COMPARISON OF SERUM FATTY ACID ETHYL ESTERS AND URINARY 5-HYDROXYTRYPTOPHOL AS BIOCHEMICAL MARKERS OF RECENT ETHANOL CONSUMPTION

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(Received 12 November 2004; first review notified 1 January 2005; in revised form 26 January 2005; accepted 9 March 2005; Advance Access publication 29 March 2005)

Abstract — Aims: To examine the effects of an acute dose of ethanol on serum fatty acid ethyl esters (FAEEs) concentration and urinary 5-hydroxytryptophol (5-HTOL)/5-hydroxyindole-3-acetic acid (5-HIAA) ratio. Methods: Sixteen (14 male, 2 female) heavy alcohol drinkers were tested in a single, 2-day long session. Six participants received 1.5 g/l of ethanol/l of body water (~0.75 g/kg of body weight, low dose group; LD) and 10 participants received 2.0 g/l of ethanol (~1.0 g/kg of body weight, high dose group; HD) in four divided doses every 20 min. Blood, urine, and breath samples were collected repeatedly over 36 h following the ingestion of ethanol and were analyzed for the presence of FAEE, 5-HTOL/5-HIAA, and ethanol, respectively. Serum γ-glutamyltransferase (GGT), a marker of chronic ethanol use, was also included. Results: The breath ethanol level peaked ~1 h after the last dose, at 95 and 120 mg/dl for the LD and HD groups, respectively. The mean ratio of urinary 5-HTOL/5-HIAA was significantly elevated 5 and 9 h after ethanol administration, but returned to baseline 13 h after ethanol administration. This ratio was twice as high for the HD group compared with the LD group. Serum levels of FAEEs were significantly elevated at 5 h, but not 13 h after ethanol administration. There were no time-dependent changes in serum GGT levels. Conclusions: Measuring the levels of FAEE and 5-HTOL/5-HIAA ratio provides a convenient method to detect recent, particularly binge-type, ethanol use, but these measures may have limited applicability in detecting ethanol use in traditional clinical trial settings.

INTRODUCTION

Objective measurements of recent drug use have been an invaluable tool in treatment programs, as well as in clinical trials of new treatment interventions where they are used as key outcome variables. Reliable and feasible biochemical methods exist for quantitating the majority of commonly abused drugs, with the exception of ethanol. The need for reliable and persistent biochemical markers of ethanol consumption has been widely recognized (Allen et al., 2001; Allen and Litten, 2001; Conigrave et al., 2002; Flannery et al., 2002; Hoyer et al., 2002).

Assessment of the presence of ethanol in the breath or body fluids has been found to be limiting in treatment trials because of the very narrow window through which this marker remains positive. Several readily available biochemical markers indirectly reflect the chronic effect of excessive amounts of ethanol on several systems, such as red blood cells and liver. Elevation of liver enzymes levels, such as γ-glutamyltransferase (GGT) and aspartate aminotransferase, reflect a prolonged use of ethanol and are used in the medical setting to screen for alcohol-related problems. Similarly, carbohydrate-deficient transferrin (CDT), a liver protein that is synthesized during heavy ethanol consumption, can be used to detect relapse in abstinent individuals, but is not sensitive enough to detect the level of ongoing use (Allen and Litten, 2003). The time frame for an effect to be observed by these markers is usually many days or weeks, and little if any changes in these parameters are seen in response to brief episodes of drinking, which is most relevant in clinical trials. Ideally, biochemical markers that would be the most useful measures of drinking behavior in clinical trials would permit the detection of a drinking episode in the previous 2–7 days, as most clinical trials involve weekly or twice weekly assessments. Self-reports of ethanol intake currently remain the primary way to assess the extent of day-to-day ethanol use patterns, despite the fact that their accuracy has been questioned for many years (Hoyer et al., 1995; Midanik, 1988).

The ideal biochemical marker that is sensitive to small changes in ethanol use would be a direct ethanol metabolite or a product of ethanol catabolism. Two biochemical markers of recent ethanol consumption have been proposed as useful in assessing ethanol intake, particularly in the setting of a clinical trial. One is 5-hydroxytryptophol (5-HTOL), a metabolite of serotonin that is formed at a higher rate in the presence of ethanol (Davis et al., 1967). Elevation of urinary 5-HTOL is proportional to the amount of ingested ethanol and persists after ethanol has been removed from the circulation and after breath and blood ethanol levels have returned to baseline levels (Beck et al., 1982; Voltaire et al., 1992; Bendtsen et al., 1998; Helder et al., 1999; Jones and Helder, 1999). Another biochemical marker is serum fatty acid ethyl ester (FAEE), a product of non-oxidative metabolism of ethanol (Laposata and Lange, 1986; Laposata, 1997). Serum FAEE concentrations rapidly increase after administration of ethanol, but persist in the serum after ethanol can no longer be detected (Doyle et al., 1996).

The purpose of this study was to compare the 36 h time-course of urinary 5-HTOL and serum FAEE levels after intoxicating doses of ethanol in heavy ethanol drinkers who were not alcohol dependent.

MATERIALS AND METHODS

Participants and procedures

Paid research participants were recruited through advertisements in newspapers and had a detailed medical and
psychiatric evaluation before study enrollment. Laboratory testing included blood and urine analysis, urine drug toxicology (for opioids, cocaine, benzodiazepines, cannabinoids and amphetamines) and an electrocardiogram. Participants were excluded from the study if they had any current physical disorder, were taking any prescription medication, had an Axis I psychiatric disorder other than nicotine dependence, or were pregnant.

In order to be eligible for the study, participants were required to be regular drinkers of alcoholic beverages and consume a minimum average of 10 and a maximum average of 40 standard drinks/week in the 1-month period before evaluation. The amount of alcohol consumption was assessed by three independent interviewers, on three separate occasions and averaged. Participants were not eligible if they met the criteria for alcohol use disorders or were seeking treatment for alcohol-related problems. Participants signed a consent form describing the aims of the study, the potential risks and the benefits of participation. This study was approved by the Institutional Review Board of the New York State Psychiatric Institute.

Sixteen participants (14 males and 2 females) completed this study. Demographic characteristics of participants were as follows: mean age 28.9 years (range 22–44 years), 58% Black, 22% White and 20% Latino. Each participant drank 24.3 drinks/week (range 12–37.5) on an average.

Participants stayed in the laboratory overnight before the administration of ethanol, and abstinence on arrival was verified using a breathalyzer. The next morning, the participants received their breakfast of choice, which varied in fat content. Then, an indwelling catheter was inserted into their forearm, and initial blood samples were collected for baseline measurements of FAEE, GGT and ethanol. One hour after breakfast, ethanol was administered in four equal doses, spaced 20 min apart. Each dose consisted of 150 ml of beverage and was consumed over the course of 30 s. Relative to the beginning of beverage consumption, blood samples for FAEE and GGT were collected from the catheter at 5, 9, 13 and 17 h, and then every 2 h up to 36 h after initiation of ethanol intake. Blood samples for ethanol determination were collected every hour for 24 h. In all cases, blood was collected into vacuum tubes without an anticoagulant. The volume of sample collected for each marker was 2 ml, and after each collection, the catheter was flushed with saline. Serum was separated and frozen at −80°C before the dry-ice shipment for FAEE and ethanol quantitation. Urine samples for 5-HTOL/5-HIAA determination were collected at baseline and at 5, 9, 13, 15 and 17 h, and then every 2 h up to 36 h. The volume of urine voided was recorded and 30 ml of each sample was sent for analysis. Breath samples for ethanol determination were collected at baseline and every hour for 24 h after ethanol administration. Alco-Sensor III Breath Testing Device (Intoximeters, St Louis, MO) was checked for calibration weekly and recalibrated when necessary.

Absolut® vodka (100 proof) was added to sufficient quantities of a reduced-calorie cranberry juice, and reduced-calorie tonic water to produce 150-ml beverages. The ethanol dose was calculated based on the estimated total body water (TBW; g ethanol/l of body water) of each participant to eliminate differences in ethanol pharmacokinetics between sexes (Watson et al., 1980). Ethanol was given in four doses, spaced 20 min apart. Two doses of ethanol were administered. The low dose (LD) group (n = 6) received 1.5 g of ethanol/l of body water (~0.75 g/kg of body weight or 3–4 drinks). The high dose (HD) group (n = 10) received 2 g of ethanol/l of body water (~1.0 g/kg of body weight or 5–6 drinks).

Biochemical markers analyses

The concentrations of 5-HTOL and 5-HIAA were determined by gas chromatography–mass spectrometry (GC–MS) at the Nathan Kline Institute for Psychiatric Research (Orangeburg, NY). 5-HTOL, HIAA and β-glucuronidase were purchased from Sigma (St Louis, MO). The internal standards, 5-HIAA-d2 was obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and 5-HTOL-d4 was synthesized in our laboratory by the method of Hessellgren and Beck (1979). Pentafluoropropionic anhydride (PFPA) was from Supelco (Bellefonte, PA) and trifluoroethanol was from Aldrich (Milwaukee, WI). All other chemicals used were reagent grade and purchased from Fisher Chemical (Springfield, NJ). The urine sample (1 ml) was combined with 5-HTOL-d4, 0.2 M phosphate buffer (pH 6.0) and β-glucuronidase. The solution was incubated at 37°C followed by an acidic precipitation with sulfosalicylic acid. The supernatant was transferred, and 1.0 M buffer (pH 10.5) and methyl t-butyl ether were added. After centrifugation, the organic phase was transferred to a 13 × 100 mm tube and evaporated to dryness through a vacuum centrifuge. The residual was derivatized with PFPA and the derivatizing mixture was dried using a vacuum centrifuge. The residue was re-dissolved in ethyl acetate and 2 µl was injected into the GC–MS system for the 5-HTOL assays. A second urine sample (0.25 ml) was combined with 5-HIAA-d2, 0.2 M phosphate buffer (pH 6.0) and methyl t-butyl ether. The organic phase was transferred to a 13 × 100 mm tube and evaporated to dryness through a vacuum centrifuge. The residual was derivatized with PFPA and trifluoroethanol and the derivatizing mixture was dried using a vacuum centrifuge. The residue was re-dissolved in ethyl acetate and 2 µl was injected into the GC–MS system for the 5-HIAA assays. The calibration standards and quality controls were run with each day’s analysis. The limit of quantification was 2.5 ng/ml for 5-HTOL and 0.1 µg/ml for 5-HIAA. The interassay precision of the method was determined by testing blank samples containing three different concentration levels of 5-HTOL and 5-HIAA on 6 separate days. The relative standard deviation (RSD%) were 3.5, 3.2 and 3.1% for 5-HTOL and 2.5, 3.8 and 3.6% for 5-HIAA, respectively.

FAEE and ethanol measurements were performed at the Division of Laboratory Medicine, Massachusetts General Hospital (Boston, MA) using GC–MS methods described previously (Best et al., 2003). Freshly thawed serum (0.5 ml) was added to a 1 nmol internal standard of ethyl heptadecanoate (E17:0). FAEE were extracted using acetone/hexane (2:8 v/v), and isolated using Bond-Elut Aminopropyl columns.

Serum ethanol concentration was determined by gas chromatography. Serum samples were mixed with an internal standard of 1-propanol, and a 1 µl sample was injected into a HP 5890 GC (Hewlett-Packard, Palo Alto, CA) containing a 5% Carbowax 20M 60/80 Carbopack B column (Supelco, Bellefonte, PA). The ethanol peak was identified and quantitated by comparison with 1-propanol as a known standard.
Breath ethanol concentration was measured using a breathalyzer (Alcosensor III Intoximeter, St Louis, MO). Serum GGT level was analyzed at a commercial laboratory (Quest Diagnostics, Teterboro NJ) using a standard method.

Results are presented as means ± SEM. For statistical analysis, a one-way repeated measures analysis of variance (ANOVA) was used. Significant interaction effects were examined using Newman–Keuls multiple comparisons procedure. Between groups comparisons were conducted using a t-test. Results were considered statistically significant at \( P < 0.05 \).

**RESULTS**

Breath ethanol concentrations peaked at \(~2\) h after the onset of drinking, at 95 and 120 mg/dl for the LD and HD groups, respectively. Breath ethanol concentrations were no longer detectable in the majority of participants at 8 and 10 h after the onset of drinking in the LD and HD groups, respectively. Serum ethanol concentrations showed peak concentrations of 110 and 163 mg/dl for the LD and HD groups, respectively. Mean ratio of urinary 5-HTOL/5-HIAA reached a maximum level at the first sampling time point, 5 h after the onset of drinking, and remained significantly elevated compared with baseline levels at 9 h in both groups \((P < 0.001)\), but not at 13 h or any time afterwards. This ratio was significantly higher in the HD group compared with the LD group \((P < 0.01)\) at 5 h.

We obtained a full time-course of FAEE concentrations for one participant in the LD group. Subsequently, we directly measured the FAEE levels only at 5 and 13 h for the remaining participants. FAEE concentrations were significantly elevated at 5 h \((P < 0.001)\), but not at 13 h after ethanol administration in both groups. The mean concentration of FAEE was 55% higher in the HD group compared with the LD group at 5 h.

Figure 1 shows FAEE concentration along with the 5-HTOL/5-HIAA ratio and breath ethanol concentration at comparable time points.

To evaluate the role of pre-study level of ethanol consumption on FAEE concentrations, a median split was performed based on the number of drinks consumed/week for the males, then FAEE concentrations at 13 h were compared between the two groups. Among the males who drank 28–37 drinks/week, 29% had detectable levels of FAEE, whereas in the males who reported drinking 12–24 drinks/week, 100% had detectable levels of FAEEs.

There were no time-related change in serum GGT levels (range 19.5–23.1 U/l).

**DISCUSSION**

Urinary 5-HTOL/5-HIAA ratio and serum FAEE concentrations increased in a dose-dependent fashion following the acute administration of intoxicating doses of ethanol. Breath ethanol concentrations, urinary 5-HTOL/5-HIAA and serum FAEE concentrations were no longer significantly elevated 13 h after ethanol administration. The levels GGT were not affected by the acute administration of ethanol.

The fact that elevated levels of FAEE were at trace or nondetectable levels in the majority of participants 13 h after ethanol administration is in contrast to earlier studies, which assessed this marker and found it detectable 24 h after ethanol administration (Doyle et al., 1996; Soderberg et al., 1999; Best et al., 2003). Results from these studies are shown in Table 1. Maximum serum FAEE concentrations were higher in the present study, but eliminated faster compared with previous studies. There are several differences between the present and previous studies that might explain the observed discrepancies in these findings. First, participants in our study...
were heavier drinkers than the participants in previous studies. In addition, participants in previous studies were asked to refrain from drinking for 5 days before the study compared with only overnight abstinence in the present study. It is therefore possible that the observed differences in FAEE elimination were related to the increased activity of enzymes involved in the degradation of ethanol, as well as increased degradation of FAEE in participants who consumed higher amounts of ethanol. Since FAEEs are generally toxic (Szczechpiorkowski et al., 1995; Werner et al., 2002), the increased activity of enzymes degrading FAEE in heavy drinkers may be an adaptation to decrease ethanol-mediated toxicity. Indeed, in the present study, detectable levels of FAEEs were more likely to be found among participants who were drinking lower levels of ethanol. Gorski et al. (1996) demonstrated that there is reduced fatty acid ethyl ester synthase activity in the white blood cells of alcoholics, which may partially explain this finding. Similar changes in enzymatic activity have been previously noted in heavy drinkers, most notably decreases in the gastric ethanol dehydrogenase activity (Parlesak et al., 2002), but also changes in the metabolism of lipids (for review see Bunout, 1999; Lieber, 2000). In addition, the present study included mostly men, who are known not only to degrade ethanol faster that women (Lieber, 2000), but to have lower FAEEs (by ~50%) than women (Soderberg et al., 1999). Another difference is that participants in the present study might have had higher serum levels of lipids before the ingestion of ethanol since they ingested breakfast with unrestricted fat content closer to the time of ethanol ingestion than in previous studies. It is known that free fatty acid levels rise during fasting and that free fatty acids may be an important pool of fatty acids for FAEE synthesis (Dan et al., 1998). Therefore, studies that restrict fat intake may be more likely to have detectable FAEE levels in the serum at 24 h.

In the present study, the 5-HTOL/5-HIAA ratio was elevated at 9 h but when tested at 13 h after ethanol administration the obtained values were not different from baseline. The detection of elevated 5-HTOL/5-HIAA ratio depended on the dose of ethanol, with higher doses of alcohol producing higher values. The duration of this elevation is somewhat shorter compared with previous studies, which found an elevated ratio at 12 h post-dose (Beck et al., 1982; Voltaire et al., 1992), with the ratio returning to baseline values 14–16 h after ethanol administration (Helander et al., 1996). Different time points of urine collection between studies prevent a direct comparison of findings, but the ranges are broadly comparable.

This study confirms that measuring urinary 5-HTOL/5-HIAA and serum FAEE levels provides a method to detect recent, particularly binge-type, ethanol use. The time frame for detection of elevated values of these markers may be a few hours longer than detection using breath ethanol concentration but is generally in the same range, i.e. 6–12 hours following the binge. The length of time over which the elevation of these biochemical markers can be detected depends on the dose of consumed alcohol. Doses consumed by alcohol-dependent individuals are usually higher than doses used in human laboratory studies (equivalent of 3–6 drinks in the present study); therefore, it is possible that these markers can be detected over longer time periods in the clinical setting. In summary, these markers may be useful in the setting of an outpatient treatment program where patients are seen on a daily basis. Tests on samples collected early in the morning may reveal the elevation of biochemical markers when alcohol would no longer be detectable in the breath sample. However, these measures offer little advantage over the traditionally used breath ethanol assessment in the setting of a clinical trial where the marker indicating use in the past 2–7 days would be most desirable.

Acknowledgements — We would like to thank Joanne E. Cluette-Brown for performance of the assays for FAEE, Catherine A. Best for obtaining measurement of serum ethanol, David Pierce for contribution to the original study design, Charise Freundlich and Jordyn Conrad for their assistance in conducting the study. This work was supported by grants RO1 AA12599 (Dr Evans) and K23 DA00429 (Dr Bisaga).

REFERENCES

Table 1. Comparison of FAEE quantitation in published studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Study population: amount of ethanol consumption (drinks/week) Mean (range)</th>
<th>Peak ethanol concentration (mg/dl)</th>
<th>FAEE concentration (pmol/ml) 5 h after the initiation of ethanol ingestion</th>
<th>FAEE at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doyle et al. (1996)</td>
<td>9 (2–27)</td>
<td>120–145</td>
<td>1100–1200</td>
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<tr>
<td>Soderberg et al. (1999)</td>
<td>Not reported</td>
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<td>750</td>
<td>Yes</td>
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<tr>
<td>Best et al. (2003)</td>
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<td>95</td>
<td>1500</td>
<td>Yes</td>
</tr>
<tr>
<td>Present study</td>
<td>24 (12–37)</td>
<td>110¹b</td>
<td>3290¹b</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163¹b</td>
<td>5113¹b</td>
<td></td>
</tr>
</tbody>
</table>

¹LD group, ²HD group.


