. Opium contains several alkaloids including morphine, codeine and papaverine. These natural products lead to the development of numerous synthetic analgesics. Narcotic analgesics are divided into 3 classes, the phenanthrenes (morphine, codeine, oxycodone, pentazocine), phenylpiperidines (meperidine, fentanyl), and the phenylheptanes (methadone, propoxyphene). As illustrated in the chart below, the effects of opiate class drugs are dependent upon interactions with specific receptor sites within the central nervous system (CNS). In addition to analgesia and cough suppression, effects of opiate use include euphoria, respiratory depression, sedation, reduced GI motility/constipation, hypothermia, dysphoria, miosis, bradycardia, nausea, and physical tolerance and dependence.

Compound	Trade Name	Receptor/ Action	Metabolites	Therapeutic uses
Buprenorphine	Buprenex®	μ agonist, κ antagonist	Norbuprenorphine	moderate-severe pain
Butorphanol	Stadol®, Stadol NS®	κ agonist, μ antagonist	3-hydroxybutorphanol, norbutorphanol	moderate-severe pain
Codeine	Tylenol 3®	μ agonist, δ agonist	morphine, norcodeine	mild-moderate
Dihydrocodeine	Paracodin®	μ agonist	dihydromorphine, nordihydrocodeine	mild-moderate
Fentanyl	Sublimaze®	μ agonist	despropionylfentanyl, norfentanyl, hydroxyfentanyl, hydroxynorfentanyl	moderate-severe
Heroin	NA in US	μ agonist	6-acetylmorphine, morphine, normorphine	----
Hydrocodone	Hycodan®, Vicodin®, Codone®, Lortab®	μ agonist	hydromorphone, norhydrocodone, dihydrocodeine hydromorphol	moderate-severe
Hydromorphone	Dilaudid®	μ agonist	hydromorphol	moderate-severe
Levorphanol	levo-dromoran®	μ agonist, κ agonist	norlevorphanol	moderate-severe
Meperidine	Demerol®	μ agonist	normeperidine	moderate-severe

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Compound	Trade Name	Receptor/ Action	Metabolites	Therapeutic uses
Methadone	Dolophine®, Methadose®	μ agonist	methadol, normethadol, EDDP, EMDP	Detoxification
Morphine	MS-IR Roxanol	μ agonist, κ agonist, δ agonist	normorphine	moderate-severe
Nalbuphine	Nubain®	κ agonist, σ agonist, μ antagonist	nornalbuphine	moderate-severe
Oxycodone	Percolone®, Roxicodone®, Oxycontin®, Oxy®	μ agonist	oxymorphone, noroxycodone	moderate-severe
Oxymorphone	Numorphan®	μ agonist	6-oxymorphol	moderate-severe
Pentazocine	Talwin®	μ agonist, κ agonist, σ agonist	cis- and trans-hydroxypentazocine, trans-carboxypentazocine	moderate-severe
Propoxyphene	Darvon®, Darvocet®	μ agonist	norpropoxyphene,	mild-moderate
Tramadol	Ultram®	μ agonist	nortramadol, O-desmethyltramadol, N-desmethyltramadol	moderate

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2.3.8.2 PRINCIPLE

This procedure outlines the use of the UCT 200 mg CLEAN SCREEN® extraction column for the extraction of Opiates from urine. The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the urine sample. The cation exchanger utilizes an anionic sorbent (-) to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption.

Opiates form glucuronide conjugates to facilitate their excretion. Prior to extraction, an enzymatic hydrolysis is required to free them from the glucuronide sugar moiety. For the extraction of opiates, the hydrolyzed urine is adjusted with a low pH acetate buffer, to maximize the ionic character of the analytes and the sorbent. The sample is then loaded onto a pre-conditioned SPE column. The conditioning creates an environment that allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the cationic functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water and a weak aqueous buffer, to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions but not the ionically bound material. Next, the column is dried to remove traces of aqueous and

organic solvents. When the column is dry the analytes of interest are recovered from the column with a basic organic solvent mixture. Following the elution from the SPE column the extract is derivatized for confirmation on the GC/MSD.

2.3.8.3 EQUIPMENT AND SUPPLIES

2.3.8.3.1 200mg CLEAN SCREEN® extraction column (ZSDAU020 OR ZCDAU020 or equivalent)

2.3.8.3.2 Drybath (Fisher or equivalent)

2.3.8.3.3 Evaporative concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.3.8.3.4 Vacuum Manifold/pump

2.3.8.3.5 Glassware
16X100 Test Tubes (Fisher 14-961-29 or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap Caps (Fisher 05-538-41N or equivalent)
GC/MS Automated Liquid Sampler (ALS) vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.3.8.3.6 pH paper (Fisher 09-876-17 or equivalent)

2.3.8.3.7 Gas chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl)

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2.3.8.4 REAGENTS

Refer to Manual section 2.6 for solution preparation

2.3.8.4.1 Methanol (Fisher A412-4 or equivalent)

2.3.8.4.2 Methylene Chloride (Fisher D37-4 or equivalent)

2.3.8.4.3 Isopropanol (Fisher A416-1 or equivalent)

2.3.8.4.4 Ammonium Hydroxide (Fisher A669-500 or equivalent)

2.3.8.4.5 Ethyl Acetate (Fisher E145-4 or equivalent)

2.3.8.4.6 Deionized/distilled water

2.3.8.4.7 1.0M Acetate Buffer (pH 5.0)

2.3.8.4.8 100mM Phosphate Buffer (pH 6.0)

2.3.8.4.9 100mM Acetate Buffer (pH 4.5)

2.3.8.4.10 1N NaOH

2.3.8.4.11 Elution Solvent
Mix 78mL Methylene Chloride, 20mL Isopropanol and 2mL Ammonium Hydroxide. ***Make fresh.***

2.3.8.4.12 β-Glucuronidase (Patella vulgata)

2.3.8.4.13 Silylation Reagent Options

- MSFTA (Pierce 48910 or equivalent)
- MSFTA + 1% TMCS (Pierce 48915 or equivalent)
- BSTFA (Pierce 38830 or equivalent)
- BSTFA + 1% TMCS (Pierce 38831 or equivalent)

2.3.8.5 CONTROL

2.3.8.5.1 Liquid Urine Control containing a minimum of Morphine and/or Codeine (BioRad 478, Utak 66812-C or equivalent)

2.3.8.5.2 Drug Mix (Alltech 601827 {Codeine, Morphine, Hydromorphone, Oxycodone, Nalorphine and Diacetylmorphine }or similar)

2.3.8.5.3 Morphine-3β-D-glucuronide (Alltech [1mg/ml] M-031, [100μg/ml] M-018, or equivalent)

2.3.8.6 STANDARDS

2.3.8.6.1 Run necessary analytical standards as indicated by examination of GC/MSD data.

Standards (in solution)	*Potential Vendors*
Codeine	Cerilliant C006, Alltech 018013
Dihydrocodeine	Cerilliant D-019, Alltech 017773
Fentanyl	Cerilliant F-013, Alltech 013993
Heroin	Cerilliant H-038, Alltech 013653
Hydrocodone	Cerilliant H-003
Hydromorphone	Cerilliant H-004, Alltech 013553
Methadone	Cerilliant M-007, Alltech 018023
Morphine	Cerilliant M-005, Alltech 018033
Oxycodone	Cerilliant O-008, Alltech 013543
Oxymorphone	Cerilliant O-004, Alltech 013983

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2.3.8.7 PROCEDURE

2.3.8.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Morphine, Codeine, and Hydrocodone. Add 10μL of stock solution to labeled tapered bottom centrifuge tube.

2.3.8.7.2 Initial set-up

Label 200 mg CLEAN SCREEN® Extraction Column, test tubes, tapered-bottom derivatization tubes and GC/MS

vials with microinserts as follows for derivatized extractions (TMS) for the negative control (NC), positive control (PC), Morphine-3β-D-glucuronide control, Standards, and appropriate laboratory numbers without prefix.

2.3.8.7.3 Sample Preparation

- Transfer 5.0mL of urine specimen, negative urine or positive control to extraction tube.

2.3.8.7.4 Sample Hydrolysis

- To each extraction tube add:
  - 2 mL β-Glucuronidase solution (pH 5.0)
- Cap and vortex *gently* to mix.
- Place in 65°C laboratory oven or waterbath for three hours.
- Allow samples to cool.
- Centrifuge for 10 minutes at 2000 rpm and discard pellet
- Adjust pH to 6.0 ± 0.5 with approximately 700μl of 1.0N NaOH

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2.3.8.7.5 Extraction

- Insert labeled 200mg CLEAN SCREEN® Extraction column in the vacuum manifold.
- Add 3mL of methanol to the column and aspirate and aspirate at ≤ 3 in. Hg to prevent sorbent drying.
- Add 3mL of deionized water to the column and aspirate at ≤ 3 in. Hg.
- 1mL of 100mM phosphate buffer (pH 6.0) and aspirate at ≤ 3 in. Hg.
- Load sample into column at 1 to 2mL/minute.
- Wash column with the following and aspirate at ≤ 3 in. Hg
  - 2mL of deionized water
  - 2mL 100mM acetate buffer (pH 4.5)
  - 3mL methanol
- Increase vacuum to ≥ 10 in. Hg (≥34 kPa) and dry extraction disc for ≥ 5 minutes.
- Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.
- Add 3mL elution solvent to the column and aspirate at < 3 in. Hg (<10 kPa).
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at ≤ 40°C.

2.3.8.7.6 Derivatization

- In fume hood add the following:
  - 50µL ethyl acetate.
  - 50µL silylating agent.
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 20 minutes in 70°C dry bath.
- Remove from heat and allow to cool.
- Transfer derivative to labeled GC/MSD ALS vial with microinsert.

2.3.8.7 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.3.8.7.1 Analysis Parameters

2.3.8.7.1.1 Inject 1 µL into GC/MS using the ALS.

2.3.8.7.1.2 Analyze sample extract in full scan acquisition.

2.3.8.7.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.3.8.7.2 Detection and Identification Criteria

2.3.8.7.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

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2.3.8.8 REFERENCES

2.3.8.8.1 UCT CLEAN SCREEN® Extraction Columns Application Manual

2.3.8.8.2 Platoff, G.E., Gere, J.A. Solid Phase Extraction of Abuse Drugs from Urine, For. Sci. Review, 3 (2):117-132; 1991.

2.3.8.8.3 Baselt RC, Disposition of Toxic Drugs and Chemicals in Man 5th ed., Chemical Toxicology Institute, 2000.

2.3.8.8.4 Hutchison TA & Shahan DR (Eds): DRUGDEX® System. MICROMEDEX, Inc., Greenwood Village, Colorado (Edition expires [12/01]).

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Toxicology Methods Manual

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Idaho State Police
Forensic Services
Toxicology Section

Section One
Screening of Blood and Urine for Drugs of Abuse

Procedure: Background and Standard Operating Procedure for Screening of Whole Blood and Urine by Enzyme Immunoassay

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- 1.5.3 *MICRO-PLATE Drugs of Abuse Series Urine Applications (MP-99-032).*
- 1.5.4 *Quick Reference - Manual Calculation Worksheet for OraSure EIA Kits (MP-99-035).*
- 1.5.5 *Importing and Exporting Protocols and Profiles on the PersonalLAB™ (IS-99-036).*

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1.1 BACKGROUND

1.1.1 Principle of ELISA Analysis

ELISA is an acronym for enzyme-linked immunosorbent assay. An ELISA is an immunoassay in which one reactant is immobilized on a solid phase and the signal generator is an enzyme. The enzyme delivers a signal to indicate a particular antigen-antibody reaction has occurred and to what extent. This reaction takes place inside of a polystyrene microtiter plate well. An enzyme commonly employed as a signal generator is horseradish peroxidase (HRP). The small size of HRP, the ease with which small conjugates can be produced through oxidation of its carbohydrate moieties to reactive aldehyde, its rapid kinetics, and reasonable price, make it popular in ELISA.

1.1.2 Description of PersonalLAB™ BioChem ImmunoSystems Instrumentation

The PersonalLAB™ is an automated microplate analyzer for processing immunoenzymatic techniques developed on 96-well microplates. The analyzer automatically dispenses samples and all reagents required for an ELISA testing procedure. In addition, the analyzer allows for the programming of incubation times and wash steps. The photometer reads the plate vertically. When the procedure is complete the PersonalLAB™ records the resulting absorbances. The instrumental software allows for the proper identification of samples.

1.1.3 Description of OraSure Technologies Micro-plate Assay

1.1.3.1 Intended Use

OraSure Micro-plate assay kits are intended for use in the qualitative determination of drugs-of-abuse in blood and urine. As described in the package inserts for each specific assay kit, the result of the assay is intended as only a preliminary analytical test result.

1.1.3.2 Overview of Assay

Each OraSure Micro-Plate EIA is a competitive micro-plate immunoassay for the qualitative determination of a specific drug, or class of drugs in blood and urine. Each of the serum assays requires a predilution step which dilutes the samples, controls and calibrators. This brings the analytes into an acceptable range for optimum performance of the bound microplate antibodies. Dilutions are either performed manually with an air displacement pipet or utilizing a Hamilton Dilutor. Samples, calibrators or controls are added to individual wells of the microplate along with the conjugate, which is the drug or hapten labeled with the enzyme horseradish peroxidase (HRP). There is a competition between the free drug in the matrix sample (blood or urine) and drug bound to enzyme (conjugate) for antibody (sheep or rabbit) fixed on the well. The wells are washed with DI water, the substrate (3,3′,5,5′-tetramethylbenzidine (TMB) with peroxide (H_2O_2) is added, and a

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color is produced. HRP catalyzes H_2O_2 oxidation of the substrate by transferring one electron from the TMB to the peroxide to yield a blue colored product. The reaction is stopped when 2.0 N sulfuric acid is added to the well. This acidic environment provides the necessary conditions for the loss of one more electron to produce the final yellow color. The acidic environment also serves to inactivate the enymatic activity of the HRP. The resulting absorbance at 450 nm is inversely proportional to the amount of drug present in the sample or standard. Consequently, a more intense yellow color results in a greater absorbance and indicates a lower concentration of drug in the sample.

The kit utilizes two calibrators, one containing no drug (negative calibrator) and one at the concentration corresponding to the accepted cut-off for the drug (cut-off calibrator). In addition, the kit utilizes negative and positive controls. The negative control contains a concentration between the negative calibrator and the cut-off calibrator and the positive control contains a concentration of drug above the cut-off calibrator. These controls are used to assure the performance of the kit. To meet specifications the following validation criteria should be met:

1. The individual replicates for the absorbance of the *negative calibrator* must be less than 1.2 times the mean *negative calibrator* and greater than 0.8 times the mean *negative calibrator*.
2. The individual replicates of the *cut-off calibrator* must be less than 1.2 times the mean *cut-off calibrator* and greater than 0.8 times the mean *cut-off calibrator*.
3. The mean absorbance for the *negative calibrator* is greater than the mean absorbance for the *negative control*.
4. The mean absorbance for the *negative control* is greater than the mean absorbance for the *cut-off calibrator*.
5. The mean absorbance for the *cut-off calibrator* is greater than the mean absorbance for the *positive control*.

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1.1.3.3 Assays in Use

Forensic Services utilizes the following assays:

Assay	*Calibrator*	*Urine Cut-off*	*Blood Cut-off*
Amphetamine Specific	d-Amphetamine	1000ng/mL	50ng/mL
Barbiturates	Secobarbital	200ng/mL	50ng/mL
Benzodiazepines	Blood: Nordiazepam Urine: Oxazepam	300ng/mL	50ng/mL
Cannabinoids	11-Nor-9-Carboxy-THC	50ng/mL	15 ng/mL
Cocaine Metabolite	Benzoylecgonine	300ng/mL	50ng/mL
Methamphetamine	Methamphetamine	1000ng/mL	50ng/mL
Opiates	Morphine	300ng/mL	50ng/mL

Refer to specific package inserts for complete details on each assay.

1.1.3.4 Protocols

1.1.3.4.1 Definition of Protocol

A protocol is a set of instructions that direct the PersonalLAB™ how to run a particular assay. Protocols exist for each of the seven assays utilized. Protocols define the volume required of standards, controls, and reagents, each assay's steps, instrument wash and incubation parameters, sample tip specifications and the validation criteria and reading parameters.

1.1.3.4.2 Standard Protocols

Additional protocols may be created and used as necessary.

1.1.3.5 Profiles

The **PROFILE** is information the software uses to actually process the samples and generate results. The **PROFILE** should not be confused with a **PROTOCOL** (1.3.4), which includes the assay procedure steps, which are programmed when the instrument is initially set-up.

1.1.3.5.1 Definition of Profile

A profile is a set of instructions, which direct the PersonalLAB,™ how to run a particular group of assays. The profile includes the order of performance, the plate location(s), the standard/reagent location and the processing mode

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(parallel or serial dispensing). Assays are grouped as noted below.

Urine

Dilution	Assays in Profile
1:60*	Amphetamine, Barbiturates, Methamphetamine and Opiates
1:5	Benzodiazepines, Cannabinoids, and Cocaine Metabolite

* One part urine in a total of 60 parts.

Blood

Dilution	Assays in Profile
1:5	Cannabinoids
1:5	Amphetamine, Methamphetamine, Barbiturates and Benzodiazepines
1:5	Cocaine, Opiates, and Cannabinoids

1.1.3.5.2 Standard Profiles

Additional profiles may be created and used as necessary.

1.1.3.6 Interpretation of Results

1.1.3.6.1 Positive Result

A positive result for a sample is indicated by an absorbance less than or equal to the OraSure analyte Cut-off Calibrator. Depressed absorbances, which are significantly less than Negative Calibrator, can be interpreted as positives if the cross-reactivity for the analyte of interest is known to be low.

In addition, at the discretion of an analyst, confirmatory techniques may be applied to samples that exhibit depressed absorbances, which *fall between the value observed for the cut-off calibrator and the negative control.* If data for confirmatory techniques supports the presence of an analyte, the analyte may be reported as present. *Examples of cases where this exception could apply include infant testing and samples collected as the result of a drug recognition examination* (DRE).

1.1.3.6.2 Negative Result

A negative result for a sample is indicated by an absorbance that is greater than the OraSure analyte Cut-off Calibrator. Special considerations may apply as outlined above.

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1.2 OPERATION OF THE PersonalLAB

1.2.1 Preliminary Considerations

1.2.1.1 Supplies Required for Sample Dilution

1.2.1.1.1 Option one: Air-displacement pipettes and appropriate tips.

1.2.1.1.2 Option two: Repeater Pipette and appropriate tips.

1.2.1.1.3 Option three: Hamilton MicroLab® 500A series dilutor equipped with appropriate syringes.

1.2.1.2 Supplies Required for Testing

Supply	*Source*	*Comments*
5 mL disposable plastic culture tubes	BioChem	Dead volume = 200µL
75 mL plastic reservoirs	BioChem	Dead volume = 1.5mL
35 mL plastic reservoirs	BioChem	Dead volume = 1.0mL
5 mL plastic cups	BioChem	Dead volume = 200µL
Caps for cups	BioChem	
Disposable plastic pipette tips	BioChem	

1.2.1.3 OraSure Technologies Assay Kits

The OraSure kits contain the following items:

- Micro-plates coated with anti-drug antibodies.
- Enzyme conjugate for specific drug/drug class.
- Refer to OraSure Technologies Micro-plate Assay Package. Insert for instruction on the preparation of the cocaine assay enzyme conjugate.
- TMB substrate reagent (universal).
- 2N H_2SO_4 Stopping reagent (universal).

1.2.1.4 Processing of New Assay Kits

When a new assay kit is opened the following should be performed:

1.2.1.4.1 Check the expiration date of all components. The specification sheet will contain the expiration dates of serum calibrators and controls, micro-plates, enzyme conjugate, substrate and stopping reagent. The manufacturer in a technical bulletin as well as on individual bottles provides the expiration date of urine calibrators and controls.

1.2.1.4.2 Date and initial kit specification sheet and indicate whether the kit contains five or a single plate.

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1.2.1.4.3 Check the revision date for the package insert. If the revision is not in the OraSure/PersonalLAB binder, place it in the appropriate section.

1.2.1.5 Quality Control (QC) Samples

1.2.1.5.1 Urine QC

The following QC samples must be included in each batch of urine specimens.

1.2.1.5.1.1 OraSure serum cut-off calibrator.

1.2.1.5.1.2 OraSure serum negative calibrator.

1.2.1.5.1.3 Negative control urine.

1.2.1.5.1.4 Positive control urine (BioRad, Utak, or equivalent).

1.2.1.5.2 Blood QC

The following QC samples must be included in each batch of blood specimens.

1.2.1.5.2.1 OraSure serum cut-off calibrator.

1.2.1.5.2.2 OraSure serum negative calibrator.

1.2.1.5.2.3 Negative control blood.

1.2.1.5.2.4 Positive control blood (In-house, Utak or equivalent).

1.2.2. General preparation for run.

Routine preparation for a run includes:

1.2.2.1 Fill wash bottles with distilled water.

1.2.2.2 Fill pipette tip tray with BioChem disposable tips.

1.2.2.3 Check printer paper supply.

1.2.3 General Rules of Operation

1.2.3.1 Care should be taken to not impede the arm action.

1.2.3.2 Run instrument with the top down. Having the top down is safer for the operator and better for the substrate.

1.2.3.3 Do not push waste button while plate washing is taking place or vapor lock may occur.

1.2.3.4 Do not open lid when the *Operation Monitor* screen indicates that the lamp is warming. Opening the lid will result in the lamp continuing to warm indefinitely.

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1.2.4 Blood calibrator and control preparation.

1.2.4.1 Calibrator Stock Standard Solutions

Drug standards (obtain as necessary from Cerilliant, Alltech, Sigma or equivalent vendor).

Stock (1.0mg/mL)	Potential Vendors
S-(+)-Amphetamine	Cerilliant A-008 1.0mg/mL
S-(+)Methamphetamine	Cerilliant M-020 1.0mg/mL
Benzoylecgonine	Cerilliant B-004 1.0mg/mL
Morphine	Cerilliant M-005 1.0mg/mL
(-)-11-nor-9-Carboxy-Δ9-THC	Cerilliant T-018 100μg/mL
Nordiazepam	Cerilliant N-905 1.0mg/mL
Secobarbital	Cerilliant S-002 1.0mg/mL

1.2.4.2 Calibrator Working Standard Solution*

Fill 10mL volumetric flask ~½ full with methanol. Add 50μL each of stock amphetamine, methamphetamine, benzoylecgonine, morphine, nordiazepam, and secobarbital. Add 150μL c-THC. Fill with methanol to 10mL line.

Solution is stable for 12 months when stored at 4 °C.

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1.2.4.3 Quality Control Stock Standard Solutions

Drug standards (obtain as necessary from Cerilliant, Alltech, Sigma or equivalent vendor).

Stock (1.0mg/mL)	Potential Vendors
(+)Methamphetamine	Sigma M-5260 1.0mg/mL
D-Amphetamine	Sigma A-3278 1.0mg/mL
Benzoylecgonine	Sigma B-8900 1.0mg/mL
Morphine	Sigma M-9524 1.0mg/mL
11-nor-Δ9-THC-9-carboxylic acid	Sigma T-6893 50μg/mL
Secobarbital	Sigma S-4006 1.0mg/mL
Desmethyldiazepam	Sigma N-3162 1.0mg/mL

1.2.4.4 Quality Control Working Standard Solution *

Fill 10mL volumetric flask ½ full with methanol. Add 50μL each of amphetamine, methamphetamine, benzoylecgonine, morphine, secobarbital, and nordiazepam. Add 300μL C-THC. Fill with methanol to 10mL line.

Solution is stable for 12 months when stored at 4 °C.

Different vendors should be used to make up the Calibrator and Quality Control Working Solutions

1.2.4.5 Blood Calibrators

1.2.4.5.1 **Positive Calibrators**

Liquid Whole Blood (Utak 44600-WB (F) or equivalent) is spiked with calibrator working standard solution at 50%, 100% and 300% of cutoff. To 1mL of negative blood add working standard solution as indicated below.*

Desired % of cutoff	µL Working Standard Solution
50% cutoff	5
100% cutoff	10
300% cutoff	30

**Calibrators may be made using serial dilutions.*

1.2.4.5.2 **Negative Calibrator**

Liquid Whole Blood Negative Control (Utak 44600-WB (F) or equivalent.

1.2.4.6 Blood Control

1.2.4.6.1 **Blood Quality Control (125% of Cutoff)**

Liquid Whole Blood (Utak 44600-WB (F) or equivalent) spiked with quality control working standard solution at 125% of cutoff. To 2mL of negative blood add 25µL quality control working standard solution.

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1.2.5 Sample collection and preparation.

1.2.5.1 Whole Blood Samples

1.2.5.1.1 Blood samples should be submitted in sodium fluoride (gray top) tubes or other tube types, as needed.

1.2.5.1.2 If particulates or clots are visible in the sample, homogenize with tissue grinder or clarify by centrifuging.

1.2.5.2 Urine Samples

1.2.5.2.1 Urine samples should be submitted in appropriate urine collection containers.

1.2.5.2.2 Samples with an unusually high turbidity should be centrifuged prior to analysis.

1.2.5.2.3 Urine samples should not contain the preservative sodium azide.

1.2.6 Off-Line Dilution of Samples

1.2.6.1 Option one: Calibrated air-displacement pipettes and appropriate tips.

1.2.6.2 Option two: Calibrated Repeater Pipette and appropriate tips.

1.2.6.3 Option three: Hamilton MicroLab® 500A series dilutor equipped with appropriate calibrated sample and reagent/diluent syringes.

1.2.6.4 Dilution of Samples

1.2.6.4.1 Prepare a 1 in 5 parts dilution in forensic diluent.

Sample	Forensic Diluent
160µL	640µL
200µL	800µL
250µL	1000µL

1.2.6.4.2 Prepare a 1 in 60 parts dilution in forensic diluent.

Sample	Forensic Diluent
91µL 1:5 dilution	1000µL
15µL	885µL

1.2.6.5 Appropriate Dilution for Each Assay

1.2.6.5.1 Urine

Dilution	Assays
1:60*	Amphetamine, Barbiturates, Methamphetamine and Opiates
1:5	Benzodiazepines, Cannabinoids, and Cocaine Metabolite

* One part urine in a total of 60 parts.

1.2.6.5.2 Blood

Dilution	Assays
1:5	Cannabinoids
1:5	Amphetamine, Methamphetamine, Barbiturates and Benzodiazepines
1:5	Cocaine, Opiates, and Cannabinoids

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1.2.6.6 Dilution of Calibrators and Controls

1.2.6.6.1 Dilution of calibrators and controls should be performed as noted under sections 1.2.6.4 and 1.2.6.5.

1.2.7 Initial Start-up / Session Preparation

1.2.7.1 Remove samples and reagents from refrigerator one hour prior to starting analysis.

1.2.7.2 Prepare samples for analysis. Dilute as indicated under sections 1.2.6.4 and 1.2.6.5.

1.2.7.3 Turn on computer.

1.2.7.4 Click on *Plab wb* icon (wb = workbench). Instrument will print-out *BIOCHEM ImmunoSystems, INC.* and the date.

1.2.7.5 From the W*orkBench – v1.1a* screen, log-on by clicking on the icon. Enter user name, press **Tab** and then enter password.

1.2.7.6 Daily maintenance can either be proceeded with at this time or at *Step* 1.2.7.26.

1.2.7.6.1 Click on icon. From the *open* screen select **⊙Session** {*Figure 1*} from list. Click **OK**

1.2.7.6.2 From *open session* screen, select *Start-up Maintenance.tpl* from "Template List" {Figure 2}. **Double click.**

1.2.7.6.3 Template will show up in lower "file list" box. **Double click** in box on selection **or** highlight selection and click **OK**

1.2.7.6.4 Click on S*tart Session* icon (far right /red arrow).

1.2.7.6.5 When *Profile –Vial Locations for Controls or Standard and Reagents* view comes up {Figure 5}, click Continue.

1.2.7.6.6 Screen will indicate *Waiting for Initialization.*

1.2.7.6.7 *Start-up* folder tab screen comes up.

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1.2.7.6.8 To select *Self Test*, Press Start. Instrument will check motors and voltages for acceptability. Display will inquire, *Print Self Test* report? Press Yes.

1.2.7.6.9 *Fill Syringes*
After priming, screen will inquire *Continue?*
Indicate Yes if bubbles are observed.
Press No, when bubbles are not longer present.

1.2.7.6.10 *Fill Lung*
Screen will instruct operator to open front cover to view lung filling.
Click **OK**.
After an initial fill, screen will inquire re: *250 µl more.* If lung is not between = lines on lung, press Yes.
Screen will continue to inquire until operator observes that lung is sufficiently full and selects No

1.2.7.6.11 ***Note: Overfilling lung can result in instrumental problems that may require a service call to remedy. The level sensing ability of the lung may be damaged.***

1.2.7.6.12 Screen will now instruct operator to *close cover to continue operation.*
Click **OK** .

1.2.7.6.13 *Buffer (Tank) 1 Prime* at least twice.
Watch tubing lines for bubbles.

1.2.7.6.14 *Buffer (Tank) 2 Prime* at least twice.

✔ A check mark will appear when each task is complete.

1.2.7.7 Click on Wrench icon (far left) to reset plastic tip counting.

1.2.7.7.1 Click on Reset

1.2.7.7.2 Click on **OK**

1.2.7.7.3 When daily maintenance is complete click ☒. Display will inquire *End-of-work has not been executed – Continue exiting?* Select Yes.

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1.2.7.8 Click on 🗁 icon. From the *open* screen select ⊙**SESSION** {***Figure 1***} from list. Click **OK**

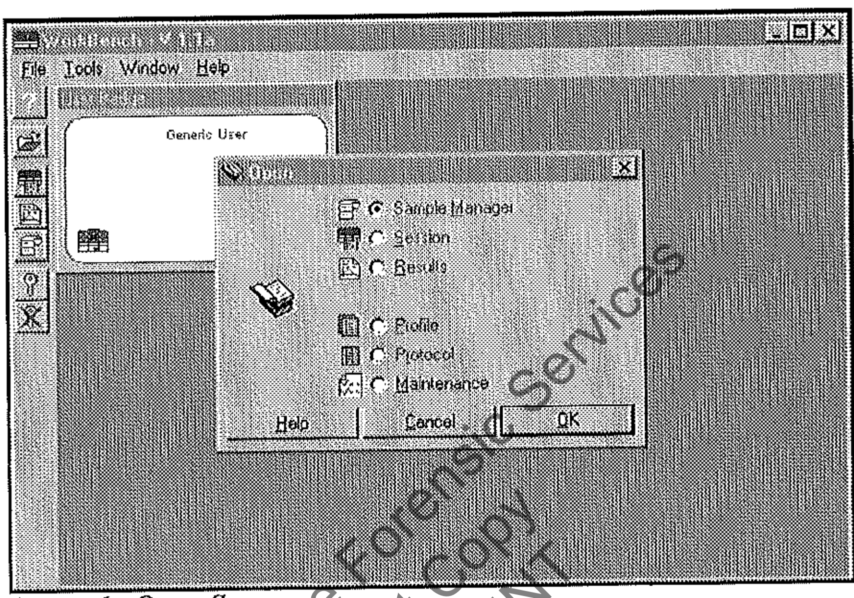

Figure 1: Open Screen

1.2.7.9 From *open session* screen, select appropriate template from "Template List" {Figure 2}.

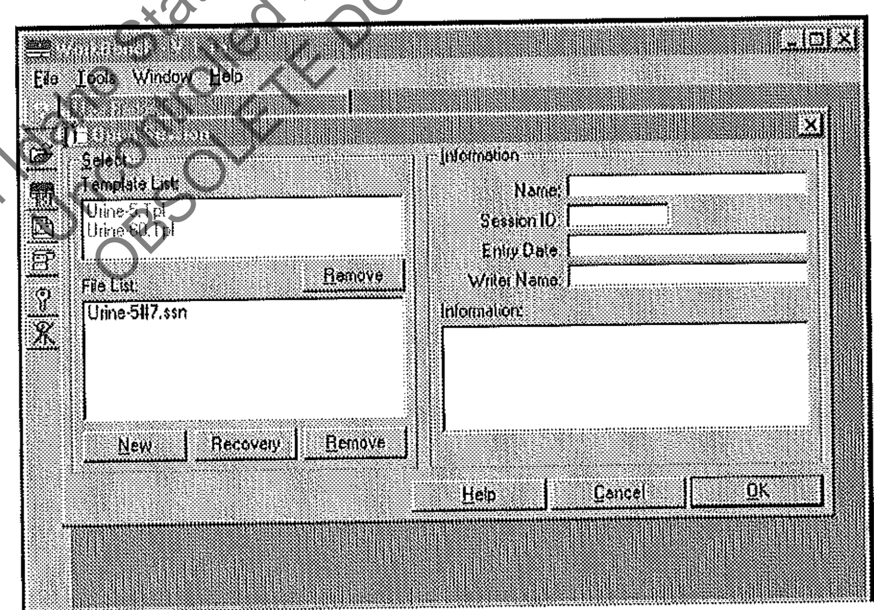

Figure 2: Open Session Screen

1.2.7.9.1 Select appropriate template.

1.2.7.9.2 Template will show up in lower "file list" box. **Double click** in box on selection or click **OK**

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1.2.7.10 *Session* screen will now come up {Figure 3}. Click on test tube icon to bring up *Sample Programming Screen* {Figure 4}.

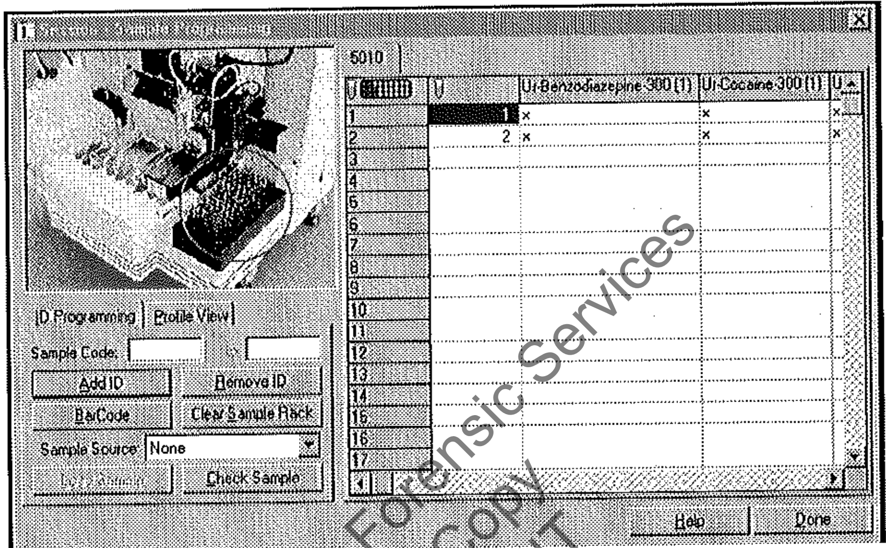

Figure 3: Session Screen

1.2.7.11 With *Session Sample Programming* screen displayed, click on sample rack of choice {Figure 4}.

1.2.7.11.1 Select appropriate rack.

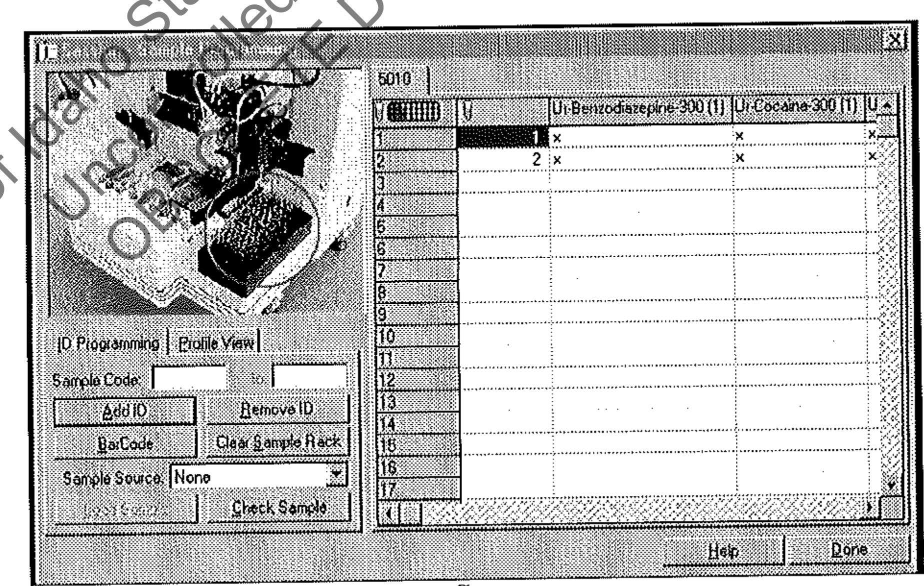

Figure 4: Sample Programming Screen

1.2.7.12 To clear previous programming, click on Clear Sample Rack.

1.2.7.13 If re-running samples DO NOT PRESS CLEAR SAMPLE RACK.

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1.2.7.14 On *Session - Sample Programming* screen enter "Sample Codes" in the box on the left portion of the screen. Number will appear on the right portion of the screen after [Enter] is pressed. Input either the laboratory number of the specimen or enter the source information for positive and negative controls.

1.2.7.15 Double click left on [rack] icon.

1.2.7.15.1 Screen will turn blue. By clicking on the [rack] icon, "X"s are placed on all assays indicating that the sample will be analyzed by all indicated assays.

-or-

Highlight desired sample boxes under assay and double click right mouse button while cursor is in highlighted area.

1.2.7.16 Click [DONE]

1.2.7.17 *Session – Protocol Position"* page then comes up {Figure 5}.

1.2.7.17.1 This view of the plate racks illustrates the number and position of the individual strips, which are necessary for each individual assay.

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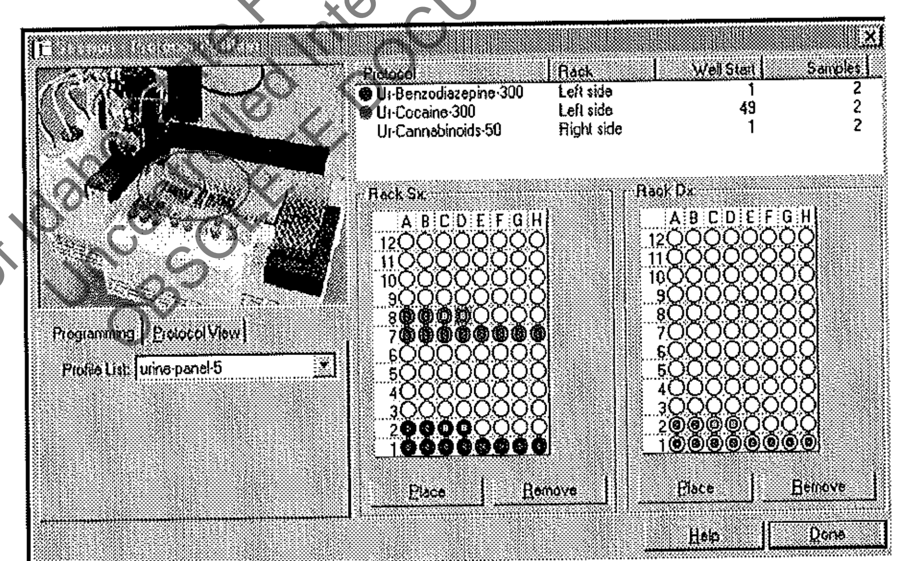

Figure 5: Session Protocol Position

1.2.7.18 <u>Load plate racks with appropriate strips</u>.

1.2.7.18.1 Press down on the strips to insure they are seated firmly into the tray. Improper strip positioning can result in the strip popping up and the instrument jamming during the washing or incubation step.

1.2.7.19 Click [DONE]

1.2.7.20 Save *Session* by clicking on save or on [save icon] (save session).

1.2.7.21 To print the sample load list

1.2.7.21.1 Click on *File View* icon from the tool bar (3rd from left). The *View File* page will come up.

1.2.7.21.2 Click on [Document] icon from tool bar (2nd icon from left).

1.2.7.21.3 Click on [Print] icon from the toolbar.

1.2.7.21.4 Click on ☒ to go back to *Session* page.

1.2.7.22 Load sample rack.

1.2.7.23 From *Session* Screen, click on *Start Session* icon (far right/red arrow).

1.2.7.23.1 *Profile –Vial Locations for Controls or Standard and Reagents* view comes up {Figure 6}.

1.2.7.24 Load cups and reagent reservoirs onto platform.

1.2.7.24.1 Using the screen template, place the 35mL conjugate containers, and the 75mL substrate and stop reservoirs according to their designated location on the platform.

1.2.7.24.2 Place appropriately diluted amount of each control (negative and positive) and calibrator (negative and cutoff) into the 5mL cups. Refer to sections 1.2.5.4 and 1.2.5.5 for appropriate dilutions. Place the cup at its designated numbered location.

1.2.7.24.3 Place serum negative calibrator and cut-off calibrator in 5mL cups at appropriate locations.

1.2.7.24.4 After loading is complete, hit [Continue].

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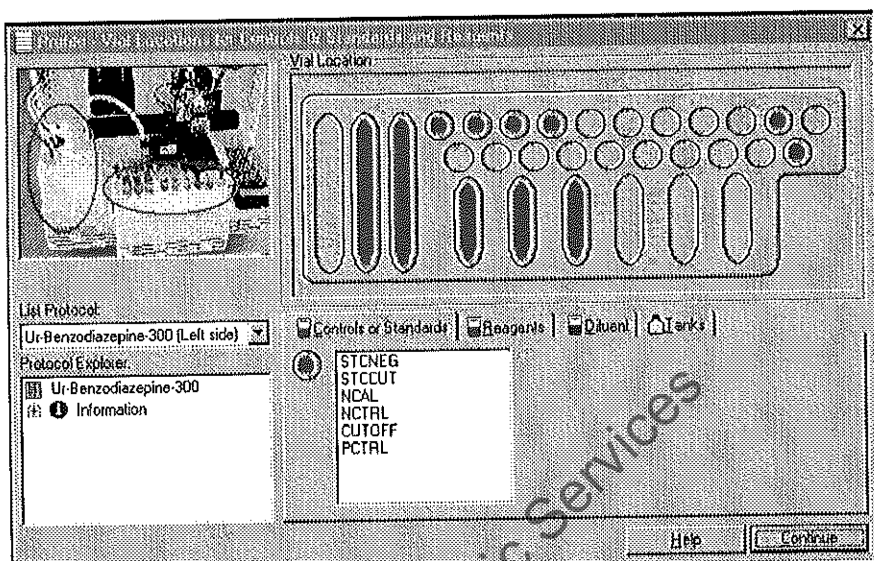

Figure 6: Vial Locations for Controls or Standards and Reagents

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1.2.7.25 If maintenance is not already complete, turn instrument on.

1.2.7.25.1 Start-up screen comes up. Screen will indicate *Waiting for Initialization.*

1.2.7.26 <u>DAILY MAINTENANCE</u>

If not performed initially, it should be completed at this time. If daily maintenance has been performed proceed to *Step 1.2.7.28.*

From *Station: 0 Processor V 1.1a* screen, click on *START-UP* folder tab to perform daily maintenance {Figure 7}. Click on appropriate boxes to:

1.2.7.26.1 Run *Self-Test.* Click on *Start.*

1.2.7.26.2 Display will inquire, *Print Self-Test* report? Press [Yes].

1.2.7.26.3 *Fill Syringes*

After priming, screen will inquire *Continue?*

Indicate [Yes] if bubbles are observed.

Press [No], when bubbles are no longer present.

1.2.7.26.4 *Fill Lung*

Screen will instruct operator to open front cover to view lung filling.

Click [**OK**].

After an initial fill, screen will inquire re: *250 µl more.* If lung is not between = lines on lung, press

Yes. Screen will continue to inquire until operator observes that lung is sufficiently full and selects No Screen will now instruct operator to *close cover to continue operation.* Click OK.

1.2.7.26.5 Buffer (Tank) 1 Prime.
Watch tubing lines for bubbles.

1.2.7.26.6 Buffer (Tank) 2 Prime.
Watch tubing lines for bubbles.

✓ A check mark will appear when each task is complete.

1.2.7.27 Click on Wrench icon (far left) to reset plastic tip counting.

1.2.7.27.1 Click on Reset

1.2.7.27.2 Click on **OK**

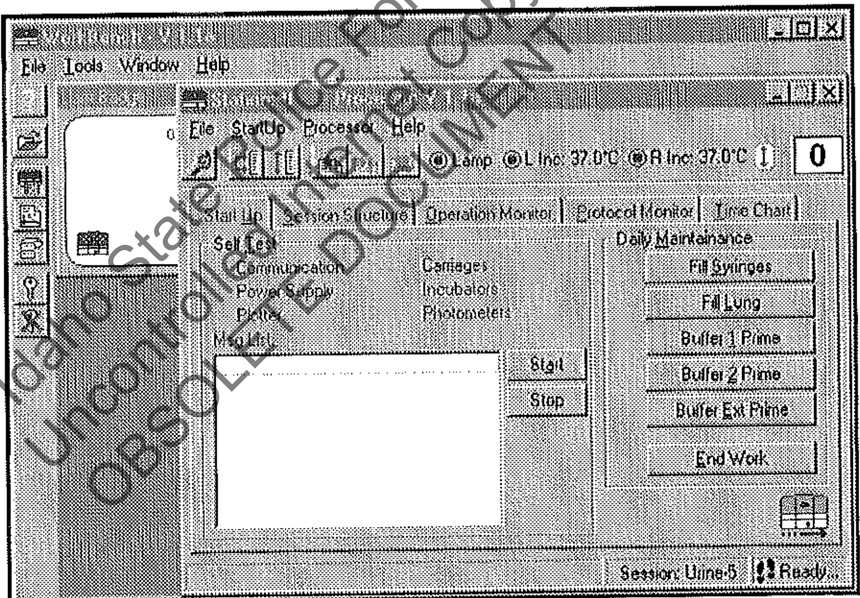

Figure 7: Daily Maintenance Screen

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1.2.7.28 From *Station: 0 Processor V 1/1a* page, click on *Operation Monitor* folder tab.

1.2.7.28.1 Click on EXE icon (4th from left).

1.2.7.29 In preparation for the run:

- Instrument will remind you about rack placement.
    - Operator can *retry* if sample rack is not in place.
- Screen will indicate *Waiting for Lamp Warm-up.*
- Instrument will check strip/racks in carriages.

1.2.7.30 Operation monitor will indicate the following tabs:

Time	*Task*	*Profile*	*Protocol*	*Rack*

1.2.7.31 To monitor run, click on profile tab.

1.2.7.32 When run is complete *Processor Screen* will indicate *Session terminated.* If end-of -work (refer to section 1.2.10) is not to be pursued at this point, click on **OK** followed by ☒.

1.2.8 Obtaining Results

1.2.8.1 From *WorkBench* screen, click on Paper (Results) icon (four down from top)

1.2.8.1.1 Highlight session name of choice under *File List.*

1.2.8.1.2 Click OK

1.2.8.1.3 Select Report icon (far right).

1.2.8.1.4 Refertation (data reduction) will commence.

1.2.8.1.5 When processing of data is complete click on Print icon.

1.2.8.1.6 After data has printed, click ☒, click ☒ to close out screens.

1.2.8.2 To access results not immediately following a run:

1.2.8.2.1 Click on Open Folder icon (folder with arrow).

1.2.8.2.2 Click on Open results.

1.2.8.2.3 Highlight Session ID, Name or Entry Date.

1.2.8.2.4 Click OK

1.2.8.2.5 Select *New* results or *Database* results (old/ previous).

1.2.8.2.6 Highlight either selection, click OK

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1.2.9 Re-Reading of a Plate

1.2.9.1 Click on icon. From the *open* screen select **⊙SESSION** {***Figure 1***} from list. Click **OK**

1.2.9.2 From *open session* screen, select appropriate template from "Template List" {Figure 2}.

1.2.9.2.1 Select previously run template.

1.2.9.2.2 **Double click.**

1.2.9.2.3 Template will show up in lower "file list" box.

1.2.9.2.4 Double click in box on selection or click **OK**

1.2.9.3 *Session* screen comes up. Click on test tube icon *(sample programming).*

1.2.9.4 Click on sample rack that samples were run in.

1.2.8.4.1 Select appropriate rack.

1.2.9.5 { } will indicate "done".

1.2.9.6 **Do not click on *Clear Sample Rack*.**

1.2.9.7 Highlight samples run. Screen will turn blue. Double click RIGHT in highlighted area. Click DONE

1.2.9.8 *Session – Protocol Position"* page then comes up {Figure 4}. Click DONE

1.2.9.9 Save *Session* by clicking on save or on (save session).

1.2.9.10 From *Session* Screen, click on the *Start Session* icon (far right/red arrow).

1.2.8.10.1 *Profile –Vial Locations for Controls or Standard and Reagents* view comes up {Figure 5}. Hit Continue.

1.2.9.11 From *Station: 0 Processor V 1.1a* page, select *Time Chart* folder tab.

1.2.9.12 On *Time Chart,* scroll down to *Reading* step. Highlight from *Reading* to *End.*

1.2.9.13 Click on Steps Select icon (second from left)

1.2.9.14 Click on EXE icon (fourth from left).

1.2.9.14.1 *Reading* Step comes up. Screen will indicate Waiting for Lamp Warm-up. Lamp will warm up for each re-read.

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1.2.9.15 When re-read is complete, display will indicate *Session Terminated.* Click **OK**.

1.2.9.16 Obtain results as described in *section 1.2.7.*

1.2.10 End-of-day Clean up

1.2.10.1 Return conjugates, stop and diluent reservoirs to refrigerator.

1.2.10.2 Dispose of used calibrator, controls, micro-plates and samples into appropriate biohazard container.

1.2.10.3 Fill plastic tip tray.

1.2.10.4 For *End-of-Work* routine, select START-UP tab.

1.2.10.4.1 Click on End of Work

1.2.10.5 Display will instruct operator *Please fill in buffer 2 with distilled water.*

1.2.10.5.1 Click OK

1.2.10.6 Screen will direct operator to *Please empty waste tank.*

1.2.10.6.1 Click OK after depressing button on left side of instrument.

1.2.10.6.2 This is a gravity flow water system and the button must be held in for it to fully empty.

1. 2.10.7 Click on ☒ to close *WorkBench* window.

1.2.10.8 Record Daily Maintenance in PersonalLAB QC binder.

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1.3 PERIODIC MAINTENANCE SCHEDULES

1.3.1 Weekly and Monthly Maintenance

1.3.1.1 Refer to the Maintenance section of the PersonalLAB™ notebook for maintenance schedules and tasks to be performed on a weekly and monthly basis.

1.3.1.2 *Start-up Maintenance Template* can be used to access priming functions.

1.6 QUALITY CONTROL

1.6.1 Assay Displacement Calculation

1.6.1.1 Calculation of Displacement
Percent displacement should be calculated based upon the values obtained from the OraSure serum cut-off calibrator and serum negative calibrator.

1.6.1.2 Calculate displacement as follows:

%Displacement to Cutoff =

$$\frac{A_{450} \text{ Value (Serum Negative Calibrator)} - A_{450} \text{ Value (Serum Cutoff Calibrator)}}{A_{450} \text{ Value (Serum Negative Calibrator)}} \times 100$$

1.6.1.3 Compare the calculated percent displacement with the acceptable range for the displacement provided on the specification sheet for the particular lot of each assay.

1.6.1.3.1 Percent displacement values should fall within ±5% of the package insert assay range.

1.6.1.3.2 If the percent displacement values do not fall within ±5% of the assay range, the particular assay should be repeated.

1.6.1.4 Record % displacement on original assay print-out.

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1.7 DISTRIBUTION OF ASSAY INFORMATION

1.7.1 Assay Printouts

1.7.1.1 Original data from each assay should be maintained in a binder on an annual basis.

1.7.1.2 A cover sheet containing the date of the run and the lot number for each assay should be included with original data.

1.7.1.3 Assay results are recorded on data page of toxicology analysis form.

1.8 REFERENCES

1.8.1 Butler, J.E. **Enzyme-Linked Immunosorbent Assay**. pp. 759-803 *In*: "Immunochemistry". Van Oss, C.J.; van Regenmortel, M.H.V., eds., Marcel Dekker, inc., New York, NY: 1994.

1.8.2 PersonalLAB™ User's Manual, 080040-001 REV.01, 1998.

1.8.3 OraSure Technologies PersonalLAB™ Training Guide.

1.8.4 OraSure Technologies Package Inserts for Serum Microplate EIA.

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Idaho State Police
Forensic Services
Toxicology Section

Section Four

Analysis of Alcohol and Common Volatile Solvents

4.1 Quantitative Analysis for Ethanol and Qualitative Analysis for Other Volatiles by Dual Column Headspace Gas Chromatography

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4.1.1 BACKGROUND

Fermented beverages such as beer and wine have been known and used by humans since prehistoric times.[6] Ethanol abuse is often manifest in driving under the influence (DUI) problems, which is a worldwide concern. The National Highway Traffic Safety Administration (NHTSA) estimates that alcohol was involved in 41% of fatal automobile crashes and 7% of all crashes in 1995.[2] chronic alcoholism also contributes to ethanol related deaths. Ethanol consumed on a regular basis can lead to the development of alcoholic hepatitis which can progress into cirrhosis, liver failure, and death.[2,6,7] Chronic excessive ingestion of ethanol is directly associated with serious neurologic and mental disorders such as brain damage, memory loss, sleep disturbances and psychoses.[7] Alcohol is also involved in a high percentage of domestic disputes many of which result in injury and/or death.

Notwithstanding the public perception that ethanol is stimulatory, ethanol is classified as a *Central Nervous System Depressant.* Ethanol is a psychoactive drug that is similar in most respects to sedative-hypnotic compounds.[4] The first mental processes to be affected are those that depend on training and previous experience.[7] The individual's memory, concentration, and insight are dulled and subsequently lost. The person may become overly confident and exhibit uncontrolled mood swings and/or emotional outbursts.[7] The effects of ethanol and other central nervous system depressants are additive, resulting in more sedation and greater impairment of driving ability.[4]

Ethanol is rapidly and completely absorbed from the stomach, small intestine and colon. The mechanism of absorption is a simple diffusion process, that is, alcohol moving from a region of higher to a region of lower concentration.[2,4,6] Alcohol is soluble in both water and fat, a property that facilitates its diffusion through biological membranes.[4] The major amount of absorption takes place in the small intestine due to its large surface area, good blood supply and thin walled membrane. The time from the last drink to peak concentrations can range between 30 and 90 minutes, depending upon the individual's stomach contents.[4,7] Alcohol absorption is slowed by the presence of food in the stomach. The time period required for gastric emptying is a prime factor that contributes to

the wide variety of absorption rates of ingested ethanol observed in different individuals and under different conditions.[2,7] Hence, the extent of absorption in the stomach and small intestine is a function of the amount of ethanol at that site, the vascularity of the site and the surface area in contract with the blood supply.[2] Other factors that affect the absorption of ethanol include the type of beverage, the alcohol content and any disease state that affects normal gastric function.[2]

Upon absorption, ethanol is distributed to all the water containing regions of the body. Within the blood there can be significant differences between arterial and venous blood depending upon the absorption status of the individual.[2] In the absorptive phase, the arterial blood ethanol concentration exceeds the venous blood ethanol concentration. Analysis of venous blood therefore, underestimates the brain alcohol concentration of the individual at this point. When absorption is complete there is little difference in ethanol concentration between and arterial and venous blood.[2]

90 to 98 percent of ethanol is completely oxidized in the liver by reacting with the cofactor nicotinamide adenine dinucleotide (NAD) facilitated by alcohol dehydrogenase to produce acetaldehyde. Acetaldehyde is then acted upon by aldehyde dehydrogenase to form acetic acid which goes onto form carbon dioxide and water (figure 1). The amount of ethanol oxidized per unit time is roughly proportional to body weight and probably to liver weight. The remaining (unoxidized) alcohol is excreted unchanged in urine, expired air, saliva and sweat. The average elimination rate of ethanol is 0.015 g/dL/hour from men and 0.018 g/dL/hour for women.[2] In addition to gender, chronic abuse, ethanol use combined with prescription drugs and certain genetic factors can also influence the elimination rate.[2,6,7]

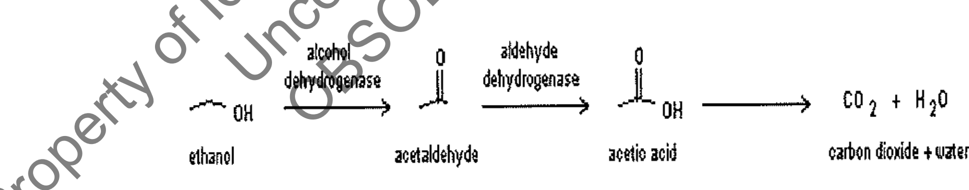

Figure 1. Metabolism of Ethanol.

Methanol (wood alcohol) causes relatively little intoxication compared to ethanol.[2,6] Its harmful affects are due to the direct result of its metabolism to formaldehyde (embalming fluid) and subsequently to formic acid. These metabolites lead to the destruction of neural cells, particularly the optic nerve, which can result in blindness.[2,6]

4.1.2 PRINCIPLE

This method describes the analysis of aqueous samples for the presence of volatile compounds including methanol, ethanol,

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acetaldehyde, acetone, isopropanol and related compounds, via a headspace sampling gas chromatographic method. Samples, controls and standards are sealed into vials that contain an aqueous 1-propanol internal standard solution and heated by the headspace analyzer. As described in Henry's Law, in a closed container at a given temperature, a direct (proportional) relationship exists between the amount of a volatile substance dissolved in a liquid and the amount of the volatile substance in the headspace vapor above the solution. An aliquot of the vapor is injected into a gas chromatograph (GC) in a dual column configuration. The GC serves to separate out the components of the solution as a function of their chemical properties. The separated components are identified on the basis of the retention time determined for each of the columns. Quantitation is accomplished through area percent data obtained from a flame ionization detector (FID). The quantitative result is based on a minimum of a three-point calibration curve, which uses the peak area ratio between the analyte and the internal standard.

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4.1.3 EQUIPMENT

4.1.3.1 Perkin Elmer Auto System XL Gas Chromatograph (GC)

4.1.3.2 Columns

4.1.3.2.1 Restek Rtx®-BAC1 (#18003: 30 meter X 0.32mm inner diameter (ID), 1.8μm film thickness (FT)) or equivalent column

4.1.3.2.2 Restek Rtx®-BAC2 (#18002: 30 meter X 0.32mm ID, 1.2 μm film thickness (FT)) or equivalent column

4.1.3.3 Perkin Elmer HS-40 or HS-110 Headspace Autosampler (figures 2 and 3)

Figure 2. HS-40

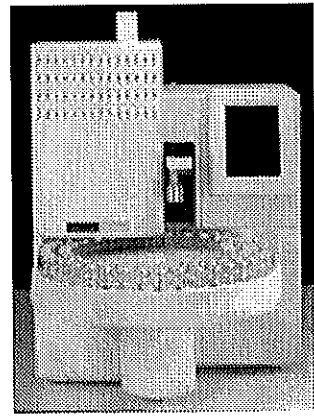

Figure 2. HS-110

4.1.3.4 PE Workstation Software, TotalChrom Version 6.2.0 or more recent version/upgrade.

4.1.3.5 Hand Crimper (P-E B003-8134 or equivalent)

4.1.3.6 Hamilton MICROLAB 503A or equivalent semi-automatic Dilutor/Pipetter equipped with sample and reagent syringes capable of dispensing 250µL and 2000µL, respectively.

4.1.3.7 <u>Glassware</u>

4.1.3.7.1 GC-Headspace vials (P-E B010-4236 or equivalent)

4.1.3.7.2 Safety Closures {PTBE septa, crimp caps and star springs} (P-E BO10-4240 or equivalent)

4.1.4 CONTROLS AND CALIBRATORS

4.1.4.1 Whole Blood Ethanol Control (LiquiSP$_x$™ or equivalent).

4.1.4.2 Aqueous Ethanol Standards (g/100mL)
0.025, 0.05, 0.08, 0.10, 0.20, 0.30, and 0.40 (Cerilliant or equivalent)

4.1.4.3 Multicomponent alcohol Calibration Kit (Cerilliant #A-054 or equivalent)

4.1.5 REAGENTS

4.1.5.1 1-Propanol (Acros/Fisher Scientific # 23207-0010, #A996-1 or equivalent)

4.1.5.2 Acetone (Fisher #A929-1 or equivalent)

4.1.5.3 Acetaldehyde (Fisher #01004-250 or equivalent)

4.1.5.4 Isopropanol (2-Propanol) (Fisher #A416-500 or equivalent)

4.1.5.5 Methanol (Fisher #A454-1 or equivalent)

4.1.5.6 Ammonium Sulfate (Fisher #A702-500 or equivalent)

4.1.5.7 Sodium Fluoride (Fisher #S299-500 or equivalent)

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4.1.6 SAFETY CONCERNS

4.1.6.1 Blood samples should be processed according to safety guidelines in the *Chemical Hygiene and Safety Manual.*

4.1.7 REAGENT PREPARATION

Record the preparation of all reagents on reagent log.

4.1.7.1 <u>Internal Standard Solution</u>
{0.03g/dL 1-propanol in 1.0M $(NH_4)_2SO_4$}

4.1.7.1.1 1.0M $(NH_4)_2SO_4$
Dissolve 132.14g $(NH_4)_2SO_4$ in distilled water. Dilute to 1L.

4.1.7.1.2 <u>0.03g/dL 1-propanol in 1.0M $(NH_4)_2SO_4$</u>

- Add approximately 800mL of 1.0M $(NH_4)_2SO_4$ to a 1000mL volumetric flask.
- Add 1g sodium fluoride *{optional}.*

- Add 375µL 1-propanol. QS to 1000mL with 1.0M $(NH_4)_2SO_4$.

4.1.7.1.3 *Solution is stable for 3 months.*

4.1.7.2 Volatile Standard Mix Solution

4.1.7.2.1 Add approximately 200 mL of DI water to a 250-mL volumetric flask.

4.1.7.2.2 Add the following volatiles, as indicated:
- 100 µL acetaldehyde
- 100 µL acetone
- 500 µL methanol
- 500 µL isopropanol
- 500 µL ethanol

4.1.7.2.3 QS to 250-mL.

4.1.7.2.4 *Solution is stable for 1 year.*

4.1.8 ANALYSIS PROCEDURE

4.1.8.1 General

4.1.8.1.1 Bring calibrators, controls, internal standard and samples to room temperature.

4.1.8.1.2 Gather necessary vials, closures and ancillary supplies in or near laminar flow hood.

4.1.8.1.3 Sample preparation should take place in a laminar flow hood.

4.1.8.2 Quality Control

4.1.8.2.1 Ethanol calibration standards must be run prior to the analysis of each batch of samples. A minimum of three points of calibration should be established.

4.1.8.2.2 An internal standard blank should follow the last ethanol calibrator.

4.1.8.2.3 A blood or aqueous control sample must be run after every 10 case samples. A minimum of two blood controls must be run per batch of samples.

4.1.8.2.4 Refer to package insert for manufacturer blood control ranges.

4.1.8.2.5 Values obtained from aqueous control and whole blood control samples must agree ± 10% of their target values.

4.1.8.2.6 Periodically run either the Volatile Standard Mix Solution or the Multicomponent Alcohol Calibration Kit solution to determine and monitor the retention of other volatiles of interest.

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4.1.8.2.7 Record values for blood control samples in B*atch Analysis QC log.*

4.1.8.2.8 On a monthly basis calculate the mean, standard deviation, relative standard deviation (CV%) and percent accuracy of the control samples. The data will be used to generate a mean quality control chart.

4.1.8.2.9 New blood control lots should be analyzed a minimum of nine times prior to official use. Calculate the mean, standard deviation, relative standard deviation (CV%) and percent accuracy of the control samples.

4.1.8.3 <u>Pipetter/Dilutor Set-up</u>

4.1.8.3.1 Switch on power.

4.1.8.3.2 Display will inquire as to the sizes of installed syringes. Select the correct size for sample syringe [right] and reagent syringe [left].

4.1.8.3.3 Scroll down to volume option. Select 250µL for sample syringe [right] and 2000µL for reagent syringe [left].

4.1.8.3.4 Scroll down to speed option. Verify that syringe speed is on desired setting.

4.1.8.3.5 Prime the fluid path. Continue priming until no bubbles are observed.

4.1.8.4 <u>Preparation of Blanks, Blood Control and Mixed Standard</u>

4.1.8.4.1 <u>Water Blank</u>

4.1.8.4.1.1 Label test vial with *water blank.*

4.1.8.4.1.2 Add 2000µL DI water to labeled test tube.

4.1.8.4.1.3 Seal **immediately** with crimp cap as illustrated in figure 4.

4.1.8.4.2 <u>Internal Standard Blank</u>

4.1.8.4.2.1 Label test vial with *ISTD blank.*

4.1.8.4.2.2 Use Pipetter/Dilutor to dispense 2000µL of internal standard (ISTD) into labeled headspace vial.

4.1.8.4.2.3 Seal **immediately** with crimp cap as illustrated in figure 4.

4.1.8.4.3 <u>Blood Control</u>

4.1.8.4.3.1 Label two headspace vials for *blood control 1 and 2.*

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internal standard (ISTD) into each labeled headspace vial.

4.1.8.5.3 Seal **immediately** with crimp cap.

4.1.8.5.4 Establish ethanol calibration plot with a minimum of three calibration points.

4.1.8.6 Initial Processing of Specimens

4.1.8.6.1 Open the sample submittal kit and remove the specimen's inner compartment. After inspecting and noting the condition of seals, open inner compartment (plastic tray or biohazard bag) and place laboratory number on each blood/urine/vitreous humor specimen.

4.1.8.6.2 When two blood/fluid samples are present, the samples should be labeled "A" and "B" or equivalent. Utilize sample "A" for analysis unless it contains insufficient sample.

4.1.8.7 Preparation of Samples for Analysis

4.1.8.7.1 Label two headspace vials with the laboratory number without the prefix.

4.1.8.7.2 Place one of the sample tubes or urine specimen bottle on tube rocker for at least two minutes.

4.1.8.8 Addition of blood, urine or vitreous humor sample to headspace vials.

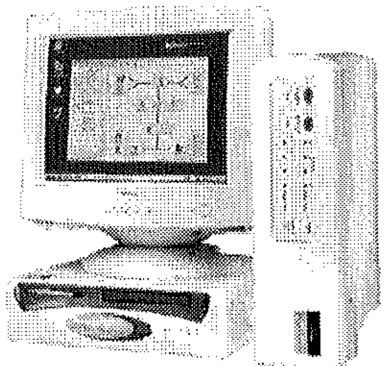
4.1.8.8.1 Use Pipetter/Dilutor dispense 250µL of sample and 2000µL of internal standard (ISTD) to a labeled headspace vial.

4.1.8.8.2 Seal headspace vials **immediately** with crimp caps as illustrated in figure 4.

4.1.8.9 Preparation for Run

4.1.8.9.1 Open **Sequence Editor**

4.1.8.9.2 Into Sequence log table, enter the sample case numbers, ethanol standards, other volatiles mix, blanks and controls.



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4.1.8.4.3.2 Use Pipetter/Dilutor to dispense 250µL of blood control and 2000µL of internal standard (ISTD) into each labeled headspace vial.

4.1.8.4.3.3 Seal **immediately** with crimp cap as illustrated in figure 4.

4.1.8.4.4 <u>Aqueous Controls</u>

4.1.8.4.4.1 Label appropriate number of headspace vials for *aqueous controls (1, 2,...).*

4.1.8.4.3.2 Use Pipetter/Dilutor to dispense 250µL of aqueous control and 2000µL of internal standard (ISTD) into each labeled headspace vial.

4.1.8.4.3.3 Seal **immediately** with crimp cap as illustrated in figure 4.

4.1.8.4.5 <u>Mixed Other Volatiles Solution</u>

4.1.8.4.5.1 Label test vial with *mixed volatiles.*

4.1.8.4.5.2 Use Pipetter/Dilutor to dispense 250µL of mixed volatile solution and 2000µL of internal standard (ISTD) into labeled headspace vial.

4.1.8.4.5.3 Seal **immediately** with crimp cap as illustrated in figure 4.

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Figure 4. Crimp cap assembly

4.1.8.5 <u>Preparation Calibration Standards</u>

4.1.8.5.1 Label vials for standards.

4.1.8.5.2 Use Pipetter/Dilutor to dispense 250µL of appropriate ethanol concentration and 2000µL of

4.1.8.9.3 Load samples, calibration standards, blank and controls into the carousel of the headspace sampler as noted in the sequence table.

4.1.8.9.4 Active headspace sampler

- Click on the **Setup** button to open the setup instrument dialog box.
- Select sequence as the setup type, and select the desired sequence file.
- On **Setup Instrument** dialog box, designate starting and ending row.
- Verify that the paths for raw and result data files specified in the sequence indicate the desired destinations.
- Select OK in the **Setup Instrument** dialog box to initialize the instrument.

4.1.8.10 Gas Chromatography Parameters

4.1.8.10.1 Refer to instrument METHOD printout for oven program and zone temperatures. Temperature program must provide for baseline separation of volatile compounds of interest as indicated by analysis of multicomponent mixtures.

4.1.8.11 Calibration

4.1.8.11.1 Ethanol calibrators should be analyzed in order of increasing concentration.

4.1.8.11.2 The least squares line resulting from the analysis of the ethanol calibrators must have a coefficient of correlation of ≥0.995.

4.1.8.12 Acceptance Criteria

4.1.8.12.1 **Accuracy**

4.1.8.12.1.1 Qualitative

The presence of ethanol can be established if there are no significant differences in the retention time between sample and standards. The relative retention times for a specimen must be within ± 0.10 minutes of the relative retention time for the compound in question. This rejection criterion should be designated in the TotalChrom analysis method.

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4.1.8.12.1.2 Quantitative
The quantitative results for a batch of samples can be accepted if the values obtained for control samples fall within 10% of their target value range.

4.1.8.12.2 **Precision**
The results obtained from duplicate analysis must agree within 0.015g/100mL. If this precision requirement is not met, the sample is reanalyzed.

4.1.8.13 Reporting of Results

4.1.8.13.1 **Blood**
Samples are quantitated to three significant figures. Report truncated mean value, of grams of ethanol per 100cc of whole blood, to two significant figures.

4.1.8.13.2 **Urine**
Samples are quantitated to three significant figures. Result obtained from blood alcohol curve should be multiplied by 0.67. Report truncated mean value, as grams of ethanol per 67 mL of urine, to two significant figures. A warning statement such as *Urine results may be of questionable value*, must be included in the report.

4.1.8.13.3 **Vitreous Humor**
Samples are quantitated to three significant figures. Report truncated mean value, as grams of ethanol per 100mL of vitreous humor, to two significant figures.

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4.1.9 QUALITY ASSURANCE

4.1.9.1 Blood or vitreous samples are to be refrigerated while at the laboratory. Urine samples can be either refrigerated or frozen.

4.1.9.2 Refer to toxicology manual section 5.1 for pipette calibration options.

4.1.9.3 Refer to toxicology manual section 5.2 for balance calibration requirements.

4.1.9.4 Refer to toxicology manual section 5.3.2 for GC-HS maintenance schedule.

4.1.9.5 Blood calibrators should be ordered prior to the current supply running out. This will allow for the analysis of new lots against existing calibrators.

4.1.10 REFERENCES

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4.1.10.3 Caplan, Y.H., *The Determination of Alcohol in Blood and Breath. in:* Forensic Science Handbook, edited by Richard Saferstein, pp. 594-648, Prentice-Hall New Jersey, 1982.

4.1.10.4 Julien,R.M., *Central Nervous System Depressants: Alcohol and the Inhalants of Abuse, in:* Primer of Drug Action, pp. 64-92, Freeman-New York, 1998.

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4.1.10.8 Idaho Administration Code, IDAPA 11.03.01, Rules Governing Alcohol Testing.

4.1.10.9 Christmore, D.S., Kelly, R.C. and Doshier, L.A. *Improved Recovery and Stability of Ethanol in Automated Headspace Analysis,* J. Forensic Sci. 29(4): 1038-1044; 1984.

4.1.10.10 Restek Applications Note #59598, Dual-Column Confirmational GC Analysis of Blood Alcohols Using the Rtx®-BAC1 and Rtx®-BAC2 Columns Optimized for the Perkin-Elmer HS-40 Headspace Autosampler, 1999.

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Forensic Services
Toxicology Section
Section Four

Blood Volatiles Determination
4.1 Quantitative Analysis for Ethanol and Qualitative Analysis for Other Volatiles by Dual Column Headspace Gas Chromatography

Revision #	Issue Date	History
0	10/01	Original Issue
1	05-15-02	Clarifications, coefficient of correlation change for system compatibility.

Approval

Technical Leader: [signature] S C Williamson **Date:** 05/15/02

Issuance

QC Manager: [signature] Rick D. Groff **Date:** June 7, 2002

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Toxicology Section

Section Two
Urine Toxicology

2.2 ANSYS® Thin Layer Chromatography (TLC) Methods

Standard Operating Procedures for TLC
Refer to methods listed below.

2.2.1 Toxi-Lab® Toxi-A Drug Detection System
- 2.1.1.1 Toxi-Lab® Toxi-A Instruction Manual

2.2.2 Toxi-Lab® Toxi-B Drug Detection System
- 2.2.2.1 Toxi-Lab® Toxi-B Instruction Manual

2.2.3 Toxi-Lab® Sympathomimetic Amine Differentiation
- 2.2.3.1 Toxi-Lab® Amine Differentiation with Acetaldehyde
- 2.2.3.2 Toxi-Lab® Amine Differentiation with Acetone

2.2.4 Toxi-Lab® THC II-PLUS 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (Δ9-THC-COOH) Detection System
- 2.2.4.1 Toxi-Lab® THC II-PLUS Instruction Manual
- 2.2.4.2 Summary Procedure with Controls

2.2.5 Appendix I. (Separate Binder for Available but Seldom Used Methods)
- 2.2.5.1 Toxi-Lab® Benzoylecgonine: Extraction and Detection
- 2.2.5.2 Toxi-Lab® Benzodiazepines: Hydrolysis Procedure
- 2.2.5.3 Toxi-Lab® Opiate Procedure
- 2.2.5.4 Toxi-Lab® Carbamates: Confirmation of Meprobamate and Carisoprodol with Furfural
- 2.2.5.5 Toxi-Lab® Methaqualone: Confirmation with Sodium Borohydride

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