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Non-linear formation of EtG and FAEES after controlled administration of low to moderate doses of ethanol

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Concentrations of ethanol non-oxidative biomarkers

Key words

Alcohol, biomarkers, ethanol, ethyl glucuronide, fatty acid ethyl esters

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Abstract

Aims: Ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEES) are non-oxidative metabolites of alcohol that can be detected in conventional and non-conventional biological matrices for longer periods than alcohol. The aim was to describe the time courses of both biomarkers after ingestion of acute low-moderate doses of ethanol.

Methods: The study design was double-blind, randomized, crossover, and controlled with placebo. Participants were distributed in three different cohorts: a) cohort-1: two doses of 18 and 30g of ethanol and placebo were administered to 12 subjects; b) cohort-2: two doses of 6 and 12g of ethanol and placebo were administered to 6 subjects; c) cohort-3: two doses of 24 and 42g of ethanol and placebo were administered to 6 subjects. Each participant received two doses of ethanol and placebo. Plasma concentrations (0-6h) of ethanol and specific FAEES (palmitic, stearic, linoleic and oleic acid ethyl esters) and urinary concentrations of EtG (0-24h) were measured.

Results: A dose-dependent increase in blood ethanol concentrations was observed. EtG excretion and FAEES plasmatic concentrations showed a disproportionate increase with the ethanol dose suggesting nonlinearity. Area under the curve ($AUC_{0\text{-}6h}$) of ethanol concentrations showed a linear trend with non-oxidative metabolites' concentrations.

Conclusion: The formation rate of ethanol non-oxidative biomarkers does not follow a linear trend, explained mainly by a disproportionate increase in $AUC_{0\text{-}6h}$ of ethanol concentrations in relation to dose. This observation should be taken into account when interpreting results in biological matrices in clinical and forensic settings.

Short summary

A double-blind, randomized, crossover, and controlled study was conducted administering ethanol (6-42g). Ethyl glucuronide (EtG) excretion and fatty acid ethyl esters (FAEES) plasmatic concentrations showed a disproportionate increase with the ethanol dose suggesting nonlinearity. This observation should be taken into account when interpreting biomarker concentrations in clinical settings.

Abbreviations

AEAT	acyl-Co/ethanol *O*-acyl-transferase
ADH	alcohol dehydrogenase;
$AUC_{0\text{-}6h}$	area under the curve from 0 to 6h
C_{max}	maximum concentration
CV	coefficient of variation
ECG	electrocardiogram
EIA	enzyme immunoassay
EtG	ethyl glucuronide
FAEES	fatty acid ethyl esters
GC/MS	gas chromatography-mass spectrometry
LC/MS	liquid chromatography-mass spectrometry
LLOQ	lower limit of quantification
UGT	UDP-glucuronosyltransferase
E_{max}	maximum alcohol effect (drunkenness)
t_{max}	time to reach maximum concentration

Introduction

Alcohol is the most worldwide consumed legal drug and alcohol use disorders are common in modern societies (WHO, 2014). Biomarkers of alcohol consumption are used to prevent health and social problems related with alcohol, allowing the identification of subjects at a higher risk of alcohol use disorders or withdrawal and to assess the efficacy of treatments for alcohol dependence (Bataille *et al*., 2003; Kip *et al*., 2008; Dahl *et al*., 2011).

The interest in non-oxidative metabolites of alcohol has grown in the last years due to the lack of accuracy or inadequacy for purpose of previously developed biomarkers and the possibility to detect them in conventional specimens and alternative biological matrices (Carbacos *et al*., 2015)

Ethanol is converted to acetaldehyde by hepatic oxidative metabolism (95-98%) in a reaction regulated by class I isozymes of alcohol dehydrogenase (ADH) and highly inducible microsomal CYP2E1. In turn acetaldehyde is converted in acetic acid (acetate) by acetaldehyde dehydrogenase. Only a small fraction of ethanol (2-5%) is excreted unchanged in urine, sweat or exhaled air, or follows non-oxidative metabolism, leading to minor metabolites such as ethyl glucuronide (EtG), ethyl sulfate, ethyl phosphate, phosphatidylethanol and fatty acid ethyl esters (FAEES).

Blood, urine or breath ethanol concentrations are useless biomarkers of abstinence because ethanol is cleared from the body at a rate of 0.1–0.25 g l^{-1} h^{-1} and becomes undetectable in blood in a few hours (Borucki *et al*., 2005; Jatlow and O'Malley, 2010). Clearance in heavy drinkers is still faster due to CYP2E1 induction (Jones, 2008).

Non-oxidative metabolites have longer biological half-lives than ethanol, accumulate in tissues after consumption and allow the detection of ethanol consumption after larger periods of abstinence. The detection window of non-oxidative metabolites may last for several hours up to some days, depending on the marker and the amount of alcohol ingested.

EtG is produced by UDP glucuronosyltransferase (UGT), it can be detected in urine up to 80h after ethanol ingestion (Wurst *et al*., 2003) and does not accumulate after repeated drinking (Sarkola *et al*., 2003). EtS has a similar excretion profile and can be used for confirmation of EtG tests (Jatlow and O'Malley, 2010).

In turn FAEES are produced by esterification of fatty acids and ethanol through FAEES synthase and acyl-Co/ethanol *O*-acyl-transferase (AEAT) (Treloar *et al*., 1996). They appear in the serum bound to albumin and in the core of lipoproteins (Laposata, 1997) and are associated with alcohol organic damage (Laposata, 1999). FAEES can be detected in blood up to 24 hours after a drinking intoxication (Doyle *et al*., 1994), and up to 44h in heavy drinkers (Borucki *et al*., 2004).

Direct ingestion of FAEES should be considered as they have been detected in food, scotch whiskey and non-alcoholic beverages (Goss *et al*., 1999). Moreover both FAAE and EtG are also formed endogenously and have been detected in ethanol abstainers and children. For FAEES a baseline range from natural human metabolism has been described while EtG could be produced from ethanol generated by intestinal bacteria (Borucki *et al*., 2005; Rosano and Lin, 2008).

Their monitoring after recent or even single consumption in law enforcement and forensic cases is common, but their application to clinical settings is still limited (Jatlow and O'Malley, 2010). EtG is the one with the most opportunities to be routinely assessed in treatment programs (high sensitivity and specificity, wide detection period, detection of low doses and availability of enzyme immunoassay commercial kits [EIA]) (Wurst *et al*., 2003; Borucki *et al*., 2005). Additionally EtG can be used in clinical trials for confirmation of self-reported alcohol abstinence (Jatlow *et al*., 2014).

We have identified several experimental studies assessing urine or blood EtG (Sarkola *et al*., 2003; Høiseth *et al*., 2007; Halter *et al*., 2008; Høiseth *et al*., 2008) or blood FAEES (Doyle *et al*., 1994, 1996; Laposata 1997; Schmitt *et al*., 1997; Soderberg *et al*., 1999; Dahl *et al*., 2002; Goll *et al*., 2002; Best *et al*; 2003, 2006) detection after administration of one dose of ethanol or a dose targeted to reach a fixed alcohol concentration. Three studies have described a dose dependent increase in urinary EtG concentrations after administration of different doses of ethanol to the same subjects (Rosano and Lin, 2008; Høiseth *et al*., 2010; Jatlow et al 2014). A non-randomized study assessed urine EtG and plasma FAEES simultaneously recommending the use of urine EtG as marker of recent alcohol intake (Borucki *et al*., 2005).

However, no randomized studies assessing both biomarkers (EtG and FAEES) in healthy subjects and covering a dose range compatible with social consumption of ethanol have been identified.

Our objective was to describe the time course of both biomarkers after ingestion of low-moderate doses of ethanol trying to avoid potential confounders by means of a randomized and placebo controlled clinical trial.

Materials and methods

Participants

The study was conducted in accordance with the Declaration of Helsinki, approved by the local Research Ethics Committee (Comitè Ètic d'Investigació Clínica Parc de Salut Mar) and registered (clinical trials.gov: NCT01788670). Eligibility criteria required to be healthy subjects with social ethanol consumption. Exclusion criteria were daily alcohol consumption of >30g or current or previous ethanol abuse or dependence. All subjects were informed and signed a consent form before the first study related procedure and were financially compensated for their participation.

Twenty four male healthy volunteers were included. All of them were randomized and finished the study. One third of them were smokers and their average alcohol consumption was 7g a day. The mean age, body weight and height were 25.8 years (range 20-36), 79.2kg (range 65.4-92.2) and 181 cm (range 172-189).

Study design and procedures

Participants were distributed in three different cohorts. Design was double-blind, randomized, crossover, and controlled with placebo. In cohort-1 drinks containing 18 and 30g of ethanol and placebo were administered to 12 subjects; in cohort-2 drinks containing 6 and 12g of ethanol and placebo were administered to 6 subjects and in cohort-3 drinks containing 24 and 42g of ethanol and placebo were administered to 6 subjects.

Recruitment and sessions took place in the Clinical Research Unit at Hospital del Mar Medical Research Institute-IMIM.

198 Sessions lasted 6h with a minimum wash out period between sessions of at
199 least three days. Participants on each cohort were randomly assigned by order
200 of recruitment to one treatment sequence using a balanced 3 x 3 Latin square
201 design. Allocation for each participant was concealed for all the staff (beverages
202 were prepared by personnel not involved in the experimental sessions).
203 Subjects were requested to abstain from ethanol ingestion and use of other
204 sources of ethanol (hand sanitizers, mouthwashes) three days before each
205 session. Breath alcohol tests (Dräger Breathalyzer 7410 Plus, Denmark) and
206 drugs of abuse tests in urine (Instant-View; ASD Inc, Poway, California, US)
207 were conducted at baseline to confirm abstinence. Xanthine-containing
208 foods/drinks were prohibited from the day before till 24h after administration.
209 On each session day an intravenous catheter was inserted into an arm
210 subcutaneous vein to obtain blood samples. Treatments were administered at
211 8:30 AM and half cheese sandwich with sunflower oil was provided 2 and 6h
212 after treatment administration. Additional water was given to volunteers at 2h
213 (300 mL) and 4h (100 mL) in order to assure urine generation in each time
214 interval. Participants left the unit 6h after administration after verifying that the
215 breath alcohol test was negative.
216 *Treatments*
217 Ethanol conditions were obtained mixing ethanol pharmaceutical grade and
218 lemonade flavored water (Fontvella Sensación sabor limón, Barcelona, Spain).
219 Placebo contains only lemonade flavored water. The total volume of the
220 beverages was 150 mL. All the beverages were consumed in fasting conditions,
221 orally, in opaque recipients, and in 5 minutes.
222 *Blood and urine samples*

Blood samples to determine ethanol and FAEES (palmitic, stearic, oleic and linoleic acid ethyl esters) in plasma were drawn at pre-dose and at 15, 30, 45 minutes, and 1, 1.5, 2, 3, 4, 6 hours after treatment administration. Urine samples were collected at baseline and at different interval periods (0-2h, 2-4h, 4-6h, 6-12h, 12-24h) for EtG quantification. Tyrosol and hydroxytyrosol urine concentrations were also measured in this clinical trial. Their relationship with ethanol concentrations have been already published in a previous manuscript (Pérez-Mañá *et al*., 2015).

Blood (6 mL) was collected in lithium heparin tubes for ethanol analysis. After centrifugation at 1700g for 10 minutes at 4° C, plasma was transferred to tubes sealed with a plastic paraffin film and frozen immediately (-20ºC) to avoid ethanol evaporation. A second collection blood tube (5 mL) containing EDTA and aprotinin was used for determination of FAEES. It was centrifuged the same way as above, and plasma was stored frozen (-20ºC) until analysis.

Urine samples were collected in different recipients and the total amount of urine generated in each time interval was registered. Three aliquots were stored from each time interval at -20ºC until analysis.

Ethanol and Ethyl glucuronide analysis

DRI® Ethyl Alcohol Assay (Thermo Fisher, Fremont, CA, USA) was used to analyze concentrations of ethanol in plasma with a cut-off of 10 mg/dL.

Ethyl Glucuronide was measured with liquid chromatography-mass spectrometry (LC/MS) using a dilute and shoot approach (see Supplementary materials for further details).

Fatty acid ethyl esters analysis

FAEES plasma concentrations were analyzed by gas chromatography-mass spectrometry (GC/MS). The method of analysis was based on a method previously described (Kulig *et al*., 2006), with some modifications (see Supplementary materials section).

Statistical analysis

Sample size was determined for cohort-1 based on the methodology of bioequivalence studies (10 subjects would be needed considering an alpha risk of 0.05, a power of 80%, a 30% of variability and an increase in FAEES concentrations of at least 40% from placebo). 12 subjects participated to increase power.

Differences from baseline were calculated for FAEES concentrations. For FAEES and ethanol the following experimental pharmacokinetic/pharmacodynamic parameters were calculated: peak concentration (C_{max}), the time to reach the maximum concentration (t_{max_c}) and the area under the concentration-time curve from 0 to 6h ($AUC_{0\text{-}6h}$). The AUC values were calculated with the trapezoidal rule. Urinary EtG excretion from 0 to 24h after administration was calculated as the sum of the total amount excreted during the different collection intervals.

The data of all three cohorts were analyzed together by means of linear mixed models, which take into account the correlation between values obtained in the same subjects. The outcomes of interest were introduced in these models as dependent variables whereas the ethanol dose was introduced as the independent variable. In the case of the $AUC_{0\text{-}6h}$ of ethanol, the FAEES concentrations and the EtG excretion (0-24h), the relations between the outcome and the ethanol dose were not always linear. For that reason, log-

transformations of both outcomes and the ethanol dose were also considered and those models that showed the most adequate fit based on graphical inspection of the corresponding residual plots were used. The analyses for the main outcomes were also performed with the weight-adjusted doses (data not shown).

In addition, Pearson's correlation coefficient was computed to study the association between ethanol and the FAEES concentrations ($AUC_{0\text{-}6h}$ and C_{max}) and between the ethanol concentrations ($AUC_{0\text{-}6h}$) and the EtG excretion (0-24h).

Results were considered statistically significant at p values <0.05 and the statistical software package R, version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria), was used for the analyses. All analyses were conducted with all the participants.

Results

There were no demographic baseline differences among participants in the three different cohorts. Also no overlap between ethanol doses was observed when the dose of ethanol was adjusted to weight of individuals.

Ethanol

There were no positive baseline samples for ethanol. Ethanol plasma concentrations and pharmacokinetic parameters calculated ($AUC_{0\text{-}6h}$, C_{max}, t_{max}) increased with the ethanol administered dose (see Figure 1). High variation in ethanol concentrations was found as the coefficient of variation (CV) of the AUC among individuals with the same dose ranged from 11.6% to 40.2%. Furthermore a nonlinear relationship was found between AUC of ethanol

297 concentrations and alcohol dose. The relationship was linearized after the log-
298 transformation of both outcomes. As a summary the pharmacokinetic
299 parameters estimated for each dose of ethanol are presented in Table 1.

300 *Ethyl glucuronide*

301 Concentrations in spot urine samples collected before drinking were below
302 LLOQ with the exception of 4 samples of 3 different subjects (values between
303 0.5 and 2.8 µg/ml). EtG was mainly excreted in the period from 0 to 6 hours for
304 all doses (2-4h for 6 to 30g, 4-6h for 42g), followed by the period from 6 to 12h
305 and finally by the period from 12 to 24h. EtG recovery (0-24h) showed a dose-
306 dependent relationship with ethanol administered doses. This relationship
307 follows an exponential trend as it can be seen in Figure 2. The logarithm of the
308 recovery of EtG increased linearly as a function of the logarithm of the ethanol
309 dose (log-dose): on average, augmenting the log-dose by one unit, the log-
310 recovery increased by 1.65 units per gram of alcohol (95%-CI: [1.49, 1.82];
311 $p<0.001$). The relative percent (molar equivalent) of ethanol excreted in the
312 urine as EtG the first 24 hours ranged from 0.004% to 0.031% in our study. The
313 values also increased with alcohol administered dose (see figure 2).

314 EtG excretion within individuals was slightly variable (CVs of EtG excretion for
315 each dose ranged from 21.2%-34.5%). The correlation between the ethanol
316 concentrations ($AUC_{0\text{-}6h}$) and the EtG excretion was 0.82 (95%-CI: [0.70, 0.90];
317 $p<0.001$). The relationship between $AUC_{0\text{-}6h}$ of ethanol and EtG excretion was
318 linear. The equation of the linear relationship calculated was the following: EtG
319 (µmols)=3.09 x $AUC_{0\text{-}6h}$ of ethanol (nmol x h /ml)+14.8.

320 *Fatty acid ethyl esters*

Palmitic acid and stearic acid ethyl esters were above LLOQ before ethanol ingestion in 6 subjects and oleic acid ethyl ester in 2 subjects. For each specific FAEES, a dose response relationship with ethanol administration was found (see Figure 3). Linoleic acid ethyl ester concentrations were undetectable for several subjects and doses (were above LLOQ for one subject with 6 and 12g, 5 subjects with 18g and 24g, 7 subjects with 30g and all subjects of 42g). FAEES could be detected till 3 hour for doses ≤ 18g and till 6h for higher doses. The concentrations of palmitic acid ethyl ester were higher than concentrations of the other FAEES. The model-based estimations of the C_{max} of the different FAAEs are shown in Table 1. In the case of the palmitic, stearic, and oleic acid ethyl esters, the logarithm of the C_{max} increased linearly as a function of the logarithm of the ethanol dose (log-dose). Augmenting the log-dose by one unit, the logarithm of the C_{max} increased, on average, in 1.06 (95%-CI: 0.88, 1.24]; p<0.001), 1.14 (95%-CI: [0.93, 1.34]; p<0.001), and 1.46 units (95%-CI: [1.03, 1.89]; p<0.001), for palmitic, stearic, and oleic acid ethyl esters, respectively.

Concerning the $AUC_{0\text{-}6h}$, the lowest range of the CV among individuals taking the same dose was observed in the case of palmitic acid ethyl ester (13.5% to 37.5%), whereas the highest range was observed in the case of linoleic acid ethyl ester (67.8% to 245.0%). The logarithm of the $AUC_{0\text{-}6h}$ of the different FAEES increased linearly as a function of the logarithm of the ethanol dose (log-dose): on average, augmenting the log-dose by one unit, the log-$AUC_{0\text{-}6h}$ increased by 1.69 units (95%-CI: [1.55, 1.83]; p<0.001) for palmitic acid ethyl ester, by 1.82 units (95%-CI: [1.60, 2.04]; p<0.001) for stearic acid , and by 2.25 units (95%-CI: [1.84, 2.66]; p<0.001) for oleic acid. Model-based estimations of the $AUC_{0\text{-}6h}$ of the different FAEES are presented in Table 1.

In the case of linoleic acid ethyl ester pharmacokinetic parameters, all models fitted showed a statistically significant increase of both measures as a function of the alcohol dose ($p<0.001$ in all cases), but none of the model fits was satisfactory. For this reason, no model-based estimations of both pharmacokinetic parameters are provided.

For palmitic, stearic and oleic acid ethyl ester median time to reach maximum concentration was 30 minutes for doses ≤18g, and 1h for higher doses. The $AUC_{0\text{-}6h}$ of the ethanol concentrations in plasma correlated with the $AUC_{0\text{-}6h}$ of the different FAEES (palmitic acid: r=0.87 (95%-CI: [0.78, 0.93]; $p<0.001$); stearic acid: r=0.88 ([0.81, 0.94]; $p<0.001$); oleic acid: r=0.80 ([0.67, 0.87]; $p<0.001$); and linoleic acid r=0.65 ([0.45, 0.79; $p<0.001$])). The relationship between $AUC_{0\text{-}6h}$ of ethanol and $AUC_{0\text{-}6h}$ of the different FAEES followed a linear trend, except for linoleic acid. For the most abundant FAEE after ethanol ingestion the equation of the linear relationship was: $AUC_{0\text{-}6h}$ of palmitic acid ethyl ester=10.33 x $AUC_{0\text{-}6h}$ of ethanol + 26.84. Also high correlation was found between the $AUC_{0\text{-}6h}$ of the ethanol concentrations with maximum concentrations of FAEES (palmitic acid: r=0.77 ([0.63, 0.87]; $p<0.001$); stearic acid: r=0.82 ([0.7, 0.89]; $p<0.001$); oleic acid: r=0.68 ([0.49, 0.81]; $p<0.001$); and linoleic acid r=0.62 ([0.4, 0.77]; $p<0.001$)).

Discussion

The administration of ethanol at low-moderate intoxicating doses (6-42g) to healthy volunteers produced detectable concentrations of ethanol non-oxidative metabolites (at least till 6h for several FAEES and ethanol doses and at least till 24h for EtG). FAEES concentrations ($AUC_{0\text{-}6h}$, C_{max}) and EtG excretion (0-24h)

showed high correlation with the ethanol administered dose. Both increased with ethanol administered dose and their formation rates do not seem to follow a linear trend. On the other hand the relationship between $AUC_{0\text{-}6h}$ of ethanol and metabolites' concentrations was linear.

Ethanol concentrations

Ethanol concentrations were similar to those obtained in previous studies (Rangno *et al*.,1981; Holford, 1987; Høiseth *et al*., 2007). Ethanol accumulation with dose (disproportionate increase in AUC) can be attributed to changes in hepatic extraction ratio with concentration and to capacity-limited elimination (Holford, 1987).

Ethyl glucuronide

EtG urinary excretion increased with the ethanol dose and as expected the highest recovery in urine was obtained with 42g. Although only <1% of the ethanol ingested is metabolized in humans to EtG (Politi *et al*., 2007) we have found a very high correlation between ethanol dose and EtG excretion. The disproportionate increase in EtG urinary excretion in relation to alcohol dose has been already described (Rosano and Lin, 2008; Jatlow *et al*., 2014). A similar trend in the relationship has been described in blood as the AUC of EtG was triplicated (2.43 vs. 8.58 mg x h/l) after just doubling the ethanol administered dose (0.5g/kg vs. 1g/kg) (Høiseth *et al*., 2010). Saturation of ethanol major metabolic pathway (ADH) and greater substrate availability for conjugation routes as the dose increases explains the results obtained (Rosano and Lin, 2008; Jatlow *et al*., 2014).

Inter-individual variability found in EtG formation in subjects taking the same dose may be explained by genetics and nutritional factors. The glucuronidation

of ethanol through UDP-glucuronosyltransferases (UGT) is produced mainly by UGT1A1 and 2B7 (Politi *et al*., 2007). Additionally diet components like flavonoids may influence the conversion of ethanol to EtG through UGT competitive inhibition (Schwab and Skopp, 2014). EtG formation follows a Michaelis-Menten kinetics (also the ethanol oxidation to acetaldehyde by ADH) so ethanol concentrations available in each subject modulate the formation rate of EtG. Furthermore, the presence of polymorphisms coding the enzymes of ethanol oxidative metabolism could explain differences between subjects (Jatlow and O'Malley, 2010).

Fatty acid ethyl esters

FAEES determined in our study were those predominant in plasma after ethanol ingestion (Dan and Laposata, 1997; Politi *et al.*, 2007). Inter-individual variability detected in FAEES concentrations was higher than for EtG. Individual differences can be attributed to the different activity of the enzymes responsible for FAAE synthesis or metabolism, but probably also to plasma triglyceride levels as a source of fatty acids for FAEES synthesis (Dan and Laposata, 1997; Soderberg *et al*., 1999). The concentration of saturated and monounsaturated FAEES showed a very high correlation with ethanol administered dose (Soderberg *et al*., 1999). On the other hand, the concentrations of the polyunsaturated linoleic acid ethyl ester showed a poorer correlation. The explanation can be related to the fact that linoleic acid is an essential fatty acid that is available only through diet, while palmitic, oleic and stearic acid are also obtained through metabolism. Breakfast 2h after ethanol administration can justify the small rise in linoleic acid ethyl ester concentrations at 4h.

The plasmatic FAEES limited detection period (6h) after ethanol doses compatible with social consumption, in comparison with 24h for EtG, and the long lasting elevations of FAEES that have been reported in heavy drinkers, due to diffusion from adipose tissue (Bisaga *et al*., 2005), makes urinary EtG more suitable to routinely monitor ethanol abstinence.

To our knowledge this is the first study reporting the lack of linearity in the relationship between ethanol dose and FAEES plasma concentrations. The explanation could be the same than for EtG, a disproportionate increase in the amount of ethanol available for FAEES formation due to saturation of ethanol major metabolic pathway. In accordance to this data FAEES concentrations increased after the inhibition of the oxidative metabolism of ethanol (Best *et al*., 2006).

It should be noted that the non-linearity in the concentrations of both non-oxidative metabolites of alcohol (urinary EtG and plasmatic FAEES) has been observed at relatively low doses of alcohol.

Strengths and limitations

The cross over design allowed the same subjects to be treated with at least two different doses. However, not all subjects received all doses and some comparisons were indirect. We enrolled only male volunteers for avoiding potential sex differences in ethanol and FAEES pharmacokinetics (FAEES formation can be two fold greater in man, according to Soderberg *et al*., 1999). Future studies with a larger sample size per dose should include women. The three day period between sessions was not enough to achieve a complete wash-out in some subjects although some FAEE could be endogenously formed. Furthermore higher doses of ethanol should be included to confirm the

trend observed in FAAE concentrations. Experimental sessions could not be planned long enough to provide a complete blood EtG pharmacokinetic profile. For this reason we decided to measure EtG only in urine, which was valuable to quantify the amount of ethanol excreted as EtG.

Conclusion

In conclusion, after administering low to moderate doses of ethanol to healthy male subjects the formation rate of EtG and FAEES does not seem to follow a linear trend and this observation should be taken into account when interpreting results in biological matrices in clinical and forensic settings.

Figure legends

Figure 1. Ethanol plasma concentrations.

Ethanol plasma concentrations from 0 to 6h after ethanol administration.

Figure 2. Ethyl glucuronide excretion.

a Ethyl glucuronide excretion 0-24 h after ethanol administration. Values are expressed as mean ± standard deviation.

b Effect of ethanol dose on percent metabolism to EtG. Individual data are presented.

Figure 3. FAEES plasma concentrations.

a Palmitic acid ethyl ester plasma concentrations from 0 to 6h after ethanol administration.

b Oleic acid ethyl ester plasma concentrations from 0 to 6h after ethanol administration.

c Stearic acid ethyl ester plasma concentrations from 0 to 6h after ethanol administration.

d Linoleic acid ethyl ester plasma concentrations from 0 to 6h after ethanol administration.

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625
626 **Supplementary material**
627 *Fatty acids ethyl esters (FAEES) analysis*
628 FAEES plasma concentrations were analyzed by gas chromatography-mass
629 spectrometry (GC/MS) based on a method previously described (Kulig *et al*.,
630 2006), with some modifications. One mL of each plasma sample was
631 transferred to 12 mL glass tubes. Plasma calibration samples were prepared
632 with blank plasma spiked at 2.5, 10, 50, 100, 200 and 300 ng/mL of each
633 FAEES (Nu-Chek, MN, USA). Plasma and calibration samples were spiked with
634 25 µL of internal standard solution (2.0 µg/mL heptanoic acid ethyl ester in
635 methanol) and 50 µL of a 50 mM butylated hydroxytoluene (Sigma-Aldrich, MO,
636 USA) solution in methanol. The plasma was extracted with 2 mL of acetone

637 (Merck, Germany). After vortex for a few seconds, the tubes were centrifuged
638 (1700g, 5 min, room temperature). Next, the plasma was extracted with 6mL
639 hexane (Merck, Germany) and placed in a rocking mixer over 20 min. After
640 centrifugation (1700g, 5 min, room temperature) the acetone:hexane organic
641 phase was separated to clean 12 mL glass tubes, placed in a water bath
642 (<30ºC) and evaporated to dryness under a stream of nitrogen. The extracts
643 were reconstituted in 1 mL hexane and applied to an aminopropyl solid phase
644 extraction column (LRC Bond Elut NH2, Agilent, CA, USA), previously
645 conditioned with 2 mL hexane. The hexane eluates were collected in 12 mL
646 glass tubes and an additional 1mL pure hexane was applied to the columns and
647 collected. The tubes were placed in a water bath (<30ºC) and the hexane eluate
648 was evaporated to dryness under a stream of nitrogen. The extracts were
649 reconstituted in 100 µL hexane and transferred to vials. Three µL of extract
650 were injected into the GC/MS system (Agilent 5973, CA, United States) in the
651 split mode with a split ratio 1:10. Chromatographic separation of FAEES was
652 achieved with a 100% methylsilicone column (Zebron ZB-1, 15m length, 250µm
653 diameter, 0.25µm film thickness) at 1 mL/min helium flow. The mass detector
654 transfer line heater and the injection port were set at 280ºC. The oven
655 temperature ramp was as follows: initial condition 150ºC for 2 min, then a ramp
656 at 15ºC/min to 295, and finally the temperature was kept at 295ºC for 4 min with
657 a total run time of 15.7 min. Quantification was done by single ion monitoring
658 (SIM) with the following m/z ions for each FAEES: m/z 88, m/z 241 and m/z 284
659 for palmitic acid ethyl ester, m/z 222, m/z 264 and m/z 180 for oleic acid ethyl
660 ester, m/z 101, m/z 269 and m/z 312 for stearic acid ethyl ester, m/z 306 and
661 m/z 261 for linoleic acid ethyl ester, and m/z 88, m/z 157 and m/z 255 for

heptanoic acid ethyl ester. The method was validated in-house. Intra-day and inter-day accuracy and precision were <15% for quality control spiked plasma samples. The lower limit of quantification (LLOQ) for palmitic, stearic, oleic and linoleic acid ethyl esters were 2.5, 3.5, 3.0 and 2.5 ng/mL, respectively.

Analysis of ethylglucuronide (EtG) by LC/MS/MS

Ethyl Glucuronide was measured with liquid chromatography-mass spectrometry (LC/MS) using a dilute and shoot approach. The LLOQ was 0.41 µg/mL. Briefly, 50 µL of centrifuged urine samples were spiked with internal standards (EtG-d5 and EtS-d5). Thereafter samples were diluted with 900 µL of 0.1% aqueous formic acid solution, and 10 µL were injected into the LC-MS/MS system. The LC-MS/MS system consisted of an Agilent 1200 series HPLC system (Agilent Technologies) coupled to a 6410 Triple Quadrupole LC-MS (Agilent Technologies) mass spectrometer with an electrospray interface. Chromatographic separation was achieved on an Acquity HSS T3 column (2.1 x 100 mm, 1.8 µm particle size) (Waters Corp.) at 40°C. The electrospray ion source was set on the negative ionization mode. The mass spectrometry detection was done by single reaction monitoring (SRM). Quantification was calculated with the slope (s), intercept and correlation coefficient (r) by weighting (1/x) least-squares linear regression of the peak area ratio (analyte/IS) versus the concentration of the standard.

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