Original Article

A Review of Alcohol Biomarkers and their Significance in Aircraft Accident Investigation and Monitoring of Alcohol Dependence Syndrome

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Abstract

Toxicological examinations of fatal aviation accident investigations routinely include analysis of ethanol levels. The possibility of alcohol being produced in the body after death via microbial contamination and fermentation is an important confounding factor in such scenarios. There have been numerous studies highlighting the important role of this kind of alcohol production after death. Increased ethanol value in post mortem samples, therefore needs to be interpreted with caution and differentiated whether the ethanol estimated is of antemortem ingestion or postmortem microbial formation.

Several indicators of microbial postmortem ethanol formation exist; however, none are completely reliable. In contrast, recent studies on sensitive potential markers of alcohol ingestion (antemortem intake) have come up with candidate biomolecules. These biomolecules can be used to confirm alcohol intake in situations of aircraft accidents or monitoring of abstinence in ADS cases. Amongst the most studied biomarker is 5-HTOL/5-HIAA ratio (5-hydroxytryptophol/ 5-hydroxyindole-3-acetic acid). It is known that the consumption of ethanol alters the concentration of two major serotonin metabolites, 5-hydroxytryptophol (5- HTOL) and 5-hydroxyindole-3-acetic acid (5-HlAA). In normal circumstances, the 5-HTOL/5-HIAA ratio is very low; however the ratio is significantly elevated upto 11-19 hours after ingestion of small quantities of ethanol. Another marker of great promise is Ethyl glucuronide (EtG), which remains elevated for several days after alcohol intake and can be detected in biological specimens like blood, urine and hair. There are other biomarkers which are also studied for their application in differentiating postmortem alcohol production.

In this review all the biomarkers and their potential applications in aviation toxicology as well as monitoring of abstinence in Alcohol Dependence Syndrome casesis discussed

Key Words: ADS, Biomarkers, Aviation Toxicology

Introduction

Ethanol analysis, most commonly accomplished by headspace gas chromatography (GC), is one of the most common and routine tests performed in forensic and aircraft accident specimens. The presence of ethanol in fatal aircraft accident victims constitutes an important part of aircraft accident investigations and its related litigation. With the precision of today's analytical techniques, there is a high degree of certainty associated with the quantitative determination of ethanol found in a biological specimen. However, whether the ethanol found in a specimen is derived from postmortem microbial formation or antemortem ethanol consumption is an important variable to consider when interpreting the quantitative ethanol results. The microbial formation of ethanol in postmortem specimens is by far the most likely complication encountered when examining ethanol results. There have been numerous studies highlighting the important role of alcohol production after death. Increased ethanol levels have been found in postmortem samples known to involve no prior antemortem ingestion. These studies indicate that simple postmortem ethanol levels cannot be used as proof of antemortem ingestion. Today, it is known that many different microbes are responsible for postmortem formation of ethanol in animals [1]. Investigations have been performed to identify the particular species of bacteria, yeast and/or fungi responsible for ethanol production and the mechanism by which it is formed [2–4]. *Candida albicans* has been the microbe most often ascribed to be responsible for postmortem production of ethanol in humans [5]. This species of yeast is commonly found in humans in vivo [9]. Located ubiquitously throughout the body, the concentrations of *C. albicans* are typically found in the mouth and on the skin [6]. Glucose is the most prevalent substrate in the human body used by these microbes to form ethanol [7]. Other endogenous compounds can also be utilized as substrates including, but not limited to, lactate, mannitol, galactose, maltose, sucrose and lactose [7–9]. However, it should be noted that approximately 100 species of bacteria, yeast and fungi have been shown capable of producing postmortem ethanol [2]. Postmortem ethanol formation is suppressed by storage at −20 ^{0}C and the addition of a

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preservative such as sodium fluoride [10]. These stabilization precautions, however, obviously do not eliminate ethanol formation prior to sample collection and preservation [11].

In Indian scenario, the Institute of Aerospace Medicine is the nodal centre authorized for investigation of all aircraft accidents both military and civil. Dept of Aviation Pathology & Toxicology has been carrying out autopsies and postmortem investigations on all aircraft accidents. At present, headspace gas chromatography (GC) available at this lab can estimate only total ethanol in post mortem samples. Being the only centre for toxicology workup for aircrash investigation in India, it is imperative that evaluation of various markers of ethanol consumption be undertaken so as to differentiate it from postmortem production in aircrashes where this scenario is common. These markers will be of further use in monitoring abstinence in Alcohol Dependence Syndrome cases in armed forces patients.

There are several biomarkers which are studied for their application in detection of acute and chronic alcoholism. This paper will discuss all the biomarkers and their potential applications in aviation toxicology as well as monitoring of abstinence in Alcohol Dependence Syndrome cases.

Biomarkers of alcohol and their significance

Serotonin metabolites

The metabolism of serotonin end products as a biological marker for ethanol consumption has begun to gain interest in the field of forensic science [12]. Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine commonly found in nature. In humans, 5-HT is found throughout the body, with substantial concentrations found in the gastrointestinal tract and blood platelets [13]. The metabolism of 5-HT initially involves oxidative deamination to form the intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate can undergo either oxidation or reduction. Oxidation of the aldehyde, catalyzed by aldehyde dehydrogenase, leads to formation of 5-hydroxyindole-3-acetic acid (5-HIAA), the predominant metabolite of 5-HT [13]. Reduction, catalyzed by aldehyde reductase, leads to formation of 5-hydroxytryptophol (5-HTOL), a relatively minor metabolite of 5-HT [12]. Ethanol consumption has been shown to result in a significantly enhanced production of 5-HTOL and to reduce the formation of 5-HIAA [12,14 15]. Therefore the 5-HTOL/5-HIAA ratio has been investigated as a marker for recent alcohol ingestion [16]. The increase in the urinary 5-HTOL levels remains elevated for several hours after ethanol itself is no longer measurable [12]. Based on the prolonged detection window for 5-HTOL compared with ethanol, determination of 5-HTOL in samples of urine has been used in different clinical and forensic settings as a sensitive biomarker for recent alcohol consumption [15].

Ethyl glucuronide (EtG)

Ethyl glucuronide is a nonoxidative minor metabolite of ethanol formed by glucuronidation, catalyzed by UDPglucuronosyltransferase (17). Numerous studies have indicated that the presence of EtG in a urine sample is a specific and sensitive indicator of recent alcohol ingestion, with a detection time spanning up to several days (18-23). Analysis of EtG furnishes a more sensitive way to monitor recent drinking because of its longer elimination half-life compared with ethanol itself [24]. Trace quantities of EtG are produced during enzymatic metabolism of ethanol (<0.1% of dose) but more importantly, EtG is not produced by the action of microbes and yeasts on glucose. Therefore if ethanol was only produced in the body after death, one would not expect to find any measurable quantities of EtG in the samples analyzed.

EtG is a stable marker in hair that can be used to detect and quantify alcohol consumption over long time periods. This alcohol metabolite remains in hair after complete elimination of alcohol [25]. EtG can be detected in the blood for up to 36 hours and in the urine for up to 5 days after heavy alcohol use. In addition to blood and urine, EtG is detectable in other body fluids, hair, and body tissues[26]. EtG in hair can be detected up to a maximum period of 10-12 weeks. Currently, there are three main analytical techniques which can be used to develop methods for quantifying EtG in hair: gas chromatography-mass spectrometry (GC-MS), gas chromatography-tandem mass spectrometry (GC-MS/MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS). No standardized protocols are yet available for the analysis of EtG levels in hair samples, and the current protocols vary in sample preparation and extraction procedures. Variables such as hair length, cosmetic treatment, gender, and pathophysiological conditions influence the final results and should be taken into account [27].

EtG in hair is sensitive to cosmetic treatment. e.g. bleached and dyed hair samples may lead to false negative EtG results. EtG was reported to be formed locally in very small and highly variable amounts[28].

Ethyl sulfate(EtS)

Another nonoxidative minorethanol metabolite, ethyl sulfate (EtS) [29],is formed by sulfate conjugation through the action of cytosolic sulfotransferase. EtS seems to have similar potential to EtG as a relapse marker [30,31].

Fatty Acid Ethyl Esters (FAEE)

The FAEEs are esterification products of ethanol and fatty acids. FAEE concentrations might be useful both as short-term markers in serum and as long-term

markers in hair. In serum, FAEEs have been reported to be detectable after ethanol consumption up to 24 hr (Doyle et al., 1994) and in heavy drinkers even for 44 hr. FAEE is a sensitive and specific marker for distinguishing social drinkers from heavy or alcohol-dependent drinkers. Further study is required to fully explore FAEE's sensitivity and specificity[26].

Other Biomarkers

Carbohydrate-Deficient Transferrin (CDT) is a version of the glycoprotein transferrin, a molecule responsible for carrying iron within the bloodstream. CDT, as its name implies, is a form of this molecule that is deficient in the carbohydrate sialic acid. Normally, txaneflure contains four to six sialic acid molecules, but research indicates that drinking disrupts sialic acid's ability to attach to transferrin as well as other molecules. Many versions of transferrin normally are found in healthy people, but studies indicate that heavy drinkers have higher amounts of the CDT version than nondrinkers.

CDT has been widely used by clinicians in recent years to screen for heavy alcohol consumption. Although it appears to be a highly specific measure of alcohol consumption, showing low rates of false positives, CDT is difficult to measure accurately. Distinguishing CDT from other forms of transferrin is challenging. Despite the disadvantages of the CDT marker, it remains a very well-characterized biomarker for heavy alcohol intake

Gamma-Glutamyltransferase (GGT). This glycoprotein—a large molecule made up of both proteins and carbohydrates—aids in digestion and is found in key liver cells (or hepatocytes) and in other cells involved in bile production, including biliary epithelial cells. Elevated GGT levels are an early indicator of liver disease; chronic heavy drinkers, especially those who also take certain other drugs, often have increased GGT levels. However, GGT is not a very sensitive marker, showing up in only 30–50 percent of excessive drinkers in the general population (Conigrave et al. 2003). Nor is it a specific marker of chronic heavy alcohol use, because other digestive diseases, such as pancreatitis and prostate disease, also can raise GGT levels.

Mean Corpuscular Volume (MCV), a person's volume of red blood cells, also is associated with heavy chronic drinking, as the MCV in heavy drinkers tends to exceed the average range. This marker is less useful clinically, however, because the MCV stays high for several months after a person stops drinking, so someone could be abstinent but still show a high MCV value. In addition, other conditions may affect MCV, reducing its specificity and further confounding any interpretation of results using this marker[26]. A summary of ethanol and their biomarkers are tabulated as table I.

Discussion

The scientific literature was searched for articles dealing with postmortem aspects of ethanol and problems associated with making a correct interpretation of the results. A person's blood-alcohol concentration (BAC) and state of inebriation at the time of death is not always easy to establish owing to various postmortem artifacts. The possibility of alcohol being produced in the body after death, e.g. via microbial contamination and fermentation is a recurring issue in routine casework. If ethanol remains unabsorbed in the stomach at the time of death, this raises the possibility of continued local diffusion into surrounding tissues and central blood circulation after death.

The problem of alcohol use by pilots is a highly contentious matter when aviation disasters are investigated and this issue continues to attract a lot of attention. During 1989–1990 the US Civil Aeromedical Institute received specimens from 975 victims of fatal aircraft crashes and 79 of these were positive for ethanol (>40 mg/100 mL) [32]. Based on the distribution of ethanol in urine, vitreous, blood and tissue it was determined that 21 of the positive cases could be attributed to postmortem synthesis of ethanol whereas 22 reflected drinking alcohol and 36 could not be interpreted in a satisfactory way. In two cases the production of ethanol postmortem reached as much as 150 mg/100 mL [32]. Similar findings were reported in a more recent compilation involving 1587 civil aviation pilot fatalities 1999–2003 [33].

Aviation fatalities are extremely difficult to deal with when it comes to recovery of bodies and obtaining the best possible samples for toxicological analysis [34]. The postmortem examination and the analytical toxicology are complicated owing to extensive trauma in victims of plane crashes including rupturing of the stomach and bursting of the bladder [35].Obtaining biological specimens for toxicological analysis after an aircraft disaster presents a great challenge and findings of positive BAC needs to be interpreted with caution because of the heightened risk of postmortem synthesis.

The use of biochemical markers to distinguish ingestion of ethanol and hepatic metabolism from microbial synthesis is urgently needed when deaths caused by blunt trauma or burns are investigated.

Recent research has focused on developing various biochemical tests or markers of postmortem synthesis of ethanol. These include the urinary metabolites of serotonin and non-oxidative metabolites of ethanol, such as ethyl glucuronide, phosphatidyl ethanol and fatty acid ethyl esters.

The 5-HTOL/5-HIAA ratio has been extensively investigated and used as potential indicator of ethanol origin in postmortem urine samples at Civil Aerospace Medical Institute, FAA, USA CAMI, developed and validated a method for the simultaneous determination

of 5-HTOL and 5-HIAA in forensic urine samples using a simple liquid/liquidextraction and LC/MS/MS and LC/MS/MS/MS [36].

Ethyl glucuronide in urine is extensively investigated and found to be sensitive marker to monitor recent drinking because of its longer elimination half-life compared with ethanol. EtG in urine is currently used as an abstinence monitoring tool in clinical and criminal justice settings. Several studies have also examined EtG in hair and concluded that EtG in hair could be used to qualitatively indicate any alcohol use. The study by G Skopp et al [28] found that a negative result for EtG in hair may not indicate that the person abstained from alcoholic beverages. However positive results were always associated with alcohol consumption. The lack of correlation between the detection of EtG in hair and drinking behavior suggests that EtG might be formed locally. The analysis of EtG and EtS together increases the sensitivity of the investigation because it is reported that EtG, but not EtS, can be produced after sampling if samples are infected with E. coli and contain ethanol formed by fermentation, for example, in samples from diabetics [27,28]. Thus, we recommend analysing EtG and EtS together to minimize false identification of alcohol consumption and possibly to identify alteration of metabolism of alcohol as described below. The use of FAEEs as a marker of ethanol intake was suggested by Doyle et al. (1996)[37], who determined FAEE levels after ethanol consumption and found detectable FAEE concentrations until 24 hr. The study by Borucki et al [38] 2005 revealed the investigation of FAEE levels over times longer than 24 hr in healthy individuals. It is evident that this marker has two weaknesses. First, the initially high FAEE concentrations decrease rapidly over 24 hr. Second, after 24 hr, they do not decline to

Table I: Ethanol & its Biomarkers

Sl No	Biomarker	Sample	Detection Window	Remarks
1	Ethanol	Blood	5-6 hrs	Useful for acute intoxication as levels indicative of consumption
		Urine	5-6 hrs	Unreliable for quantification as bacterial interference may be present
2	Gamma GlutamylTransferase (GGT)	Blood	No direct relation to time of alc intake, tends to rise within 24 hrs after a binge of heavy drinking	Elevated levels are an indication of Liver damage used for monitoring ALD patients/ Chronic alcohol abuse
3	AST, ALT	Blood	No direct relation to time of alc intake tends to rise slowly due to cellular damage in liver.	Elevated levels are an indication of Liver damage used for monitoring ALD patients/ Chronic alcohol abuse
4	Carbohydrate Deficient Transferrin (CDT)	Blood	2-3 wks	Marker of regular heavy (> 60 gms/day) intake of alcohol for more than 2 wks
5	MCV	Blood	Changes after Long term abuse (months to yrs)	Indicative of long term abuse
6	Ethyl Glucuronide (EtG)	Blood	5- 6 hrs	A highly specific and recent marker of alcohol abuse
		Urine	5-7 days	Protocol development has been achieved in few labs worldwide, can be used for clinical evaluation.
		Hair	10 wks	No clear data exists on correlation between consumption and hair conc of EtG.
7	The ratio of 5 Hydroxy Tryptophol (5HTOL) to 5 Hydroxy Indole Acetic Acid (5HIAA)	Urine	4-48 hrs	A new and highly specific marker for ante mortem alcohol intake. The ratio is very useful in forensic toxicology to differentiate post-mortem production
8	Fatty Acid Ethyl Esters (FAEE)	Blood	6-24 hrs	Marker of recent heavy alcohol intake
9	Ethyl sufate	Blood	6-24 hrs	Marker of recent heavy alcohol intake

zero or to the LoQ but remain stable in a low range with no further decrease even when the individuals have been abstinent for up to 4 days.

The other biomarkers like GTT, MCV, AST& ALT are not directly related to ethanol consumption and they are specific markers of ethanol consumption**.** Elevated levels are an indication of long term abuse and Liver damage used for monitoring ALD patients/ Chronic alcohol abuse. CDT is a good marker for chronic alcohol abuse in routine use. Many studies on CDT examined the difference in serum levels between heavily dependent alcoholics and teetotalers or social drinkers.

Conclusion

The ratio of 5HTOL to 5HIAA in urine and increased levels of EtG in urine are very specific indicators of acute alcohol consumption can used for differentiating postmortem production in aircraft accident investigation and forensic toxicology setup. The combined analysis of a panel of markers like EtG along with Ets and other biomarkers like FAEEs , CDT are more useful in monitoring abstinence in Alcohol Dependence Syndrome cases

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