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Markers of Ethanol Use
Blood and Urine Can Both Be Used

By Matthew H. Slawson, PhD, and Kamisha L. Johnson-Davis, PhD

People have consumed ethanol for at least as long as history has been recorded. In the Middle Ages, when Arabs introduced distillation to Europeans, many of the latter believed the elixir of life had been discovered as a remedy for all diseases. However, excessive ethanol consumption can lead to a host of social and medical problems. Ethanol is primarily a central nervous system depressant, and although it has some stimulatory effects, they are primarily due to depressed inhibitory brain mechanisms (1). Ethanol can also be administered as an antidote for methanol and ethylene glycol poisoning.

Absorption

Ethanol is absorbed rapidly in the stomach, small intestine, and large intestine. Maximal blood concentrations are measured about 30–90 minutes after the last drink. Ethanol vapor can be absorbed through the lungs. Slower gastric emptying (as with the presence of food) can delay absorption through the small intestine. Once in the small intestine, however, ethanol absorption is complete, rapid, and generally independent of the presence or absence of food. These variables often result in very different absorption profiles among individuals or within the same individual under different circumstances (1,2).

Distribution

Once absorbed, ethanol is evenly distributed in the body water to the point that a blood concentration can be estimated given a known dose, body weight, gender, and percentage body fat (2). The placenta is permeable to ethanol, which enters the fetal circulation (1). Females have a smaller volume of distribution than males (2).

Metabolism

Ethanol metabolism is independent of dose (zero-order kinetics), although first-order kinetics have been described at low (<0.02 g/dL) or very high blood concentrations. Females have also been shown to metabolize ethanol faster than males (1,2). About 90–98% of an ingested dose is metabolized by oxidation, primarily in the liver by alcohol dehydrogenase to produce acetaldehyde. Cytochrome P450 enzymes also convert ethanol to acetaldehyde. Acetaldehyde is converted to acetyl coenzyme A (via acetate) for fatty acid synthesis through the citric acid cycle or elimination.

The dehydrogenase enzymes responsible for alcohol metabolism exhibit genetic polymorphisms that are expressed with different frequencies in different racial populations. These polymorphisms can also contribute to variable rates of metabolism. A very small percentage of an ethanol dose (<0.1%) is conjugated to either glucuronic acid or sulfonic acid to form ethyl glucuronide and ethyl sulfate, respectively (1–3). These minor metabolites are of interest as biomarkers of ethanol exposure with a longer window of detection than the parent drug in urine.

Elimination

As mentioned above, acetaldehyde generated from ethanol oxidation can be used in the citric acid cycle leading to increased fatty acid synthesis or elimination (3). Oxidation products and ethanol conjugates are excreted in the urine; the remaining dose is eliminated via the lungs or feces (1,2).

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Markers of Ethanol Use

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Toxicology

Ethanol toxicity is largely related to its mechanism of action and its metabolism. Ethanol is a small molecule that can pass through the blood-brain barrier to exert its effects. Ethanol use can increase fatty acid synthesis and lead to fatty deposits in the liver. Protein and nucleic acid adduct formation due to the reactive nature of acetaldehyde is also possible.

Chronic alcohol abuse leads to a large spectrum of symptoms, including liver damage due to a buildup of acetaldehyde, neurologic disorders (such as seizures), nutritional disorders, and more. Use of ethanol concurrent with other central nervous system depressants can exacerbate the effects of both, leading to severe adverse reactions and even death (1,2).

Analysis

Ethanol can be measured in a variety of biological matrices. Breath analysis is commonly used by law enforcement officers in suspected cases of driving under the influence. The test involves the oxidation of expired ethanol to acetic acid and water in the flow cell of the analyzer. The acetic acid produces an electrical current, with the alcohol concentration extrapolated from its strength.

Ethanol is also commonly measured in blood by extracting vaporized ethanol from the headspace of a sealed vessel containing the blood sample or by direct injection without headspace (2). This vapor (or prepared sample) is injected onto a gas chromatograph and the detector response (typically flame ionization) is proportional to the concentration of ethanol in the sample. This method can also be used to distinguish specific alcohols in the system (for example, ethanol, methanol, or isopropanol). A major disadvantage of measuring ethanol directly is its short half-life of 2 to 14 hours.

Markers of Ethanol Use

Table 1 summarizes some of the traditional blood markers of ethanol use. Several of these markers have low specificity for ethanol and false positives can occur from other diseases or physiological conditions. Newer direct markers of ethanol use have proven more specific.

Phosphatidylethanol (PEth) is a direct ethanol marker in blood that detects chronic heavy drinking with high specificity (4). PEth is an abnormal phospholipid formed in the red blood cell membrane in the presence of ethanol, catalyzed by the enzyme phospholipase D. PEth is not detectable in blood after a single administration of ethanol, but forms from chronic or binge use, usually exceeding 50 g. Its half-life is 3 to 5 days, with a window of detection of about 28 days. It is not affected by liver disease. PEth can be analyzed by mass spectrometry and is useful for detecting ethanol use during pregnancy or when a longer window of detection is needed.

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are urinary markers that provide a window of detection of up to a week after alcohol consumption (5). They are not affected by the presence of liver disease. Ethanol is primarily metabolized by alcohol dehydrogenase (92–95%); however, <0.1% is metabolized by UDP-glucuronosyltransferase to form EtG and <0.1% is metabolized by sulfotransferase to form EtS. These markers can be used in forensic investigations to determine alcohol use days after ingestion and in abstinence programs in which blood collection is undesirable or impractical.

A recent study by Himes et al. demonstrates that EtG is a better marker for maternal drinking during pregnancy in meconium than fatty acid ethyl esters (6). EtG and EtS can be readily analyzed using liquid chromatography and mass spectrometry (7–10).

Interpretive Issues

Urine concentrations of alcohol or its metabolites cannot be used to extrapolate a dose of alcohol or degree of impairment (2). However, urine alcohol concentrations have been used in conjunction with blood concentrations to help determine a timeframe of drinking, which can help in driving-under-the-influence cases (11). Direct analysis of ethanol in urine can be complicated by the fact that fermentation of glucose in the bladder by bacteria can produce ethanol and thus lead to a false result (12,13).

Recent studies have investigated the occurrence of positive EtG results caused by exposure to hand sanitizers, mouthwash, or other "incidental" exposures (13). The concomitant presence of EtS in the urine can help distinguish actual ethanol consumption from dermal exposures to these alcohol-containing products.

In summary, ethanol testing is important for patient care. However, ethanol itself has a short half-life in serum and urine. Direct metabolites with a longer window of detection can enhance the ability to detect drinking days or weeks after use.

Learning Objectives

After completing this article, the reader will be able to list several major clinical biomarkers for ethanol use in blood and urine as well as describe which markers are most useful depending on whether blood or urine is being analyzed.

Table 1. Traditional Markers of Ethanol Use

Marker	Clinical Use	False-Positive Potential	General Comments
Gamma-glutamyl transferase (GGT)	Chronic alcohol abuse	Not specific: liver, biliary disease, and medications can induce increase in enzymes	Elevations caused by excessive drinking (100g/day) for up to 2 weeks
Alanine Aminotransferase (ALT) Aspartate Aminotransferase (AST)	Chronic alcohol abuse	Not specific: liver, biliary disease, and medications can induce increase in enzymes	AST/ALT > 2.0 is specific for ethanol-related liver disease
Carbohydrate-deficient transferrin (CDT)	Heavy alcohol use Indicator of relapse	Not specific: iron deficiency, fulminant hepatitis C virus, and inborn errors of glycogen metabolism can interfere	Altered form of iron transport protein when drinking is continued for >2 weeks Caused by drinking 60g/day for >2 weeks
Mean corpuscular volume (MCV)	Heavy alcohol use	Not specific: hemolysis, anemia, liver disease, and vitamin B12 deficiency can interfere	MCV increases with excessive ethanol intake Caused by drinking 60g/day for >2 weeks
Acetaldehyde adducts (AA)	Hemoglobin-bound AA distinguishes heavy drinkers from abstainers First metabolite of ethanol	Not specific: Diabetics have 2X the concentration of hemoglobin-bound AA than alcoholics	Testing methods are complex
Fatty acid ethyl esters (FAEE)	Alcohol abuse Analyzed in serum, meconium, hair, and tissues	Limited specificity in hair: FAEEs are found in the hair of non-drinkers as well as drinkers	Light- and heat-sensitive

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The authors have nothing to disclose.

Future of Mass Spectrometry: *New Techniques Could Offer Faster and Simpler Sample Prep*

By Jennifer Powers, PhD

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is highly valued in clinical and forensic toxicology labs because of its high precision, specificity, and sensitivity not generally found with immunoassay techniques or with LC alone. However, when complex mixtures are involved, as is the case for most blood and urine samples, it can require tedious and time-consuming sample preparation. Typical sample preparation can involve solid-liquid extraction, liquid-liquid extraction, or precipitation steps using equipment available only in a laboratory setting. These steps can remove protein, pre-concentrate the analytes, or minimize ion suppression.

Advances in Mass Spectrometry

Research in the field of mass spectrometry has led to many advances, including the miniaturization of mass spectrometers and development of ambient ionization methods requiring minimal sample preparation. All this points the way to one day using MS in the field or at the bedside in point-of-care devices. A recent article by scientists at Purdue University highlights their progress with one such device (1). These advances are of greatest interest to emergency departments and for therapeutic drug monitoring, although techniques that reduce sample preparation time would interest any toxicology lab trying to improve throughput.

Ambient Ionization and On-Line Solvent Extraction

Ambient ionization refers to a direct ionization technique with minimal sample preparation and soft ionization that can interface to mass spectrometers with an atmospheric pressure inlet (2). It generally also implies surface sampling. There are many such techniques and related publications.

Most give credit to Graham Cooks and coworkers for making this concept widely known. Cooks published a paper in 2004 showing how one could obtain mass spectra from solid objects such as a flower, a seed, a piece of leather, or even skin using desorption electrospray ionization (3). In this technique, a liquid is sprayed onto the surface of the object. Desorbed ions are then directed into an atmospheric pressure inlet of a mass spectrometer. Analytes that have been examined by this method include amino acids, drugs, terpenoids, steroids, peptides, and proteins.

Cooks' group obtained a mass spectrum by spraying aqueous alcohol on the skin of a person who had taken an over-the-counter antihistamine. They detected loratadine about 40 minutes after a subject took a tablet, and it remained above the detection limit for an additional 50 minutes.

New techniques on the horizon for use in toxicology and therapeutics include paper spray mass spectrometry, an ambient ionization technique, and slug-flow microextraction, an on-line solvent extraction method. Both methods reduce sample preparation time and use very small amounts of sample. For a more thorough review of these and other ambient ionization techniques, see the recent review by Monge et al. (2).

Paper Spray Mass Spectrometry

Wang et al. have described a method for monitoring the blood concentration of the leukemia drug imatinib (Gleevec) using dried blood spots on filter paper (4). Their simple technique involves applying less than 1 µl of blood then drying the paper. Wetting that paper with a solvent allows movement of the analyte, and a voltage is applied while the wet paper is held in front of a mass spectrometer. The measured concentration was linear from 62.5 ng/mL to 4 µg/mL, which includes the therapeutic range.

The authors added deuterated imatinib to the blood as an internal standard for quantitation. They applied the technique to a wide variety of analytes, including epinephrine, methadone, cocaine, atenolol, angiotensin I, and phosphatidylcholine, using concen-

trations of 0.1 to 10 µg/mL. Signals lasted from one to several minutes.

A nice advantage of paper as a medium is that it both separates components and serves as the ion source. The group illustrated this by separating two dyes, then performing MS on each spot separately.

Paper Spray with Blood

In a more recent paper, Espy and co-workers provide insight into the requirements of paper spray when using blood as the matrix (5). Although less than 1 µL of blood can be used, in some cases a larger volume can improve detection limits and reproducibility. With spots greater than 2 µL, the wicking carries hemoglobin (and presumably other components) toward the tip of the paper triangle and inhibits its formation of a stable Taylor cone. This problem can be avoided by drying the sample, but larger blood volumes can take a long time to dry. Volumes of 10–15 µL require approximately two hours to dry.

Espy et al. showed that the long wait time can be avoided by the addition of alum (potassium aluminum sulfate) to the paper prior to spotting, which causes immediate blood clotting. To achieve precision better than 15%, they used an isotopically labeled internal standard. A blood volume of 10 µL improved precision, and a voltage of 3.0 kV reduced background noise.

The researchers examined a wide variety of oncology drugs that spanned the range of molecular weights for this drug class. The lower limits of detection were in the same order of magnitude as those for dried blood spots and within one order of magnitude of previously reported LC-MS/MS methods for these drugs. Total run time after spotting was less than one minute.

Slug-Flow MicroExtraction with Nano-Electrospray Ionization

Slug-flow microextraction (SFME) is an on-line version of liquid-liquid extraction that has recently been coupled with nano-electrospray ionization (nanoESI) (6). Ren et al. used a disposable glass capillary (0.8 mm internal diameter) with a pulled tip. Inside the capillary, they created two adjacent liquid plugs by sequentially injecting small volumes (5 µL) of organic solvent and sample. The extraction speed can be increased by either tipping the capillary up and down or by using a push-and-pull mechanism to force air pressure through a pipette tip. These actions, combined with the friction on the capillary wall, mix the solutions effectively.

The researchers obtained the maximum analyte signal after only five cycles of SFME for urine samples containing methamphetamine, benzoylecgonine, or nicotine. Blood samples required more cycles unless they were first diluted to reduce viscosity. After extraction, the researchers inserted a stainless steel wire to push the solvent plug to the tip of the capillary and applied a high voltage to generate the nanoESI for MS analysis.

As one would expect, the selection of an appropriate solvent for sample extraction is critical. The solvent needs to be compatible with nanoESI and miscible with the sample fluid, but also have good solubility for the target analyte. For the compounds used in this study, ethyl acetate was the best solvent across multiple spray voltages.

The authors obtained sensitivity and precision with both blood and urine samples for molecules commonly analyzed in a toxicology lab setting, including methamphetamine, benzoylecgonine, verapamil, amitriptyline, and various steroids. Although extraction of benzoylecgonine into ethyl acetate required a dilution process, the limit of detection was still 0.08 ng/mL.

With this technique, one can spike a deuterated internal standard into the extraction phase, allowing it to mix during the slug-flow extraction process. For methamphetamine, the analyte to internal standard ratio was linear over the range of 1–100 ng/mL, and the relative standard deviations were less than 10% for concentrations above 10 ng/mL.

SFME can also be used when derivatization is required. For the reaction of epitestosterone with hydroxylamine, the researchers simply injected the derivatization agent as a liquid plug between the ethyl acetate and urine sample. The derivatized product formed when components mixed during the slug-flow extraction process.

Measurement of Enzymatic Activity

The same researchers also used SFME to measure enzymatic activity (6). They examined pseudocholinesterase activity using diluted whole blood samples by adding substrate to a diluted sample and then mixing 5 µL of this solution with 5 µL extraction phase in the glass capillary. After room-temperature incubation, they analyzed the substrate (acetylthiocholine, ATCh) and reaction product (thiocholine, TCh) at varying time points. In-line and off-line incubation gave identical results for the TCh/ATCh ratio. Ethyl acetate performed markedly better than chloroform as the extraction solvent.

Summary

Ambient ionization and on-line extraction are exciting new developments in the analysis of complex

mixtures by MS. Paper chromatography MS combines sample collection, extraction, and ionization in one step. SFME used with nanoESI can combine extraction and ionization into a single step. When these techniques are integrated with miniaturized mass spectrometers with atmospheric pressure inlets, MS can be freed from the traditional laboratory setting for the identification and quantitation of a wide variety of analytes. The sample preparation steps are much faster than current techniques and require less solvent. These advances could allow point-of-care MS devices with high specificity and sensitivity to be available in the near future.

Learning Objectives

After completing this article, the reader will be able to describe paper spray mass spectrometry and slug-flow microextraction as well as list their advantages over sample preparation techniques currently used in toxicology laboratories.

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The author has nothing to disclose.

Case Study
The Visine Prank Can Have Serious Consequences in Children

By He S. Yang, PhD

An 18-month-old male was brought to the emergency department at San Francisco General Hospital. He presented with diarrhea, vomiting, lethargy, and pinpoint pupils. His blood pressure was high (125/77 mmHg) and his heart rate low (70/min) for his age. Electrocardiography showed bradycardia with first degree heart block. Body temperature (36.5 °C), respiratory rate (25/min), and pulse oximetry saturation on room air (99%) were normal. Other initial laboratory results were unremarkable.

His urine drug screen, performed by immunoassay, was negative for opiates, oxycodone, amphetamine, barbiturates, benzodiazepines, cocaine, and methadone. In addition, tests for ethanol, acetaminophen, and aspirin were negative.

Given his presentation with pinpoint pupils and somnolence, the treating team suspected opioid intoxication. Despite the negative urine screen for the opiates oxycodone and methadone, the boy could have been exposed to other synthetic or semisynthetic opioids that are not detected by traditional immunoassays. However, he did not respond to the opioid antagonist naloxone.

Key Information

Further questioning of his parents revealed that a fight had occurred at his home between his father and maternal grandfather. His father had adulterated a half-gallon of milk with a bottle of Visine eye drops (about 30 mL of 0.05% tetrahydrozoline HCl) intended for his grandfather. Unfortunately, the child was given an unknown amount of the milk.

Urine, serum, and leftover milk samples were sent to the hospital toxicology laboratory for analysis. The urine, serum, and milk samples were treated by dilution, protein precipitation, and phospholipid removal, respectively.

The samples were analyzed by a liquid chromatography quadrupole time-of-flight mass spectrometry system (AB Sciex TripleTOF 5600) operating in full-scan positive ion mode for untargeted data acquisition. Full-scan data were collected over a range of masses (50–700 Da) and product ion spectra were collected for every ion that met a specified frequency threshold. The data were analyzed using targeted analysis in which the accurate mass, retention time, isotope pattern, and product ion spectrum for all compounds were compared with a database containing

around 170 compounds validated in our laboratory. This approach identified no compounds.

Because tetrahydrozoline was not in our compound database, we performed a suspect analysis based on its formula ($C_{13}H_{16}N_2$) and accurate mass (200.13135). That analysis detected a significant peak with the accurate mass and formula of tetrahydrozoline in all the samples associated with this case. On the following day, we purchased a reference standard of tetrahydrozoline and confirmed the findings. Subsequently, our laboratory developed and validated a quantitative method for tetrahydrozoline. Quantitative analysis revealed a tetrahydrozoline concentration in the child's urine sample of 2430 ng/mL, in the serum of 22 ng/mL, and in the milk of 2995 ng/mL.

Twenty hours after presentation, the child had a heart rate of 90/min and a blood pressure of 90/50 mmHg. His mental status was back to baseline. Other than supportive care, he received no additional treatment. Child protective services and the police department investigated the circumstances surrounding the child's exposure.

The Visine "Prank"

Although it has been known for decades that adding Visine eye drops to a drink can induce diarrhea, the prank was popularized by the 2005 comedy movie "Wedding Crashers." In the movie, the main character spiked a competitor's drink with a few drops of Visine, and induced sudden bouts of severe diarrhea. This scene and the urban legend that this use of Visine is a harmless prank are misleading because the real side effects can be dangerous, especially in children.

Oral ingestion of tetrahydrozoline can produce profound central nervous system depression ranging from drowsiness to coma (1). Nausea, vomiting, hypertension and rebound hypotension, and cardiovascular and hemodynamic effects are also common (2). These adverse affects can occur because tetrahydrozoline is an imidazoline derivative with alpha-2 adrenergic receptor agonist activity (3).

Laboratory Response

Clinicians should be aware of the potential for significant toxicity with the ingestion of a relatively small amount of tetrahydrozoline-containing eye drops.

Laboratory professionals should consider including tetrahydrozoline in their targeted general unknown drug screen. Alternatively, a method with untargeted data acquisition can allow for retrospective analysis of uncommon intoxicants such as tetrahydrozoline, as described in this case.

Learning Objectives

After completing this article, the reader will be able to describe the potential central nervous system and cardiovascular system toxicities of orally ingested tetrahydrozoline-containing eye drops.

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The author has nothing to disclose.

Liquid Chromatography-Mass Spectrometry Guide Released

By William Clarke, PhD, MBA, and James Ritchie, PhD

The Clinical and Laboratory Standards Institute (CLSI) recently released a consensus guidance document, "Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline (C62-A)" on quantitative liquid chromatography-mass spectrometry (LC-MS) method development and validation. This document covers quantitative methods, and doesn't include qualitative screening, proteomics, or any other research applications.

The target audience is broad, including both novice and experienced users. A secondary goal is to provide a resource for clinical laboratory accrediting organizations and regulatory agencies as MS use continues to increase.

The document builds on the informational document, "Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance; Approved Guideline (C50-A)," released in 2007 as a broad discussion of mass spectrometry in clinical laboratories. The new guidance begins with an introduction to LC and MS instruments commonly used in clinical laboratories, then focuses on best practices in the devel-

opment and validation of robust LC-MS methods. It discusses performance targets and monitoring tools along with other aspects of quality assurance specific to clinical LC-MS analyses.

Guideline C62-A provides guidance on the reduction of interlaboratory variance and the evaluation of interferences, assay performance, and other characteristics of clinical assays. It presents a standardized approach for method verification specific to MS. Sample pages and the table of contents can be viewed at http://shop.clsi.org/c.1253739/site/Sample_pdf/C62A_sample.pdf.

The document follows on the heels of another recently released guidance, "Mass Spectrometry for Androgen and Estrogen Measurements in Serum (C57-Ed1)" from the same organization. Both guides can be purchased from the CLSI website (http://shop.clsi.org/chemistry-documents/C62.html).

William Clarke, PhD, MBA, chaired the committee that developed the document and is associate professor of pathology and director of clinical toxicology and point-of-care testing at the Johns Hopkins University School of Medicine in Baltimore. Email: wclarke@jhmi.edu. James Ritchie, PhD, is with Emory University Hospital in Atlanta and is a member of the Clinical & Forensic Toxicology News *editorial board. Email: jritchi@emory.edu*

Disclosure: Dr. Clarke chaired the committee that developed the C62-A guidance document and has both research and consulting relationships with Thermo Fisher Scientific. Dr. Ritchie has nothing to disclose.

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