Preanalytical Variables Affecting the Quantification of Fatty Acid Ethyl Esters in Plasma and Serum Samples

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**Background:** Fatty acid ethyl esters (FAEEs) are cytotoxic nonoxidative ethanol metabolites produced by esterification of fatty acids and ethanol. FAEEs are detectable in blood up to 24 h after ethanol consumption. The objective of this study was to assess the impact of gender, serum or plasma triglyceride concentration, time and temperature of specimen storage, type of alcoholic beverage ingested, and the rate of ethanol consumption on FAEE concentrations in plasma or serum.

**Methods:** For some studies, subject were recruited volunteers; in others, residual blood samples after ethanol quantification were used. FAEEs were isolated by solid-phase extraction and quantified by gas chromatography–mass spectrometry. FAEE concentrations were twofold greater for men than for women (P = 0.05). Accounting for triglycerides improved the correlation between blood ethanol concentrations and FAEE concentrations for both men (from r = 0.640 to r = 0.874) and women (from r = 0.619 to r = 0.673). FAEE concentrations did not change when samples were stored at or below 4 °C, but doubled when stored at room temperature for 24 h. The type of alcoholic beverage and rate of consumption did not affect FAEE concentrations.

**Results:** For weight-adjusted amounts of ethanol intake, FAEE concentrations were twofold greater for men than women (P = 0.05). Accounting for triglycerides improved the correlation between blood ethanol concentrations and FAEE concentrations for both men (from r = 0.640 to r = 0.874) and women (from r = 0.619 to r = 0.673). FAEE concentrations did not change when samples were stored at or below 4 °C, but doubled when stored at room temperature for 24 h. The type of alcoholic beverage and rate of consumption did not affect FAEE concentrations.

**Conclusion:** These studies advance plasma and serum FAEE measurements closer to implementation as a clinical test for ethanol intake.

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Fatty acid ethyl esters (FAEEs) are nonoxidative ethanol metabolites that are esterification products of ethanol and fatty acids. FAEEs have been implicated as mediators of ethanol-induced cell injury. Previous studies have shown both in vitro (1, 2) and in vivo (3, 4) that FAEEs are cytotoxic. Importantly, however, FAEEs are detectable in the blood for at least 24 h after ethanol intake in individuals who have consumed ethanol to intoxication. A major disadvantage of blood ethanol as a marker of ethanol intake is the limited period for its detection. The mean rate of decline of blood ethanol is, on average, 3.3–3.9 mmol·L⁻¹·h⁻¹ (15–18 mg·dL⁻¹·h⁻¹), with wide inter-individual variability [range, 2.2–4.3 mmol·L⁻¹·h⁻¹ (10–25 mg·dL⁻¹·h⁻¹)] (5). Therefore, the blood ethanol is usually undetectable within 6 h after ethanol intake is stopped in individuals who achieve a peak blood ethanol of 21.7 mmol/L (100 mg/dL). Thus, there is substantial value in FAEEs as longer-term markers of ethanol intake.

The present study analyzes how gender, plasma or serum triglyceride concentrations, specimen storage conditions, the type of alcoholic beverage, and the rate of ethanol ingestion affect serum or plasma FAEE concentrations. In our studies with FAEEs purified from plasma or serum by solid-phase extraction (6) and quantified by gas chromatography–mass spectrometry (GC-MS) (7), we determined that (a) men have higher serum FAEE concentrations than women for weight-adjusted amounts of ethanol ingestion (P ≤ 0.05); (b) FAEE/triglyceride correlates better with blood ethanol than FAEE alone, improving the correlation coefficient for men from r = 0.640 to r = 0.874 and for women from r = 0.619 to r = 0.673; (c) storage of plasma samples at room temperature for longer than 1 day can lead to artifactual formation of FAEEs; (d)
the plasma FAEE concentration is not affected by the type of alcoholic beverage consumed; and (e) the rate of ethanol ingestion does not influence the FAEE concentrations in serum. Taken together, these findings provide essential information to appropriately interpret the clinical significance of an FAEE concentration in the blood of an individual presenting for assessment of ethanol intake.

### Subjects and Methods

#### IMPACT OF GENDER ON FAEE CONCENTRATIONS

The results of FAEE quantification in samples obtained in a previously published clinical trial were reevaluated to determine whether gender influences FAEE concentrations after ethanol intake. Briefly, in that study, seven volunteers, four men and three women, were given measured amounts of 100-proof vodka mixed with fruit juice in a 1:3 ratio. Based on their weight, the subjects were given enough ethanol to increase their blood ethanol concentrations to \(~26.1-32.0\) mmol/L (120–147 mg/dL). The vodka-fruit juice beverage was divided in nine equal aliquots, which the subjects drank over a 90-min period, one aliquot every 10 min. Once the drinking commenced, blood was collected from a catheter every 15 min for the first 2.5 h. Between 2.5 and 4.0 h, samples were collected every 30 min; and from 4 to 8 h, they were collected every 60 min. A final blood sample was also collected 24 h after the ethanol ingestion commenced. At all time points, the blood was collected both into vacuum tubes without anticoagulant for serum FAEEs and for serum ethanol (8).

#### IMPACT OF TRIGLYCERIDES ON FAEE CONCENTRATIONS

For studies assessing the influence of triglyceride concentrations on FAEE concentrations, 85 residual plasma or serum samples (from 64 men and 21 women) were obtained from the toxicology laboratory at the Massachusetts General Hospital. Samples were positive for blood ethanol and, in essentially every case, positive for FAEEs as well. The samples were stored at 4 °C until analysis. The triglycerides were quantified using a Hitachi 917 automated chemistry analyzer (Boehringer Mannheim Diagnostics).

#### CHANGES IN FAEE CONCENTRATIONS UNDER DIFFERENT STORAGE CONDITIONS

To evaluate the effect of time and temperature on sample storage, frozen residual plasma from individuals in the study investigating type of alcoholic beverage on plasma FAEE concentrations (described below) was used. Samples were thawed, pooled, divided into five 0.5-mL aliquots, and stored under conditions that differed in duration and temperature. Samples were analyzed after 24 and 48 h of storage at \(-80^\circ\)C, 4 °C, and room temperature (25 °C). The control sample was thawed, processed, and analyzed immediately.

### IMPACT OF TYPE OF ALCOHOLIC BEVERAGE ON FAEE SYNTHESIS

The effect of alcoholic beverage type on FAEE concentrations was analyzed in eight healthy volunteers (seven women, one man) who were social drinkers. Volunteers were admitted to the General Clinical Research Center (GCRC) on the day of the study. Subjects were required to complete a brief food survey form, recalling the past 24 h of dietary intake, and a drinking history survey (Khavari Alcohol Test) (9). The researchers recorded each subject’s weight and height. Volunteers were required to abstain from any ethanol 5 days before and 72 h after the study. The eight participants were equally divided into two groups, four for beer ingestion and four for vodka intake. Alcohol type was chosen at random.

The amount of ethanol that each person received was based on his or her body weight and was intended to increase the blood alcohol concentration to at least 21.7 mmol/L (100 mg/dL). The total ethanol dose was divided into nine aliquots and consumed over a 90-min period. Five blood samples were drawn from each subject. One vial (10 mL) of blood was collected before ingestion of ethanol to establish a baseline; the second and third samples were drawn 15 and 30 min after drinking was completed (105 and 120 min after the onset of drinking, respectively); and the fourth and fifth samples were collected 24 and 72 h after the beginning of ethanol ingestion. Food was provided during the study by staff dieters immediately after the 120-min time point.

Subjects remained in the GCRC until they could be safely discharged, as determined by the blood alcohol concentration and subjective sense of sobriety. Subjects later returned to the GCRC for the 24- and 72-h blood collections.

At each time point, blood was collected in 10-mL Vacutainer Tubes containing 0.117 mL of 150 g/L potassium EDTA solution, and the tubes were placed immediately on ice. Tubes were centrifuged at 3420 g for 20 min at 4 °C, and the plasma was isolated. An aliquot of plasma was reserved for ethanol analysis, and another aliquot was used for subsequent FAEE isolation and quantification.

Plasma ethanol concentrations were determined by GC. Briefly, the plasma sample was mixed with an internal standard, 1-propanol, and a 1-μL sample was injected into a Hewlett Packard 5890 GC equipped with a 5% Carbowax 20 M 60/80 Carbopack B column. The oven program was isothermal at 100 °C, and the ethanol peak was identified by comparison with a known standard.

#### INFLUENCE OF RATE OF ETHANOL CONSUMPTION ON FAEE SYNTHESIS

In studies to determine whether the rate of ethanol intake affects FAEE pharmacokinetics in the blood, six young healthy male Caucasian volunteers were studied. The mean ± SE age was 24.2 ± 0.7 years; the mean ± SE body weight was 73.4 ± 1.7 kg the mean ± SE body mass index was 22.3 ± 0.3 kg/m²; and the mean ± SE lean body mass...
was 85.8% ± 1.4% total body weight. All were nonsmokers, and their habitual ethanol intake, based on a representative 1-week dietary recall, was 46 ± 22 g/week. All men had negative clinical histories, and serum aminotransferase, alkaline phosphatase, bilirubin, and albumin concentrations within the appropriate reference intervals, and none had any serological evidence of viral hepatitis.

The subjects fasted for at least 12 h before the study. At the initiation of the study, subjects were placed on a bed in a semirecumbent position, and an indwelling catheter (kept open with normal saline) was inserted into an antecubital vein for blood sampling. After 1 h of baseline measurements (three blood drawings spaced exactly 20 min apart), the subjects were given 31.9 ± 0.6 g of ethanol (0.43 ± 0.004 g/kg body weight) to ingest in a 2-min oral bolus. The subjects ingested the ethanol as a 10% volume solution (diluted with tap water only), containing 2 drops of a concentrated flavored extract used for baking. Venous blood was then sampled at 20-min intervals over a total period of 5 h. Blood ethanol concentrations were determined by GC, using head space injection. Serum was isolated from blood by centrifugation at 2800 g for 10 min at 4 °C and frozen immediately at −70 °C for subsequent measurement of FAEEs. The rates of FAEE increase and decrease in the blood from this study were compared with those reported previously by our laboratory from a study studying alcohol metabolism (8).

FAEE ISOLATION AND QUANTIFICATION

In the above experiments, serum or plasma samples were thawed, and a 1-mL aliquot was removed for analysis. There was no statistically significant difference in FAEE concentrations quantified from serum vs plasma (data not shown). Ethyl heptadecanoate (E17:0; 1 nmol) was added as an internal standard. Using a method we have described previously (4), samples were extracted with acetone-hexane (2:8, by volume) and dried under nitrogen vapor to an ~300 μL volume, and FAEEs were isolated by solid-phase extraction using Bond Elut-LRC aminopropyl columns. A complete analysis of FAEE isolation by solid-phase extraction was performed in our laboratory, and the results of this analysis have been published previously (6). Columns were prewashed with dichloromethane followed by hexane; the sample was then applied to the column and eluted by successive washes of hexane and dichloromethane. Combined eluates were concentrated, and FAEEs were then quantified by GC-MS using a Hewlett Packard 5890 Series II gas chromatograph equipped with a Supelcowax SP-2330 capillary column coupled to an HP-5971 mass spectrometer. The injector and detector were maintained at 260 °C and 280 °C, respectively. The oven program was initially maintained at 130 °C for 2 min, then ramped to 160 °C at 5 °C/min, ramped again at 2 °C/min to 180 °C, held for 7 min, and finally, ramped to 230 °C at 15 °C/min and maintained for 2 min. Carrier gas flow rate was maintained at a constant 0.8 mL/min throughout. Single-ion monitoring was performed, quantifying appropriate base ions for individual FAEE species [i.e., ions m/z 67, 88, and 101 for ethyl palmitate (E16:0), ethyl heptadecanoate (E17:0), ethyl stearate (E18:0), ethyl oleate (E18:1), and ethyl linoleate (E18:2); and ions m/z 79 and 91 for ethyl arachidonate (E20:4), ethyl eicosapentaenoate (E20:5), and ethyl docosahexaenoate (E22:6)]. FAEE quantification was determined by interpolation of the slope generated from individually prepared calibration curves comparing areas of varying concentrations of E16:0–E22:6 to fixed concentrations of the internal standard (E17:0). Mass relationships were obtained for each FAEE using its individual calibration curve. Total FAEE mass was determined by the addition of masses of individual FAEEs (E16:0–E22:6).

Results

IMPACT OF GENDER ON FAEE CONCENTRATIONS

The time courses for serum FAEE and blood ethanol after ethanol ingestion by four men and three women in a controlled clinical trial are shown in Fig. 1. All of the subjects drank a weight-adjusted amount of ethanol as a mixture of vodka and fruit juice to increase their peak blood ethanol to similar concentrations >21.7 mmol/L (100 mg/dL). As shown by the nearly overlapping lines for ethanol and FAEEs in Fig. 1 in the region where ethanol concentrations were <21.7 mmol/L (100 mg/dL), the presence of FAEEs does not require that a subject has a blood ethanol concentration that produces intoxication. Fig. 1A illustrates that the men in the study had a twofold higher serum FAEE concentration than the women (P ≤0.05). Fig. 1B shows the time courses for blood ethanol concentration in the men and the women. The women showed a modestly higher blood ethanol concentration, but only at time points after the peak blood ethanol concentration (P ≥0.05) was attained, which was the same for men and women. Thus, the higher serum FAEE concentrations in the men were not explained by correspondingly higher ethanol concentrations.

IMPACT OF TRIGLYCERIDES ON FAEE CONCENTRATIONS

The impact of serum or plasma triglyceride concentrations on FAEE formation is shown in Fig. 2. The results for blood ethanol vs FAEE concentration for men and women are shown in Fig. 2A and Fig. 2B, respectively, and the correlation between blood alcohol concentration and the ratio of the FAEE concentration to triglyceride concentration for men and women is shown in Fig. 2C and Fig. 2D, respectively. There was a similar degree of correlation between ethanol and FAEEs for men (r = 0.640) and women (r = 0.619). In Fig. 2C (for men) and Fig. 2D (for women), the units for the y-axis are nmol FAEE/mg triglyceride. (The volume units were canceled in the development of the y-axis parameter.) The inclusion of the triglyceride concentration in the denominator greatly increased the correlation coefficient between blood ethanol
and FAEEs for men, from 0.640 to 0.874, and had a modest effect for women, increasing the value from 0.619 to 0.673.

CHANGES IN FAEE CONCENTRATIONS UNDER DIFFERENT STORAGE CONDITIONS

Because the currently available method for FAEE quantification involves isolation of the lipid by solid-phase extraction and quantification by GC-MS, samples for FAEE quantification are likely to be sent to a limited number of laboratories for analysis. It is for this reason that we determined the impact of temperature and time of storage on FAEE concentrations. The sample was pooled serum or plasma frozen within 30 min of collection from multiple patients in a clinical trial involving ethanol ingestion, with blood alcohol concentrations in the individual samples ranging from 21.7 to 32.6 mmol/L (100 to 150 mg/dL). The FAEEs in the pooled baseline sample were isolated immediately after thawing and then quantified (mean ± SE, 2317 ± 458 nmol/L; n = 5). Storage of this same sample at −80 °C for up to 2 days had no impact on the FAEE concentrations [mean ± SE, 2812 ± 184 nmol/L (n = 5) at 24 h, and 2940 ± 288 nmol/L (n = 5) at 48 h; Fig. 3]. In addition, storage of the sample at 4 °C for up to 2 days did not alter the FAEE concentration [mean ± SE, 2741 ± 63 nmol/L (n = 5) at 24 h, and 3014 ± 234 nmol/L (n = 5) at 48 h]. Importantly, however, when samples were stored at room temperature, the FAEEs increased significantly over control concentrations [mean ± SE, 6378 ± 274 nmol/L (n = 5) at 24 h, and 7091 ± 160 nmol/L (n = 5) at 48 h]. This artifactual production of FAEEs may account for the limited number of high outliers in Fig. 2 because some of the samples used for this study may have been held at room temperature for extended periods in the clinical laboratory before we received the specimens. There was little difference in FAEE concentrations between 24 and 48 h at room temperature, suggesting that most of the artifactual synthesis occurred within the first day of storage at room temperature.

IMPACT OF TYPE OF ALCOHOLIC BEVERAGE ON FAEE SYNTHESIS

We also performed a controlled study in our clinical research unit in which individuals drank either beer or vodka in weight-adjusted intoxicating amounts of alcohol to determine whether the type of alcoholic beverage influences the FAEE concentrations in the blood. Fig. 4 shows that individuals drinking beer and vodka had the same peak plasma FAEE concentrations. The peak occurred in the range of 105–120 min, and at both of these times, the subjects ingesting beer and those drinking vodka had very similar plasma FAEE concentrations.

INFLUENCE OF RATE OF ETHANOL CONSUMPTION ON FAEE SYNTHESIS

We measured the FAEE concentration in samples from a study in which individuals were given alcohol to ingest in a 2-min oral bolus. We previously reported the results of a study in which subjects ingested ethanol over a 90-min period (8). We compared the pharmacokinetic data from this investigation with those from the 2-min bolus FAEE concentration study. As shown in Fig. 5, all six subjects showed a significant overlap over the time course for blood ethanol and FAEE concentrations. These pharmacokinetics were essentially identical to those we reported in our earlier study in which individuals ingested ethanol over 90 min. The time for attaining peak ethanol concentration was predictably shorter in this study because the time of ingestion was only 2-min. In both studies taken together, it appears that the blood ethanol peaks ~20–40 min
before the FAEE concentrations. This finding is consistent with the fact that FAEEs are metabolites of ethanol.

There was no interference in FAEE measurements by hemoglobin <0.12 mmol/L (200 mg/dL), bilirubin <0.34 mmol/L (20 mg/dL), or cholesterol <10.3 mmol/L (400 mg/dL).

The CV for replicate analysis of samples for FAEE was <5% for specimens processed with solid-phase extraction and then quantified by GC-MS and <3% for samples quantified by GC-MS without prior extraction.

Discussion

This series of studies brings clinical testing for FAEEs as markers of ethanol intake closer to clinical application. In summary, we have found that men have higher FAEE concentrations than women for weight-adjusted amounts of ethanol ingestion; that FAEE/triglyceride correlates better with blood ethanol than FAEEs alone; that there is

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**Fig. 2.** Correlation between individual blood ethanol and plasma or serum FAEE concentrations in men (A) and women (B). Correlations adjusted for individual triglyceride concentrations for men (C) and women (D) are also shown.

**Fig. 3.** Plasma FAEE concentrations for samples stored under various conditions of temperature and duration.

Plasma from intoxicated individuals was thawed, pooled, aliquoted, and stored under the various conditions shown. The control sample was analyzed immediately. Data represent the mean ± SE of five measurements per condition.
artifactual formation of FAEEs when samples are stored at room temperature for 1 day or more; and that the type of alcoholic beverage and the rate of ethanol ingestion have no impact on FAEE concentrations.

We found in one series of experiments that there was a gender difference in serum FAEE concentrations. For a weight-adjusted amount of ethanol ingested, we observed that men had peak serum FAEE concentrations approximately twofold higher than the peak values for women. The presence of FAEEs in the serum or plasma does not necessarily indicate alcohol intake to intoxication. In a previously published clinical trial, it was shown that FAEE concentrations parallel ethanol concentrations and that FAEEs are detectable when ethanol is well below concentrations associated with intoxication (8).

It has long been known that women attain higher blood ethanol concentrations than men for equal amounts of ethanol ingested (10). In addition, women carry a higher risk for developing cirrhosis than their male counterparts (11, 12). One of the explanations that has been offered to explain this observation is that women have decreased first-pass metabolism of ethanol in the stomach, thereby permitting the transport of larger amounts of ethanol from the gastrointestinal tract into the blood (10). There are several possible explanations as to why women

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**ALCOHOLIC BEVERAGE INGESTED**

Fig. 4. Plasma FAEE concentrations at two representative peak time points for ethanol ingestion of beer and vodka. Individuals consumed equivalent amounts of ethanol adjusted for body weight. Data represent mean ± SE for four individuals per group.

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**Fig. 5. Serum FAEE and blood ethanol concentrations for six individual subjects over time.** Subjects in this study ingested ethanol within a 2-min period as an acute bolus.
may have lower peak FAEE concentrations after ethanol intake. It could in part be because the women in this study were all premenopausal, with relatively high HDL-cholesterol concentrations and correspondingly low triglyceride concentrations. From the experiment concerning the effects of triglycerides on FAEE concentrations, it was found that by incorporating triglyceride concentrations into FAEE concentrations, the correlation between blood ethanol and FAEE did not improve as much for women as it did in men. Alternatively, it could be that in women there is a decreased activity of enzymes required for the synthesis of FAEEs or an increased activity of enzymes involved in the degradation of FAEEs. With in vitro (1) and in vivo (4) evidence that FAEEs are toxic, this could represent an attempt to reduce the ethanol-mediated toxicity because women already suffer greater toxic effects from ethanol ingestion than men (11, 12).

It has been reported that lipoprotein lipase has FAEE synthetic activity (13, 14). This enzyme also degrades triglycerides with release of free fatty acids from the glycerol backbone. Another enzyme that degrades triglyceride, carboxylester lipase, has also been shown to have FAEE synthetic activity (15). Because these two enzymes that degrade triglycerides have the ability to synthesize FAEEs, it has been speculated that the rate-limiting step in FAEE synthesis is a hydrolytic event that liberates a fatty acid from the triglyceride in the presence of ethanol in cell water. The result could be a nonenzymatic esterification of fatty acid and ethanol to create FAEEs. The data in Fig. 2 provide support for this hypothesis. Fig. 2 shows that including the triglyceride concentration as the denominator on the y-axis, the r value increases, especially in male subjects.

This study also gives an initial indication of specimen handling requirements for FAEE quantification. There was no FAEE degradation even when the samples were kept at refrigerator temperatures for up to 2 days. Thus, it should be possible to collect samples, isolate the serum or plasma, freeze or refrigerate it, and send it for analysis. There was artifactual FAEE formation in vitro when samples were maintained at room temperature for at least 1 day. This is most likely because ethanol is still present in the sample and there is some FAEE synthetic activity in the plasma sample, possibly from residual intact or disrupted white blood cells or platelets, which are known to have FAEE synthase activity (16).

It was anticipated that because FAEEs are metabolites of ethanol, the type of alcoholic beverage would not influence FAEE concentrations. This report demonstrates that this is true, at least for a comparison between beer and vodka, but most likely for all alcoholic beverages. The evidence indicates that ethanol, and not other ingredients in the alcoholic beverage, is the major component that determines the amount of FAEE synthesized.

Finally, the rate of ethanol ingestion did not impact FAEE concentrations when subjects who drank a 2-min bolus were compared with those that consumed alcohol over a 90-min period. This is consistent with the conclusion that FAEE concentrations are dependent on the amount of ethanol consumed and not the rate of consumption.

The FAEE concentrations in the individual studies in this report varied widely for several reasons. First, as shown in Fig. 5, there is significant interindividual variation in FAEE production with identical amounts of ethanol intake. Second, the samples for Fig. 2 were stored for variable amounts of time, mostly at 4°C, before analysis for FAEE, whereas samples were processed immediately after collection for the clinical studies shown in Figs. 1, 4, and 5. Third, the amount of ethanol ingested by individuals whose serum or plasma was also assayed for FAEEs was different in the various clinical studies. In addition, the amount of ethanol ingested in the experiment shown in Fig. 2 was not intended to be a controlled variable, and therefore differed widely between individuals.

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