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**Involvement of UDP-glucuronosyltransferases UGT1A9 and UGT2B7 in ethanol glucuronidation, and interactions with common drugs of abuse**

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**Running title:** Ethanol glucuronidation: enzymes and potential interactions

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**Abbreviations:** CBN: Cannabinol; CBD: cannabidiol; EtG: Ethylglucuronide; HLM: Human Liver Microsomes; HKM: Human Kidney Microsomes; HIM: Human Intestinal Microsomes; UGT: UDP-glucuronosyltransferase; RAF: Relative Activity Factor;  $Cl_{int}$ : Intrinsic Clearance; UDPGA: Uridine 5'-diphosphoglucuronic acid; DMSO: Dimethyl Sulfoxide; CDCA: Chenodeoxycholic Acid; MEF: Mefenamic Acid; TFP: Trifluoperazine; AZT: Azidothymidine; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LOQ: Limit of Quantification.

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### ABSTRACT

**Background:** Ethylglucuronide (EtG) determination is increasingly used in clinical and forensic toxicology to document ethanol consumption. The enzymes involved in EtG production, as well as potential interactions with common drugs of abuse, have not been extensively studied.

**Methods:** Activities of human liver (HLM), kidney (HKM) and intestinal (HIM) microsomes, as well as of twelve major human recombinant UDP-glucuronosyltransferases (UGTs), toward ethanol (50 and 500 mM) were evaluated *in vitro* using liquid chromatography-tandem mass spectrometry. Enzyme kinetic parameters were determined for pooled microsomes and recombinant UGTs with significant activity. Individual contributions of UGTs were estimated using the relative activity factor (RAF) approach, proposed for scaling activities obtained with cDNA-expressed enzymes to HLM. Interaction of morphine, codeine, lorazepam, oxazepam, nicotine, cotinine, cannabinol and cannabidiol (5, 10, 15 mg/L) with ethanol (1.15, 4.6, 11.5 g/L; i.e. 25, 100, 250 mM) glucuronidation was assessed using pooled HLM.

**Results:** Ethanol glucuronidation intrinsic clearance ( $Cl_{int}$ ) was 4- and 12.7- times higher for HLM than for HKM and HIM, respectively. All recombinant UGTs, except UGT1A1, 1A6 and 1A10, produced EtG in detectable amounts. UGT1A9 and 2B7 were the most active enzymes, each accounting for 17% and 33% of HLM  $Cl_{int}$ , respectively. Only cannabinol and cannabidiol significantly affected ethanol glucuronidation. Cannabinol increased ethanol glucuronidation in a concentration-dependent manner, whereas cannabidiol significantly inhibited EtG formation in a non-competitive manner ( $IC_{50}$ =1.17 mg/L;  $K_i$ =3.1 mg/L).

**Conclusions:** UGT1A9 and 2B7 are the main enzymes involved in ethanol glucuronidation. In addition, our results suggest that cannabinol and cannabidiol could alter significantly ethanol glucuronidation.

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### INTRODUCTION

Alcohol abuse is one of the most frequent addictions worldwide. It is the third leading cause of preventable death in the United States (Mokdad et al., 2004) and also causes many serious social and health problems (Ferreira and Willoughby, 2008). After oral administration, ethanol is readily absorbed by passive diffusion through the stomach wall (approx. 20%) and the intestine wall (approx. 80%) (Norberg et al., 2003). Its elimination occurs primarily through metabolism (95–98%), with minor fractions of the administered dose being excreted unchanged in breath (0.7%), sweat (0.1%), and urine (0.3%) (Holford, 1987). Ethanol metabolism occurs primarily in the liver, mainly through oxidation catalyzed by alcohol dehydrogenases, aldehyde dehydrogenases, cytochrome P450 2E1 (CYP2E1), and catalase (Zakhari, 2006; Agarwal, 2001). Besides these pathways, glucuronidation by UDP-glucuronosyltransferases (UGTs) represents a minor detoxifying pathway for ethanol (< 0.1% of complete alcohol elimination) (Jaakonmaki et al., 1967; Dahl et al., 2002; Goll et al., 2002). Ethanol itself is a good marker of alcohol consumption. However, it has a rapid elimination rate from blood (0.10 to 0.20 g/L/h in occasional drinkers and 0.25 to 0.35 g/L/h in chronic drinkers) (Jones, 2010) which makes its detection in body fluids only possible during a relatively short time after alcohol intake. Ethylglucuronide (ethyl- $\beta$ -D-6-glucuronic acid, EtG) can be detected in various body fluids, tissues, and hair. It is eliminated with a terminal half-life of 2 to 3 h (Schmitt et al., 1997) and presents a particularly interesting detection window in body fluids (up to 8 h and 5 days after complete ethanol elimination, in serum and urine, respectively (Schmitt et al., 1997 ; Wurst et al., 1999). EtG determination is also much more sensitive and specific than conventional markers (e.g. gamma-glutamyl transferase, alanine aminotransferase, aspartate aminotransferase, mean corpuscular volume and carbohydrate-deficient transferrin). It has recently become of more

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interest in clinical and forensic toxicology. Cutoff for its determination in urine and hair have been reported (Kintz, 2010; Rohrig et al., 2006). However, considering the numerous factors that could affect glucuronidation (e.g. genetic polymorphisms, inhibition or induction by xenobiotics, age ...), the relevance of these cutoffs in clinical and forensic practices can be questioned in certain circumstances (Kiang et al., 2005). Some reports have indeed documented a high interindividual variability in EtG production (Halter et al., 2007; Paul et al., 2008). Halter *et al.* (2007) showed that the administration of a conventional dose of ethanol to 13 individuals resulted in highly variable (8-fold) maximum concentrations of serum EtG, and that EtG concentrations did not correlate with blood ethanol concentrations (Halter et al., 2007). This marked interindividual variability in EtG levels could be attributed to variable activities of UGTs involved in ethanol metabolism. As these enzymatic activities are controlled by genetic polymorphisms and can be affected by concomitantly administered drugs (Kiang et al., 2005), the impact of these factors on EtG production deserves further investigation. The enzymology underlying glucuronidation of ethanol has not received much attention to date. Only one *in vitro* study by Foti and Fisher (2005) showed that multiple UGTs are likely to be responsible for catalyzing EtG formation, with UGT1A1 and UGT2B7 predominating (Foti and Fisher, 2005). The purpose of the present *in vitro* study was first of all to evaluate qualitatively and quantitatively the specific contribution of UGT enzymes in EtG formation and secondly to study the impact of the co-administration of drugs frequently used by consumers of ethanol (opioids: morphine, codeine; benzodiazepines: oxazepam, lorazepam; tobacco: nicotine, cotinine; and cannabis: cannabinal (CBN) and cannabidiol (CBD)) on the production of EtG.

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### MATERIALS AND METHODS

#### Chemicals and reagents

Ethylglucuronide (EtG; ref: EGL-332-10) and its deuterated analogue EtG-D5 (ref: EGL-780-10), used as an internal standard, were obtained from Lipomed (Souffelweyersheim, France). Bovine serum albumin (BSA; product number A7906), uridine 5'-diphosphoglucuronic acid (UDPGA), dimethyl sulfoxide (DMSO), chenodeoxycholic acid (CDCA), mefenamic acid (MEF), trifluoperazine (TFP), alamethicin, glafenine, Morphine, codeine, lorazepam, oxazepam, CBN, CBD, nicotine, and cotinine were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ethyl alcohol (17 M, 99.8%) and MgCl<sub>2</sub> were purchased from Merck (Darmstadt, Germany). Tris-HCl was obtained from Qbiogene (Illkirch, France), and azidothymidine (AZT) was a kind gift from GlaxoSmithKline (Nanterre, France). Milli-Q water and all solvents were of HPLC grade.

#### Microsomes and enzymes

Human microsomes were prepared from individual liver (n = 44), intestine (n = 19) and kidney (n=7) samples derived from surgical specimens. Samples were obtained from Biopredic International (Rennes, France) for liver and intestine, and from the pathology department of Limoges University Hospital for kidney. All samples were collected after donors had given their informed consent, in accordance with the French bioethics law. The tissues were microscopically examined by a pathologist to document normal histology, immediately frozen in liquid nitrogen, and then stored at -80°C until use. Microsomes were obtained by differential centrifugation, as described previously (Picard et al., 2004). Protein concentration of the microsomal suspensions was measured according to the Bradford's method (Bradford, 1976). Three pools derived from

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all individual human liver, intestine, and kidney microsomes (HLM, HIM, and HKM) were prepared.

Membrane fractions from baculovirus-infected insect cells expressing human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 (Supersomes<sup>®</sup>), as well as a control preparation, were purchased from BD Biosciences Gentest (Woburn, MA, USA).

### Incubation procedures

In preliminary experiments, linearity of EtG formation rate *versus* microsomal protein concentration (0.5-1 mg/mL) and incubation time (10-75 min) was checked by incubating 250 mM ethanol (diluted in Tris-HCl) with HLM (data not shown). It was also assessed that no major evaporation of ethanol occurred during incubation by comparing ethanol concentrations (25; 100; 1000 mM) prior to, and after 45 min at 37°C in the incubation buffer, using a validated gas chromatography-flame ionization detection method (Clarus 480, Perkin-Elmer, Courtaboeuf, France). Control incubations without substrate, microsomes or co-substrate (UDPGA dissolved in Tris-HCl) were also performed.

As  $K_m$  values for substrates of UGT1A9 and 2B7 can be over-estimated in the absence of BSA (Rowland et al., 2007; Rowland et al., 2008), incubations with HLM, as well as with recombinant UGT1A9 and 2B7, in the presence of 2% BSA were also performed. The incubation mixture (100  $\mu$ L) contained 0.5 mg/mL microsomal proteins (microsomes or Supersomes<sup>®</sup>), 2 mM UDPGA, 10 mM MgCl<sub>2</sub> and 0.1 M Tris-HCl buffer (pH 7.4). Microsomes were first activated by incubation with the pore-forming peptide alamethicin (100  $\mu$ g/mg microsomal proteins) for 15 min on ice. UDPGA and microsomes were then pre-incubated at 37°C for 5 min before starting the reaction by addition of the substrate (5-1000 mM ethanol). After 45 min

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incubation at 37°C, the reaction was stopped by addition of ice-cold acetonitrile (50 µL). After centrifugation (10 000 g; 5 min) to spin down proteins, the supernatant was stored at -20°C until analysis.

### LC-MS/MS analysis

Sample preparation consisted in the addition of 20 µL of internal standard EtG-D5 solution (at 2 mg/L in acetonitrile) to 150 µL of incubation supernatants. An aliquot (15 µL) of each sample was then injected into the HPLC-tandem mass spectrometry (MS/MS) system.

*Liquid chromatography:* Chromatographic system consisted of a Shimadzu SIL20 AC high-pressure gradient pumping system equipped with two LC20 AD binary pumps, a SIL20-ACHT injector and a CTO-10ASvp column oven and an upisphere 100A°, 3 µm (100 x 2.1 mm) column (Interchim, France), maintained at 25°C. The mobile phase, delivered at a constant flow-rate of 200 µL/min, consisted of a linear gradient of solution A (5 mM ammonium acetate pH 6.8) and solution B (acetonitrile / 5 mM ammonium acetate pH 6.8, 98/2; v/v), as follows: 0-0.1 min, 5% B; 0.1-1 min, 5% to 20% B; 1-5 min, 20% to 30% B; 5-6 min, 30% to 80% B; 6-9 min, 80% B; 9-10 min, decrease from 80% to 5% B; 10-16 min, 5% B.

*Mass spectrometry:* Detection was performed using a 4000 QTRAP™ LC-MS/MS System (ABSciex, Foster City, CA) tandem mass spectrometer equipped with a Turbo V™ Ionspray source and controlled by Analyst® 1.5 software. Negative ionization was performed with the following settings: ion spray voltage, -4500 V; curtain gas, 20; ion source gas flow rates 1 and 2 at 15 and 30 units, respectively; declustering potential, -70 V; and temperature set at 500°C.

*Acquisition conditions:* Acquisitions were performed in multiple reaction monitoring (MRM) mode using three transitions for EtG (m/z 221.0→75.0; m/z 221.0→85.0, and m/z 221.0→112.9)



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and two for EtG-D5 ( $m/z$  226.0→75.0;  $m/z$  226.0→85.0). Transition  $m/z$  221.0→75.0 was retained for EtG quantification, whereas transitions  $m/z$  221.0→85.0 and  $m/z$  221.0→112.9 were used for identification.

*Analytical validation:* The validation parameters were based on the following criteria: specificity, linearity, precision (repeatability and intermediate precision), reproducibility, accuracy, limit of detection, and limit of quantification (LOQ).

### Extrapolation of recombinant UGT activities

Relative Activity Factor (RAF) approach is proposed for scaling enzymatic activities obtained using cDNA-expressed enzymes to human microsomes. This approach is well established for cytochromes P450 (Crespi and Miller, 1999) and has also been used for UGTs (Toide et al., 2004; Rouguieg et al., 2010). In this study, we calculated RAFs for UGT1A3, 1A4, 1A9, and 2B7 in order to scale velocities obtained using UGT Supersomes<sup>®</sup> to HLM. CDCA (15  $\mu$ M), TFP (40  $\mu$ M), MEF (0.5  $\mu$ M), and AZT (70  $\mu$ M) were used as selective substrates for UGT1A3, 1A4, 1A9, and 2B7, respectively. In the case of UGT1A7, 1A8, 2B4, 2B15, and 2B17, RAFs could not be calculated as no adequate probe has been identified so far. Briefly, incubations were performed using the same incubation procedure as described above except that reactions were terminated by addition of 80  $\mu$ L of ice-cold methanol containing glafenine (13.5  $\mu$ M) as an internal standard. Samples were centrifuged at 10 000 g for 5 min at 4°C to pellet the precipitated proteins. CDCA, TFP, MEF, and AZT glucuronides were determined using LC-MS/MS, as described previously (Gagez et al., 2012). RAF values were calculated by dividing the mean activity of glucuronide formation ( $n = 2$  experiments) obtained in HLM by that obtained in Supersomes<sup>®</sup> (both expressed in pmol/mg protein/min). The scaled velocities of UGT1A3, 1A4,

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1A9 and 2B7 were calculated by multiplying the rate of metabolism observed with these enzymes ( $V_i$ ) by the corresponding RAF. The contribution (%) of each isoform (contribution<sub>*i*</sub>) was calculated using the following equation:

$$\text{Contribution}_i (\%) = (V_i \times \text{RAF}_i) / V_{\text{pooled HLM}} \times 100$$

### Chemical inhibition experiments

The glucuronidation of ethanol by HLM in the presence of niflumic acid (2.5  $\mu\text{M}$ ) and fluconazole (2.5 mM), used as a specific inhibitor of UGT1A9 and UGT2B7, respectively, was investigated (Gaganis et al., 2007; Uchaipichat et al., 2006). These experiments were conducted in triplicate, as described in “incubation procedures” using 250 mM ethanol. Results are reported as percentages of inhibition of control activities determined in the absence of inhibitor.

### Interaction Experiments

The impact of the co-incubation of eight compounds (morphine, codeine, lorazepam, oxazepam, CBN, CBD, nicotine, and cotinine) on HLM-catalyzed ethanol glucuronidation was assessed at three ethanol concentrations (25, 100, and 250 mM; *i.e.* 1.15, 4.6, and 11.5 g/L). Potentially-interacting drugs were tested at three concentrations (5, 10 and 15 mg/L. The effect of CBN and CBD (15 mg/L) on UGT1A9- and UGT2B7-catalyzed ethanol (25 mM) glucuronidation was also assessed. Nicotine and cotinine were dissolved in methanol. Other substrates were dissolved in DMSO. Solvent concentration in the incubation mixture did not exceed 1% (v/v). Incubation and analytical conditions were as described above. Enzyme activities were compared with those determined in the absence of potentially-interacting drugs (control incubation which contained

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the same volume of solvent). Differences are reported as percentage of control activity derived from triplicate measurements.

### Data Analysis

All data points represent the mean of either duplicate or triplicate experiments. Enzyme kinetic parameters were estimated by fitting Michaelis-Menten equation to the data using nonlinear regression (Prism version 5.01; GraphPad Software Inc., La Jolla, CA, USA) and are presented as a regression parameter estimate  $\pm$  S.D. of the estimate. The fit of the model to the data was assessed from the Akaike information criterion.

The concentration of the inhibitor required to produce 50% inhibition of the enzymatic activity ( $IC_{50}$ ) was determined from the curves plotting enzymatic activity versus inhibitor concentrations. The inhibition mechanism was estimated from the figure plotting  $1/V$  versus  $1/S$ . The  $K_i$  and the mode of inhibition were determined by fitting competitive, uncompetitive, noncompetitive, and mixed inhibition models to the kinetic data using nonlinear regression analysis. The mode of inhibition that best described the data was determined by comparing the fit of various models using the Akaike information criterion. Differences between metabolic rates with and without potential inhibitors were considered significant when p value was less than 0.05. All statistical analyses were performed using non parametric tests (Kruskall-Wallis or Mann-Whitney) using GraphPad Software.

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## RESULTS

### Analytical validation

Ethylglucuronide was detected at a retention time of 5.83 min following three specific MRM transitions (Fig. 1). The quantification limit (LOQ) was 5 ng/mL. Good linearity was observed from the LOQ up to 500 ng/mL ( $r > 0.999$ ). Intra- and inter-day precisions and mean relative errors showed relative standard deviations (RSD) lower than 10% over the calibration range.

### Ethanol glucuronidation in human microsomes

The specific formation of EtG by HLM, HIM, and HKM was confirmed by retention time and MS/MS spectra (Fig. 1). EtG was not detectable at substrate concentrations lower than 5 mM. Kinetics of ethanol glucuronidation was best fitted by the Michaelis-Menten equation (Fig. 2). Substrate concentration higher than 1000 mM resulted in a drastic decrease of EtG formation rate, presumably caused by the denaturation of the enzymes (data not shown). Kinetic parameters including  $K_m$ ,  $V_{max}$ , and intrinsic clearance ( $Cl_{int}=V_{max}/K_m$ ) for HLM, HIM, and HKM are reported in Table 1. The three preparations showed very low affinity for ethanol. Maximal formation rate ( $V_{max}$ ) and  $K_m$  were about 1.4-fold and 5.8-fold higher, respectively, with HKM compared to those with HLM.  $V_{max}$  and  $K_m$  were about 27-fold and 2.1-fold lower, respectively, with HIM compared to those with HLM. As a result, the hepatic intrinsic clearance of ethanol appeared 4-fold and about 13-fold higher than that observed with HKM and HIM, respectively.

Regarding the potential albumin effect on HLM activities, 2% BSA increased the  $Cl_{int}$  of ethanol glucuronidation by about 80%.  $K_m$  (and  $Cl_{int}$ ) values determined in the absence and presence of BSA were 1082 mM (0.70 nl/mg/min) and 999.6 mM (1.25 nl/mg/min), respectively.

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### **Ethanol glucuronidation in recombinant human UGTs**

In order to identify the human UGTs involved in ethanol glucuronidation, incubations with 12 recombinant UGT enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) were performed using 50 mM and 500 mM of ethanol. Our primary results showed that all tested isoforms, except UGT1A1, 1A6, and 1A10, can produce EtG in significant amounts (i.e. > LOQ; Fig. 3). UGT2B7 exhibited the highest activity at both concentrations (50 mM and 500 mM) with an EtG production of 5.1 and 27.5 pmol/mg protein/min, respectively. EtG production followed the classical Michaelis–Menten equation in all cases; Enzyme kinetics of ethanol glucuronidation by recombinant hepatic UGT enzymes (Fig. 2). The kinetic parameters of EtG production were then determined for the nine UGTs that produced detectable amounts of EtG (Table 1). Regarding the potential albumin effect on UGT1A9 and 2B7 activities, 2% BSA had a negligible effect on the kinetics of ethanol glucuronidation by recombinant UGT1A9 and 2B7.  $K_m$  (and  $Cl_{int}$ ) values determined in the absence and presence of BSA were 501 mM (0.103 nl/mg/min) and 407 mM (0.147 nl/mg/min) for UGT1A9, respectively, and 619 mM (0.109 nl/mg/min) and 548 mM (0.152 nl/mg/min) for UGT2B7, respectively.

The relative contribution of UGT1A3, 1A4, 1A9, and 2B7 to EtG production in HLM could be extrapolated using the RAF approach. UGT1A9 and 2B7 were the major contributors (Table 1); together these two enzymes were found to account for about 50% of EtG hepatic production. The contribution of UGT1A3 and 1A4 was about 3%.

### **Effect of niflumic acid and fluconazole on the glucuronidation of ethanol by HLM**

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Niflumic acid and fluconazole resulted in a limited inhibition of ethanol glucuronidation in HLM ( $9\pm 5\%$  and  $18\pm 9\%$ , respectively).

### **Effect of common drugs of abuse on the glucuronidation of ethanol by HLM**

Among all the drugs tested (morphine, codeine, lorazepam, oxazepam, CBN, CBD, nicotine, and cotinine), only CBN and CBD significantly affected ethanol glucuronidation (Fig. 4). CBN significantly increased the glucuronidation of ethanol in a concentration-dependent manner (Fig. 4C; Kruskal-Wallis test:  $p < 0.05$ ), whereas CBD significantly inhibited EtG production with an  $IC_{50}$  value of 1.17 mg/L (Fig. 4C; Kruskal-Wallis test:  $p < 0.05$ ). Inhibition kinetics demonstrated a noncompetitive inhibition with a  $K_i$  of 3.1 mg/L (Fig. 5). In order to identify which UGT was involved in these effects, the impact of CBN or CBD (15 mg/L) on the glucuronidation of ethanol (25 mM) by recombinant UGT1A9 and 2B7 was assessed. UGT1A9 was inhibited by both CBN and CBD ( $-34\pm 12\%$  and  $-49\pm 14\%$ , respectively;  $p = 0.05$ ). On the other hand, the activity of UGT2B7 was significantly increased by CBN ( $+429\pm 112\%$ ;  $p = 0.05$ ), whereas it was inhibited by CBD ( $-70\pm 7\%$ ;  $p = 0.05$ ; data not shown).

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### DISCUSSION

Using human microsomes, we showed that liver, intestine and kidney can produce EtG. The  $Cl_{int}$  of HLM was 4- and 13-times higher than those of HKM and HIM, respectively, which strongly suggests that ethanol is primarily glucuronidated by the liver, and that kidney and intestine tissues play only minor roles in this metabolic pathway.

The low affinity of ethanol for liver UGTs observed here is in accordance with data published by Jurowich *et al.* (2004), who found comparable values of  $K_m$  using HLM ( $373.4 \pm 47.6$  mM vs  $1082 \pm 343.3$  mM here) (Jurowich *et al.*, 2004). It is also in the range of the affinities obtained with recombinant enzymes (from 133 mM for UGT2B17 to 1706 mM for UGT1A4). In contrast, Foti and Fisher (2005) reported an apparent  $K_m$  of  $0.17 \pm 0.08$  mM using HLM. We have no clear explanation for this discrepancy. It could possibly be attributed to differences in experimental conditions, such as the type of reagent use to circumvent UGT latency (alamethicin vs CHAPS), the absence of normalization of enzyme activity with UGT content, and more importantly, the range of tested ethanol concentrations.

In humans, EtG represents less than 0.1% of complete ethanol elimination. It seems thus logical that the affinity for UGT enzymes is less than that for hepatic alcohol dehydrogenase (ADH;  $K_m = 0.2$  to  $2.0$  mM) and CYP2E1 ( $K_m = 10$  mM), two enzymes with a major role in ethanol metabolism (Zakhari, 2006; Hamitouche *et al.*, 2006).

Although multiple UGTs (especially UGT1A1 and 2B7) have already been highlighted as competent isoforms for EtG hepatic formation (Foti and Fisher, 2005), their specific contributions were unclear. Using the RAF approach, we evaluated quantitatively the role of UGT1A3, 1A4, 1A9, and 2B7. RAFs were calculated from metabolic rates obtained by

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incubating CDCA, TFP, MEF, and AZT with recombinant enzymes and pooled HLM. The choice of these substrates and their tested concentrations was supported by the literature (Trottier et al., 2006; Court, 2005), as well as by a recent study conducted in our group (Gagez et al., 2012). We found that UGT1A9 and UGT2B7 were responsible for approx. 17% and 37% of liver ethanol glucuronidation, respectively.

Niflumic acid and fluconazole are considered as specific inhibitors of UGT1A9 and 2B7, respectively (Gaganis et al., 2007; Uchaipichat et al., 2006). They resulted only in a moderate inhibition of ethanol glucuronidation by HLM, suggesting an implication of the two UGTs of approx. 30%. However, ethanol was tested at a concentration 10 000- and 100-fold higher than those of UGT1A9 and 2B7 inhibitor, respectively. It is possible that ethanol could overcome competitive inhibitions.

BSA is known to affect the kinetics of UGT1A9 and 2B7 (Rowland et al., 2007; Rowland et al., 2008). Consistent with previous observations, we observed a decrease in enzyme  $K_m$  and a consecutive increase of  $Cl_{int}$  but this effect was limited. The nonspecific binding of ethanol to BSA could result in a falsely elevated estimation of the  $K_m$  but this seems unlikely because ethanol has not been reported to bind to albumin (at least *in vivo*). Another possible explanation is that we did not use fatty-acid free BSA, which would presumably have resulted in a more accurate estimation of the kinetic parameters. Nonetheless, a more pronounced BSA effect would only have strengthened our conclusion on the predominant role of UGT1A9 and 2B7.

The RAF approach predicted a limited role of UGT1A3 and 1A4 (about 3%) in EtG hepatic production. Because of the absence of specific substrates for UGT2B4, 2B15, and 2B17, we could only hypothesize that these isoforms would account for the remaining part of ethanol glucuronidation in HLM. As UGT1A7 and 1A8 are not expressed in the liver (Court et al., 2012;



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Ohno and Nakajin, 2009) and as no specific substrates are available for these UGTs, their contribution to ethanol glucuronidation could not be precisely assessed. Furthermore, as some human UGTs are not commercially available as recombinant enzymes, several other hepatic and extra-hepatic UGTs (including UGT1A5, 2B10, 2B11, 2B28, 2A1, 2A2, and 2A3) were not studied and their contribution toward EtG formation cannot be ruled out. Considering their low expression levels in human liver, kidney, and intestine, their contribution to ethanol metabolism is likely to be minor (Court et al., 2012; Ohno and Nakajin, 2009; Sneitz and others, 2009).

We found almost no activity with UGT1A1 (EtG formation rate < LOQ), which contradicts the data of Foti and Fisher (2005). This discrepancy could possibly be attributed to the different commercial sources of UGT used. We could not test this hypothesis as the Panvera UGT1A1 Baculosomes® (used by Foti and Fisher, 2005) was no longer available at the time of the present study.

Another possible confounding factor is the effect of ethanol on UGT activities. Ethanol (> 0.5% v/v) can substantially inhibit UGT2B17 and 1A6 (Uchaipichat et al., 2004). Our initial screening for competent UGTs was performed using 0.3 and 3% v/v ethanol (i.e. 50 and 500 mM). We cannot exclude that the activity of UGT2B17 and UGT1A6 had been under-estimated. However, it is unlikely that other UGTs, and specifically UGT1A1, had been affected in particular when using 0.3% v/v ethanol. On the other hand, kinetic experiments required up to 6% of ethanol (v/v), which possibly influenced the estimation of kinetic parameters for other UGTs.

Several drugs are inhibitors of UGT-mediated glucuronidation (Kiang et al., 2005), but drug interactions with ethanol glucuronidation have not been studied so far. Paul *et al.* (2008) compared the mean concentration of EtG in hair samples between a group of narcotic and drug

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consumers (opiates, cocaine, amphetamines, methamphetamine, benzodiazepines, and cannabis) and a group of non-consumers. The mean concentration of EtG was significantly higher in the drug-negative group than that found in the drug-positive group (0.107 vs 0.011 ng/mg;  $p < 0.05$ ) (Paul et al., 2008). To better understand such possible interactions, we performed *in vitro* co-incubations of ethanol with various compounds frequently used by alcohol consumers, using pooled HLM. The eight potential inhibitors tested here were known UGT substrates. Morphine and codeine are metabolized by UGT2B7 (Coffman et al., 1997), and lorazepam and oxazepam by UGT2B7 and 2B15 (Court et al., 2002; Chung et al., 2008). CBN is metabolized with high affinity by UGT1A9 and is also substrate for UGT2B7 and the extra-hepatic UGT1A7 and 1A10 (Mazur et al., 2009). CBD is metabolized less intensively than CBN by UGT1A9, UGT2B7, and UGT2B17 (Mazur et al., 2009). Finally, nicotine is a substrate for UGT1A4, 2B10, and 2B7, and cotinine for UGT1A4 and 2B10 (Kaivosari et al., 2007).

Inhibition screening studies were performed at ethanol concentrations of 25, 100, and 250 mM (*i.e.* 1.15, 4.6, and 11.5 g/L) which are lower than HLM apparent  $K_m$  and close to expected blood concentrations in the context of abusive intake of alcoholic beverages. Morphine, codeine, and benzodiazepines were tested at concentrations ranging from 1- to 30-times the blood levels usually encountered in clinical or toxicological settings. Morphine, codeine, nicotine, and cotinine did not modify EtG *in vitro* formation rate. Lorazepam and oxazepam produced a minor, but not significant, increase of EtG formation by HLM. These results tend to exclude a potential effect of these drugs on ethanol glucuronidation *in vivo*, in particular at lower therapeutic or toxic levels. In contrast, CBD exhibited a strong non-competitive inhibition of ethanol glucuronidation which could be attributed to the inhibition of both UGT1A9 and 2B7. On the other hand, CBN increased ethanol glucuronidation in a concentration-dependent manner, which appears to result

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from the modulation of UGT2B7 activity. Although heterotropic activation of UGT2B7 has been demonstrated (Uchaipichat et al., 2008), the precise mechanism of interaction between UGT2B7-catalyzed CBN and ethanol glucuronidations remains to be further investigated. In addition, it is noteworthy that the cannabinoid concentrations used here are very likely exceeding expected *in vivo* concentrations. It is well known that CBN and CBD contents vary widely in various cannabis sources, but scarce information is available on their blood and tissue concentrations in cannabis consumers. Recently, median plasma maximum concentrations were reported to be about 2.0 µg/L for CBD and 3.6 µg/L for CBN after smoking one 6.8% THC cannabis cigarette (Schwope et al., 2011). However, plasma or whole-blood cannabinoid concentrations do not reflect tissue concentrations, in particular liver concentrations, which can be largely higher for CBD and CBN (Gronewold and Skopp, 2011). Consequently, the relevance of this cannabinoid/ethanol interaction on EtG formation remains uncertain and needs to be further investigated.

Other sources of interaction with EtG levels might also exist and were not studied here. One can hypothesize that EtG renal elimination involves active efflux transporters and that competitive inhibition of these transporters by glucuronides of other drugs of abuse can occur.

In conclusion, UGT1A9 and 2B7 are predominantly involved in ethanol glucuronidation (50% of the overall EtG formation) and appear to be differently affected by high concentration of cannabinoid compounds. Additional studies are required to assess the potential contribution of UGT1A9 and 2B7 genetic polymorphisms, as well as of cannabis consumption, to the inter-individual variability of EtG production.

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**Authorship Contributions**

*Participated in research design:* Al Saabi, Allorge, Tournel, Gaulier, Marquet, and Picard.

*Conducted experiments:* Al Saabi, Sauvage.

*Performed data analysis:* Al Saabi, Allorge, and Picard.

*Wrote or contributed to the writing of the manuscript:* Al Saabi, Allorge, and Picard.

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**Footnotes**

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### FIGURE LEGENDS

**Figure 1:** Multiple reaction monitoring (MRM) chromatograms of ethylglucuronide (EtG) after incubation of human liver microsomes with ethanol (100 mM). Transition of  $m/z$  226→75 was used for IS (A), 221.0→112.9 and 221.0→85.0 were used for EtG identification (B, C), and transition of 221.0→75.0 was used for EtG quantification (D).

**Figure 2:** Enzyme kinetics of ethanol glucuronidation by HLM, HKM, and HIM (a); and recombinant hepatic UGT enzymes (b). Glucuronidation activities of microsomes and recombinant proteins were measured by incubating membrane fractions with increasing concentrations of ethanol at a constant concentration of UDP-GA (2 mM). Each point represents mean  $\pm$  SD of duplicate experiments (triplicate experiments for HLM, UGT1A9, and UGT2B7).

**Figure 3:** Ethylglucuronide (EtG) production following incubation of ethanol (50 mM and 500 mM) with 12 recombinant human UGTs (Supersomes). All tested isoforms, except UGT1A1, 1A6 and 1A10, produced EtG in detectable amounts ( $>$  limit of quantification; LOQ). Data (mean  $\pm$  SD of triplicate experiments) are expressed as activities relative to the most active isoform (UGT2B7) for each ethanol concentration (EtOH 50 mM  $\rightarrow$  5.1 pmol/(min/mg); 500 mM  $\rightarrow$  27.5 pmol/(min/mg)). EtG concentrations were not detectable at substrate concentration lower than 5 mM.

**Figure 4:** Effect of morphine, codeine, oxazepam, lorazepam (A), nicotine, cotinine (B), cannabiniol and cannabidiol (C) on ethanol (25 mM) glucuronidation. Results are expressed as the mean ( $\pm$  SD) percentage of inhibition compared to control incubation (triplicate experiments). \*, significantly different from control ( $p < 0.05$ ).

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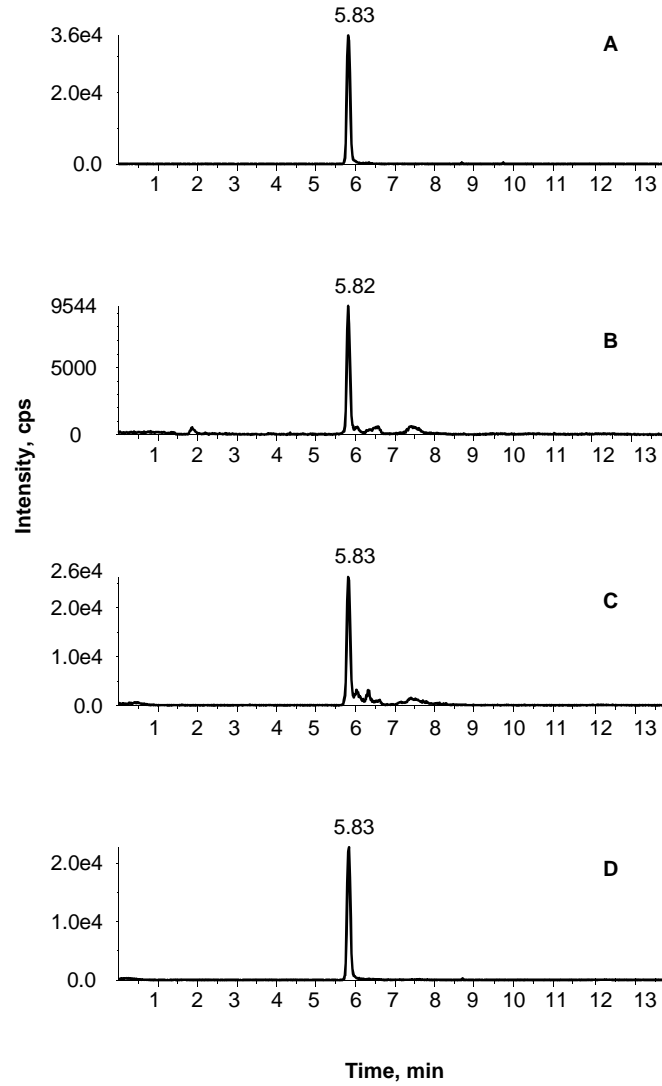
**Figure 5:** Lineweaver-Burk plots for the inhibition of ethanol glucuronidation by various concentrations of cannabidiol in human liver microsomes. Ethanol was incubated at 6 concentrations (range of 5 to 1000 mM). Each plot is derived from duplicate experiments.

**Table 1** Kinetic parameters ( $\pm$  S.E. of parameter fit) of ethylglucuronide formation by pooled human liver, kidney, and intestinal microsomes, and recombinant UGTs (Results are the mean of duplicate experiments). Contributions of UGT to ethanol glucuronidation at the hepatic level were calculated using the relative activity factor (RAF) approach.

	$K_m$	Apparent $V_{max}^a$	$Cl_{int}^a$	RAF	HLM scaled $Cl_{int}$	Contribution to the overall hepatic microsomal metabolism
	mM	pmol/mg/min	nl/mg/min		nl/mg/min	
HLM	1082 $\pm$ 343.3	757.9 $\pm$ 150.1	0.7004	na <sup>b</sup>		
HKM	6224 $\pm$ 6843	1081 $\pm$ 1049	0.1736	na		
HIM	508.6 $\pm$ 102.7	28.34 $\pm$ 2.73	0.0550	na		
UGT1A3	385.1 $\pm$ 106.7	10.71 $\pm$ 1.4	0.0278	0.31	0.0086	1.2%
UGT1A4	1706 $\pm$ 699.1	33.64 $\pm$ 9.6	0.0197	0.67	0.0132	1.9%
UGT1A7	193 $\pm$ 74.88	3.05 $\pm$ 0.41	0.0155	nd <sup>c</sup>		
UGT1A8	312.1 $\pm$ 78.57	6.73 $\pm$ 0.68	0.0214	nd		
UGT1A9	501 $\pm$ 87.77	52.1 $\pm$ 4.43	0.1039	1.15	0.1196	17.1%
UGT2B7	619.7 $\pm$ 157.7	67.68 $\pm$ 8.97	0.1092	2.1	0.2293	32.7%
UGT2B4	442.1 $\pm$ 112.2	20.95 $\pm$ 2.41	0.0472	nd		
UGT2B15	203.1 $\pm$ 89.67	5.2 $\pm$ 0.81	0.0256	nd		
UGT2B17	132.9 $\pm$ 50.08	5.6 $\pm$ 0.66	0.0421	nd		

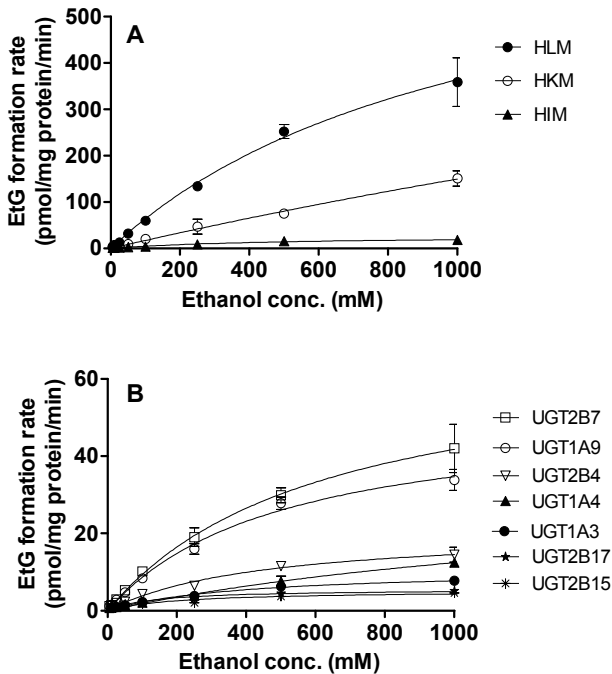
<sup>a</sup> Activities are not normalized to microsomes or supersomes UGT contents;

<sup>b</sup> na, not applicable; <sup>c</sup> nd, not determined.

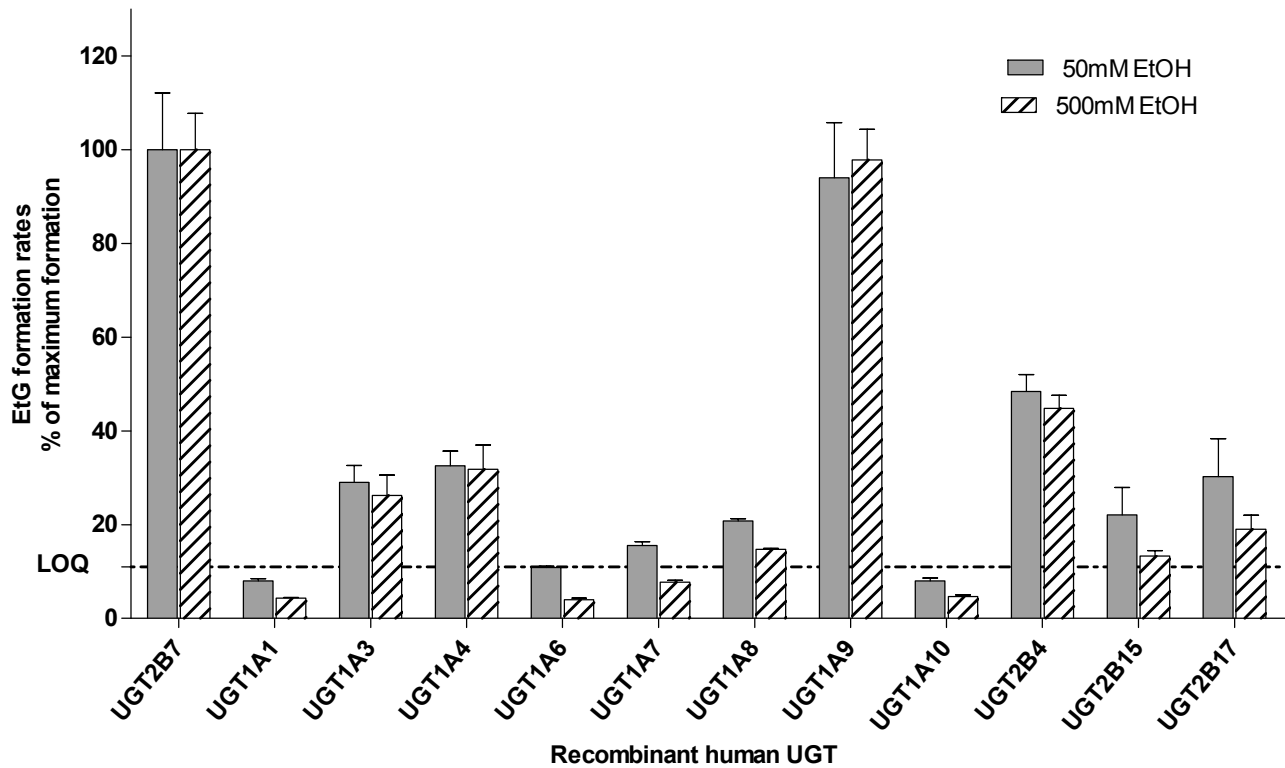


**Figure 1**

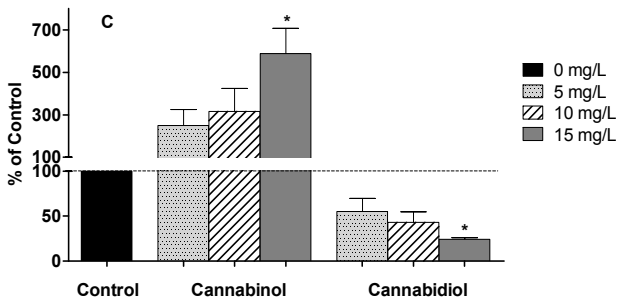
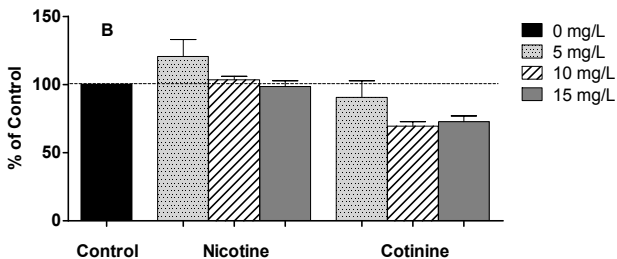
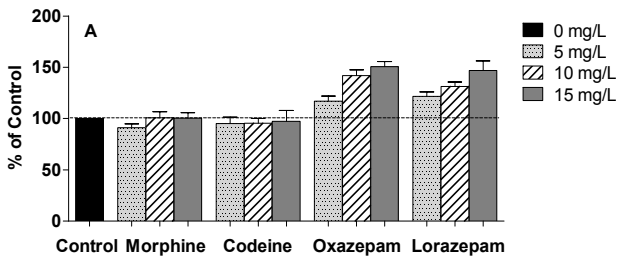




**Figure 2**



**Figure 3**



**Figure 4**

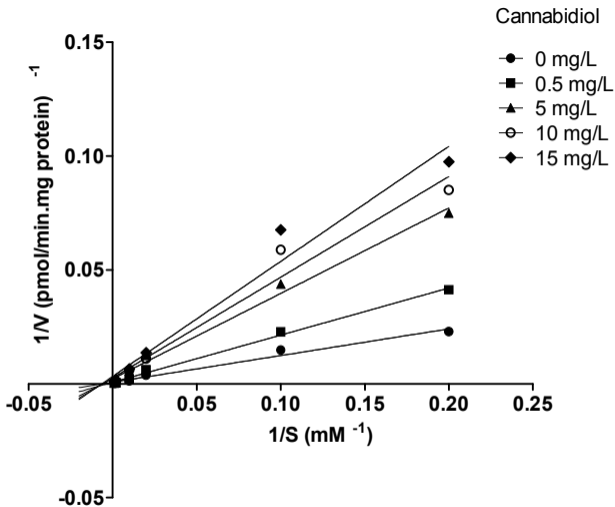


Figure 5