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Relevance of Ethylglucuronide as a marker of alcohol consumption Development of dosage methods and study of factors potentially affecting its production

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A mes parents,

à ma sœur et à mon frère…

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3. Al Saabi A., Picard N., Tournel G., and Allorge D. Influence of UGT1A9 and UGT2B7 genetic polymorphisms on ethylglucuronide (EtG) production *in vitro* (article in preparation).

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Other scientific publications

1. Ducroquet A., Leys D., Al Saabi A., Richard F., Cordonnier C., Girot M., Deplanque D., Casolla B., Allorge D., Bordet R. Influence of Chronic Ethanol Consumption on the Neurological Severity in Patients With Acute Cerebral Ischemia. Stroke. 2013 May 16. [Epub ahead of print].

2. Soichot M., Hennart B., Al Saabi A., Leloire A., Froguel P., Levy-Marchal C., Godefroy O.P., and Allorge D. Identification of a Variable Number of Tandem Repeats Polymorphism and Characterization of LEF-1 Response Elements in the Promoter of the IDO1 Gene. PLoS One. 2011;6(9):e25470.

ABBREVIATIONS

5-HIAA	5-hydroxyindole–3–acetic acid
5-HT	5-hydroxytryptamine (Serotonin)
5-HTOL	5-hydroxytryptophol
ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
ALDH	acetaldehyde dehydrogenase
ALT	alanine aminotransferase
APA	acetaldehyde-protein adduct
Apo J	apolipoprotein J
AST	aspartate aminotransferase
AUDIT	alcohol use disorders identification test
AZT	zidovudine
BAC	blood alcohol concentration
β-HEX	beta hexosaminidase
CBD	cannabidiol
CBN	cannabinol
CDT	carbohydrate deficient transferrin
CYP	Cytochrome P450
EIA	enzyme immunoassay
ER	endoplasmic reticulum
ESI	electrospray ionization
EtG	ethylglucuronide
EtOH	ethanol
EtS	ethylsulfate
FAEE	fatty acid ethyl ester
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
FPM	first pass metabolism
g/d	gram per day
GC-FID	gas chromatography-flame ionization detection
GC-MS/MS	gas chromatography-tandem mass spectrometry
GGT	gamma-glutamyl transferase
HAA	hemoglobin–acetaldehyde adducts
HIM	human intestinal microsomes
HKM	human kidney microsomes
HLM	human liver microsomes
IU	international unit
L	liter

LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-PED	liquid chromatography with pulsed electrochemical detection
LOD	limit of detection
LOQ	limit of quantification
MCV	mean corpuscular volume
MeOH	methanol
MRM	multiple reaction monitoring
NCI	negative chemical ionization
PA	phosphatidic acid
PCR	polymerase chain reaction
PEth	phosphatidylethanol
PFPA	pentafluoropropionic acid
PLD	phospholipase D
RAF	relative activity factor
RSD	relative standard deviation
SA	sialic acid
THC	Δ-9-tetrahydrocannabinol
SIJ	sialic acid index of apolipoprotein J
SNP	single-nucleotide polymorphism
SPE	solid-phase extraction
SULT	sulfotransferase
TSA	total sialic acid
UDP	uridine diphosphate
UDP-GA	uridine 5’-diphosphate glucuronic acid
UGT	UDP-glucuronosyl transferase
VH	vitreous humor

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Relevance of ethylglucuronide as a marker of alcohol consumption: Development of dosage methods and study of factors potentially affecting its production

Abstract: Alcohol abuse is one of the most frequent addictions worldwide. It is frequently associated with an increased risk of accidents, violence, and can also lead to serious social and health problems. Therefore, the use of reliable markers to detect excessive punctual and/or chronic consumption of alcohol is necessary. Ethylglucuronide (EtG) has been proposed as a marker of alcohol consumption in a variety of clinical and forensic contexts. Compared with the indirect markers (e.g. CDT, GGT), this minor metabolite of ethanol is very sensitive and specific, and is quantifiable in diverse biological matrices. It is formed by conjugation of ethanol with uridine 5'-diphosphate glucuronic acid (UDP-GA) *via* the action of UDP-glucuronosyl transferase (UGT) enzyme family. However, the knowledge of the UGT isoforms involved in the glucuronidation of ethanol, and the potential sources of interindividual variability in the production of EtG are still not clearly established in humans. The aims of our work were (1) to develop and validate a method for the determination of EtG in different biological matrices by gas chromatography coupled with tandem mass spectrometry, (2) to identify and to study of the relative contribution of human UGT isoforms in the hepatic, renal and intestinal glucuronidation of ethanol, as well as to study of the impact of substances frequently used by consumers of ethanol on the hepatic production of EtG, (3) to study the impact of functional genetic polymorphisms of implicated UGTs on the hepatic production of EtG, and (4) to study the impact of *ante-mortem* consumption of cannabis and other illegal and/or medicinal drugs on EtG levels in *post-mortem* blood samples.

The main results of our study showed that (1) ethanol is primarily glucuronidated by the liver, and that kidney and intestine tissues play only minor roles in this metabolic pathway, (2) UGT1A9 and 2B7 were clearly identified as the main human UGTs involved in ethanol glucuronidation, (3) among the tested substances (opiates, benzodiazepines, cannabinoids, nicotine, and cotinine), only cannabidiol and cannabinol significantly affect the *in vitro* production of EtG, (4) the *UGT1A9* SNPs, c.-275T>A and IVS1+399T>C, significantly affect the *in vitro* production of EtG, and (5) drugs consumption (mainly benzodiazepines) seem to be associated with ratios of blood concentrations of EtG/ethanol significantly higher than those observed among only alcohol consumers or co-consumer of ethanol and cannabis. Taken together, these results show the existence of several factors that could potentially influence the production of EtG, and must be taken into account when interpreting its concentration *in vivo*.

Intérêt de l'éthylglucuronide comme marqueur d'alcoolisation : développement de méthodes de dosage et étude des sources de variabilité de sa production

Résumé: La consommation excessive d'alcool est associée à un risque accru d'accidents de la voie publique et d'actes de violence, et peut également conduire à plus ou moins long terme à des affections pathologiques graves, telles que des cancers. De nombreux marqueurs d'alcoolisation sont disponibles pour détecter une consommation excessive d'alcool, aiguë ou chronique et sont utilisés dans des domaines variés comme le suivi du sevrage alcoolique ou encore pour la restitution du permis de conduire. L'éthylglucuronide (EtG) est un marqueur d'alcoolisation de développement récent, utilisable en toxicologie clinique (alcoologie) et médicolégale. Par rapport aux marqueurs indirects d'alcoolisation (CDT, γ-GT), ce métabolite mineur de l'éthanol est très spécifique et est quantifiable dans diverses matrices biologiques. La production d'EtG est catalysée par des enzymes de la famille des UDP-glucuronosyl-transférases (UGT). Cependant, les UGT impliquées dans la glucuronoconjugaison de l'éthanol, ainsi que les sources potentielles de variabilité interindividuelle de la production d'EtG, sont encore mal connues. Nos travaux ont ainsi consisté à (1) développer et valider une méthode de dosage de l'EtG dans différentes matrices biologiques par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem, (2) identifier les UGT humaines impliquées dans la glucuronoconjugaison de l'éthanol et étudier leur contribution relative au niveau hépatique, ainsi qu'étudier l'impact de substances fréquemment utilisées par les consommateurs d'alcool sur la production d'EtG *in vitro*, (3) étudier l'impact de polymorphismes génétiques fonctionnels des UGT sur la production hépatique d'EtG, et enfin (4) évaluer l'impact de la consommation de cannabis et d'autres drogues sur la production d'EtG à l'aide de prélèvements *post-mortem*.

Ces travaux ont notamment permis de montrer que (1) l'éthanol est glucuronoconjugué principalement par le foie et, dans une moindre mesure, par les reins et l'intestin, (2) les UGT1A9 et UGT2B7 sont les deux enzymes majoritairement impliquées dans la glucuronoconjugaison de l'éthanol, quel que soit l'organe considéré, (3) parmi les substances testées (opiacés, benzodiazépines, nicotine et cotinine et cannabinoïdes), seuls le cannabidiol et le cannabinol affectent significativement la production d'EtG *in vitro*, (4) les SNP c.-275T>A et IVS1+399T>C affectant l'*UGT1A9* modifient significativement le taux de formation d'EtG *in vitro*, et (5) le rapport des concentrations EtG/éthanol apparaît significativement plus élevé chez des co-consommateurs de drogues que chez des consommateurs d'alcool seul, ou des co-consommateurs d'alcool et de cannabis. L'ensemble de ces résultats démontre l'existence de plusieurs facteurs pouvant potentiellement influencer la production d'EtG, et qui devraient donc être pris en considération lors de l'interprétation de sa concentration *in vivo*.

INTRODUCTION

The use of ethanol is a major global factor contributing to death (Fig.1), disease and injuries to the drinker through direct health impacts or to others through dangerous behaviors (*e.g.* alcohol consumption during pregnancy, child neglect and abuse, violence, traffic accidents, domestic or professional accidents, absenteeism in the workplace, or also suicides, etc.) *(WHO report, 2011)*.

Alcoholism is defined by the World Health Organization (WHO) and by the American Society of Addiction Medicine as "*a primary, chronic disease with genetic, psychosocial, and environmental factors influencing its development and manifestations. It is characterized by continuous or periodic impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking, most notably denial*". In fact, from a medical point of view, there is no clear definition of excessive alcohol consumption, but it is usual to consider that the consumption of alcohol is excessive if it exceeds three alcoholic beverages (~30 g of ethanol) per day. Heavy alcohol consumption is generally defined as an ethanol daily intake exceeding 50 g for several weeks. An ethanol intake reaches or exceeds 21 alcoholic beverages per week for men and 14 for women may also be a definition of chronic and excessive alcohol consumption.

Approximately 4.5% of the global burden of disease and injury can be attributed to alcohol.

Certain diseases are completely and exclusively attributable to an excessive consumption of alcohol (*e.g.* alcoholic hepatic cirrhosis, alcoholic psychosis, alcohol dependence). For other pathologies, alcohol constitutes a risk factor, but is not the only causal one. It is often involved in certain cancers (oral cavity and lips, pharynx, larynx, esophagus, colon and rectum, liver, and also breast cancer), in certain cardiovascular diseases (arterial high blood pressure, ischemic heart disorder) and in digestive diseases, such as pancreatitis (Fig. 1). Furthermore, alcohol contributes to traumatic outcomes that kill or disable people at a relatively young age.

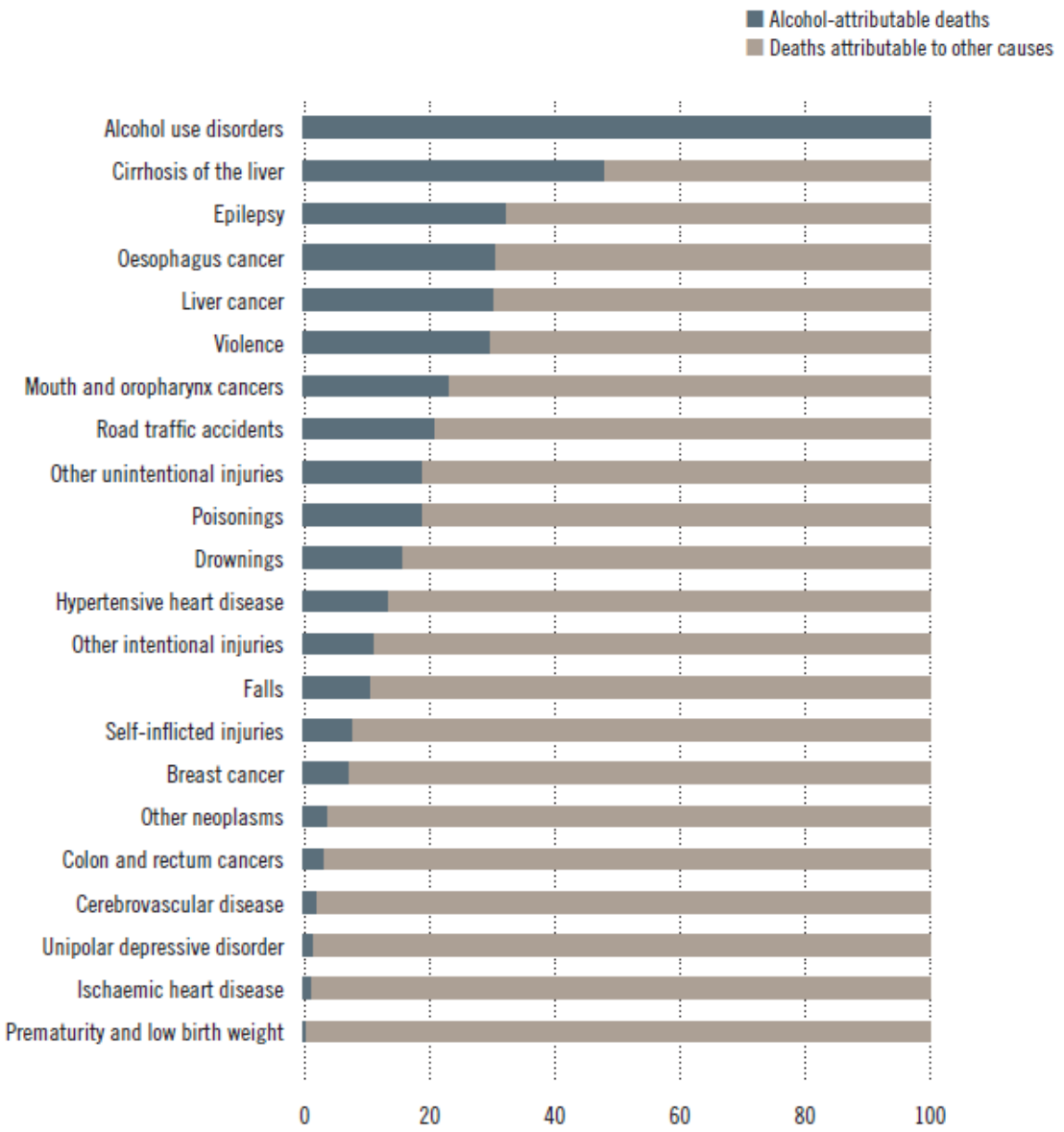


Figure 1. Global alcohol-attributable deaths as a percentage of total deaths by disease or injury, in 2004. From (WHO report, 2011).

The Global Status Report on Alcohol from the WHO estimated that more than 76 million people worldwide have recognizable alcohol misuse *(WHO report, 2004)*. The morbidity and mortality associated with alcohol misuse is significant (*i.e.* 2.5 million deaths worldwide each year, with one-third due to accidents) *(WHO report, 2011)*. It accounts for more deaths than caused by HIV/AIDS or tuberculosis. The harmful use of alcohol is especially fatal in younger age groups; it is considered as the world's leading risk factor for death among males aged 15–59 *(Rehm et al., 2009)* (Fig. 2).

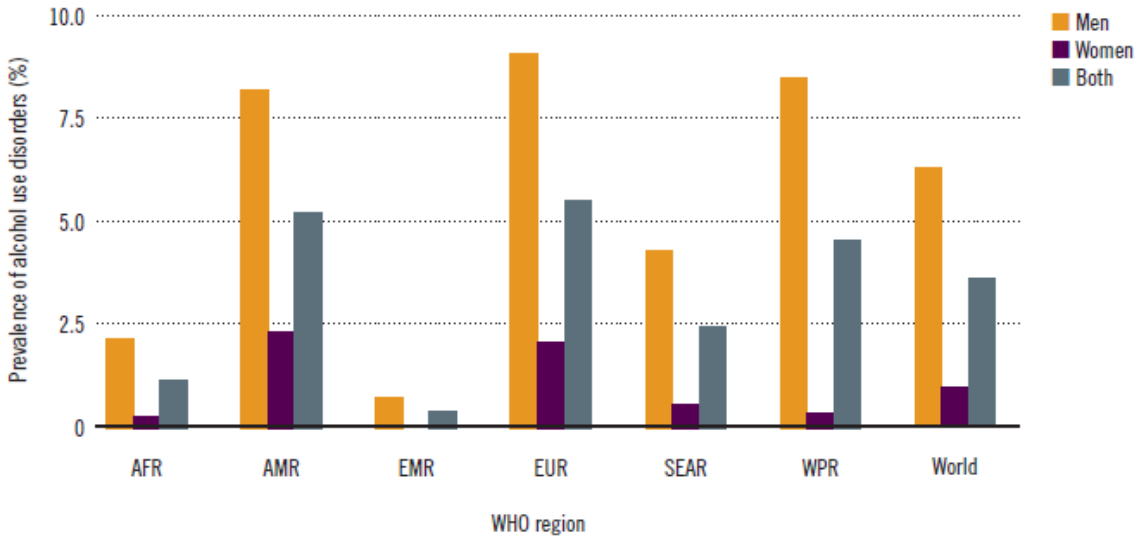


Figure 2. Alcohol-attributable deaths as a proportion of all deaths by sex and WHO region in 2004. AFR=African region. AMR=American region. EMR=Eastern Mediterranean region. EUR=European region. SEAR=Southeast Asian region. WPR=Western Pacific region.
From (Rehm *et al.,* 2009).

The highest alcohol consumption levels are found in the developed countries, including Western and Eastern Europe, and in high-income countries *(WHO report, 2011)*. Historically, the heaviest drinking countries were the wine producer ones: for many years, France had one of the highest known levels of alcohol consumption in the world, and one of the highest levels of alcoholism. Whereas, in recent years, alcohol consumption has stabilized or fallen in most European countries, France is still one of the countries with the most drinkers (9th place, with about 12.3 liters of pure ethanol per person annually; Fig. 3). The French ministry delegated to the Social Cohesion and to the Parity revealed that during the first nine months of 2006, 113 homicides between intimate partners had been committed, with alcohol present in the quarter of these cases. Similarly, students aged 15 to 18 who participated in the European Schools Project on Alcohol and other Drugs (ESPAD) survey reported that they were "often violent or criminal after drinking large quantities of alcohol on a regular basis, or when they have been drunk" *(Legleye et al., 2007)*.

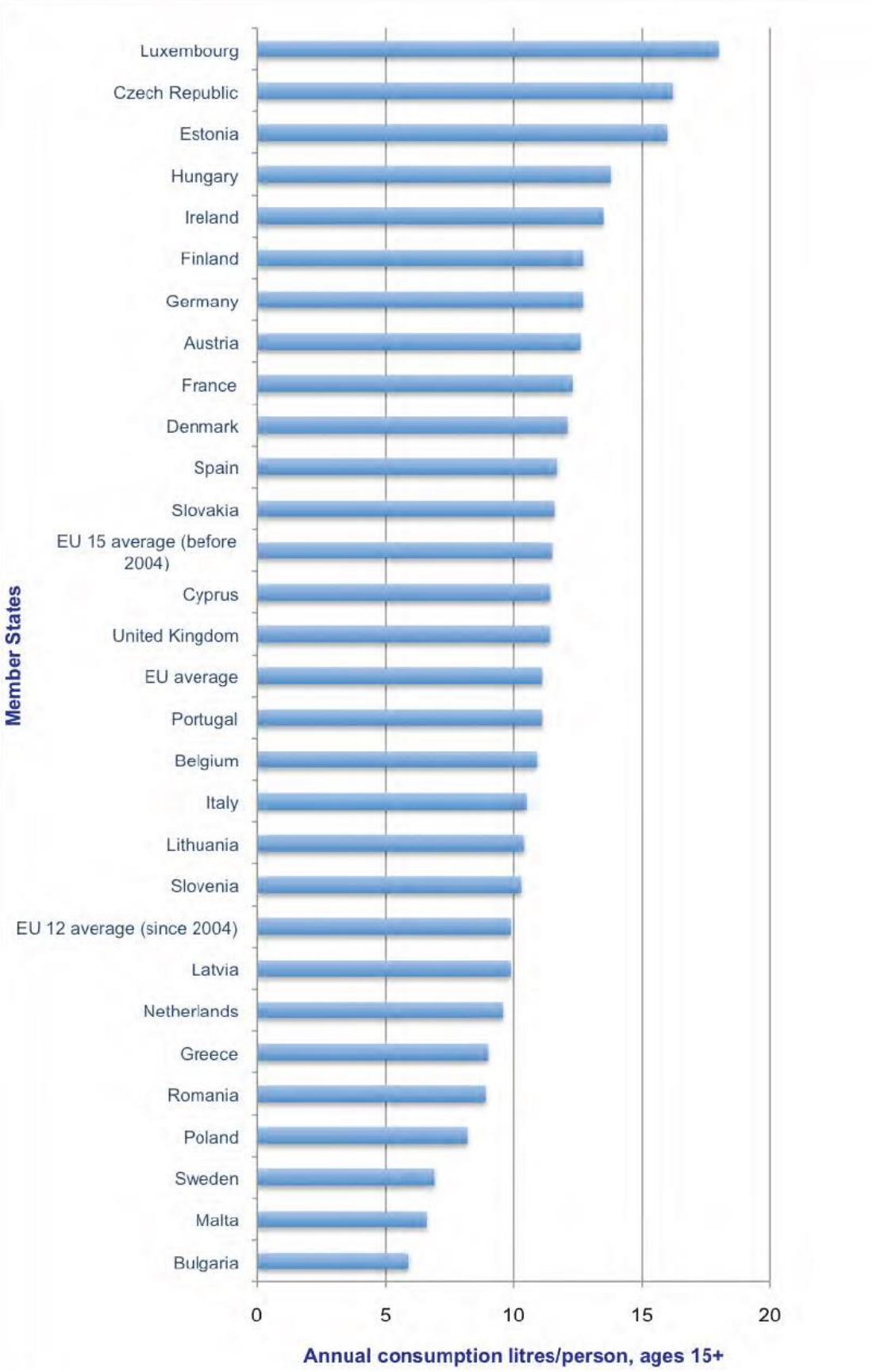


Figure 3. Annual consumption of pure alcohol (L/person) in individual aged >15 years. From (Institute of Alcohol Studies report, 2009).

Furthermore, the problems related to alcoholism in France affect about 5 million people, among whom approximately 2 million are dependent. The Gustave Roussy Institute (IGR) estimated that a total of 49 000 deaths (36 500 deaths for men *versus* 12 500 for women) were attributable to alcohol in France in 2009. Overall, this included 15 000 deaths from cancer, 12 000 from circulatory diseases, 8 000 from digestive system diseases, 8 000 from external causes and 3 000 from mental and behavioral disorders *(Guérin et al., 2013)*. The alcohol-attributable fractions were 22% and 18% in the population aged 15 to 34 and 35 to 64, respectively, *versus* 7% among individuals aged 65 or more *(Guérin et al., 2013)*. Moreover, fetal alcohol syndrome concerns every year between 5 and 7 000 newborns. The social cost of alcohol in France is about 17.4 billion euros *(Kintz et al., 2009)*. It is thus important to have laboratory tests, sensitive and specific, allowing better assessment of ethanol consumption. Various biomarkers for the detection of alcohol misuse have been described.

These last years, the research has focused on the use of markers allowing simultaneously the identification of the consumption profile of a subject and a specific and sensitive, thus reliable, diagnosis. As a direct metabolite of ethanol, formed by conjugation of ethanol with uridine 5'-diphosphate glucuronic acid (UDP-GA) *via* the action of UDP-glucuronosyl transferase (UGT), ethylglucuronide (EtG) presents a high specificity. It is detectable in the body only further to alcohol consumption. Its fast appearance, its presence in the body during a long time after the elimination of ethanol, and its peculiarity to fix in hair, make EtG a relevant and promising marker in clinical and forensic toxicology.

The first part of this thesis describes the metabolism of ethanol and the various biological markers of alcoholism, together with their advantages and limits. In a second part, we address more specifically ethylglucuronide, its fields of application in clinical and forensic medicine, and the enzymes involved in its production (UGTs). We also describe the methods used to study the glucuronidation of ethanol, as well as those used to study the associated variability factors. The third part presents all the work achieved in this thesis. Finally, a general discussion considers the possible impact of these results on clinical and forensic current practices.

Part 1: Biomarkers of alcohol consumption

Facing the previously mentioned problems related to alcoholism, the diagnosis of alcohol consumption remains an important issue from a medical or forensic point of view. It is still difficult because a majority of the subjects denies or minimizes alcohol abuse and also because diagnosis parameters with both high sensitivity and specificity are lacking.

Most studies on alcohol consumption patterns have relied on patient self-reports as records of drinking, although it is recognized that this information is often inaccurate *(Fuller et al., 1988; Helander et al., 1999)*. Similarly, detection of prenatal alcohol exposure is based on maternal report, or suspicion of it, which is based on interviews of the mother or her history (lack of prenatal care, alcohol related life events, abuse, and alcohol impaired driving infractions) *(Chang, 2001)*. However, one of the major disadvantages of self-reports is that women tend to underreport the amount and frequency of their alcohol intake and they are reluctant to reveal gestational alcohol use, because of the stigma and fear of punishment *(Russell et al., 1996)*. Furthermore, while screening questionnaires, such as TWEAK (Tolerance, Worried, Eye-opener, Amnesia, Kut down), or Alcohol Use Disorders Identification Test (AUDIT), can be quite sensitive for the identification of risky drinking behavior (≥ 14 drinks/week; *Bradley et al., 1998; Chang, 2001; Chang et al., 1999; Russell et al., 1996)*, their validity for the identification of more moderate alcohol use is largely unknown.

Concerning forensic toxicologists and pathologists, they are often called on to interpret the alcohol concentrations found in specimens. Correct interpretation of ethanol detection in forensic autopsy samples can be difficult due to the possibility of *post-mortem* production of ethanol *(O'Neal and Poklis, 1996)*, which may occur both in the body *post-mortem* and in the sample after autopsy. Preserving the specimens with fluoride after autopsy inhibits ethanol formation *(Blackmore, 1968; Jones et al., 1999; Lewis et al., 2004)*, but production in the body before sample collection might constitute a problem.

In all cases, determining whether an individual has consumed alcohol is often the critical information needed to make significant clinical and legal decisions. Clinicians can use several

biochemical measurements to objectively assess patients' current or past alcohol use. However, none of these currently available biomarkers—including measures of various liver enzymes and blood volume—are ideal.

1. What is an ideal biomarker of alcohol consumption?

In recent years, significant advances have been made in biological assessment of heavy drinking. These advances include development of new laboratory tests, formulation of algorithms to combine results of multiple measurements, and more extensive applications of biomarkers in alcoholism treatment and research. The accuracy of biomarker information is rarely a function of sample collection, but is rather closely related to (1) sample handling, storage, and transmittal, (2) quality assurance of laboratory procedures for isolation of the biomarker and (3) methods for quantifying and interpreting the results.

Selecting the proper biomarker for a particular application involves several issues. Ideally, the biological test would yield values that would directly correspond to the amount of alcohol consumed over a defined period of time. The sample for the test would be easy to obtain, readily testable, and inexpensive to quantify. Results would be quickly available. Further, the procedure would be highly acceptable to patients and therapists. No currently available biomarker has all of these features. Tests that directly or indirectly measure alcohol blood levels approach these goals, but are useful only in situations of acute alcohol ingestion. They do not provide information regarding drinking status prior to acute ingestion.

Several additional considerations should guide the choice for a biological test. First, the window of assessment (*i.e.* the period of time when the marker remains positive following drinking) needs to be understood. In emergency room settings, as well as in occupational contexts, to include transportation, public safety, delivery of medical care, or even in forensic cases, level of alcohol consumption in the immediate past is often the primary concern. On the other hand, in insurance and general health care treatment screening contexts, as well as in alcoholism treatment efficacy trials, the emphasis is likely to be particularly on chronic heavy drinking.

An additional concern that should guide selection of the biomarker is the nature of the population being assessed. Biomarkers often perform differently as a function of age, gender, ethnicity, and health status of the respondent. In addition, biomarkers are likely to perform more accurately in distinguishing extreme groups than in determining at–risk or harmful use of alcohol in a heterogeneous population with respect to drinking behavior.

Direct and indirect biomarkers of alcohol consumption could improve or supplant patient's report; they represent an objective measurement of alcohol exposure better than that reported by patients. A number of biological markers, considered far more reliable for documenting ethanol use, have been introduced and validated.

Indirect biomarkers rely on alterations in a metabolic or organ function caused by long-term alcohol consumption. These markers include gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), mean corpuscular volume (MCV), and carbohydrate-deficient transferrin (CDT). Challenges of their interpretation consist of the fact that they lack specificity, as results may be influenced by age, gender, medications, or other non-alcohol-related disease processes (false-positive) *(SAMHSA Advisory, 2012)*. Further, indirect marker assessments also lack sensitivity for alcohol intake, considering some individuals may drink heavily for months or years without developing abnormal test results.

Direct markers are tests with sufficient sensitivity to detect even a single ethanol exposure and include ethanol measurement (blood or urine), 5-hydroxytryptophol (5-HTOL), fatty acid ethyl esters (FAEEs), ethylsulfate (EtS), and ethylglucuronide (EtG) *(Helander, 2003; Niemelä, 2007)*. Moreover, the specificity of these markers is greater than that of indirect markers.

EtG can successfully fill the assessment void between the few hours following exposure in which direct markers are valid and the weeks to months before suggestive changes in indirect markers occur *(SAMHSA Advisory, 2012; Wurst et al., 2003)*.

All these biomarkers, including direct measurements of ethanol and measurements of the products of oxidative and non-oxidative ethanol metabolism, are presented in Fig. 4.

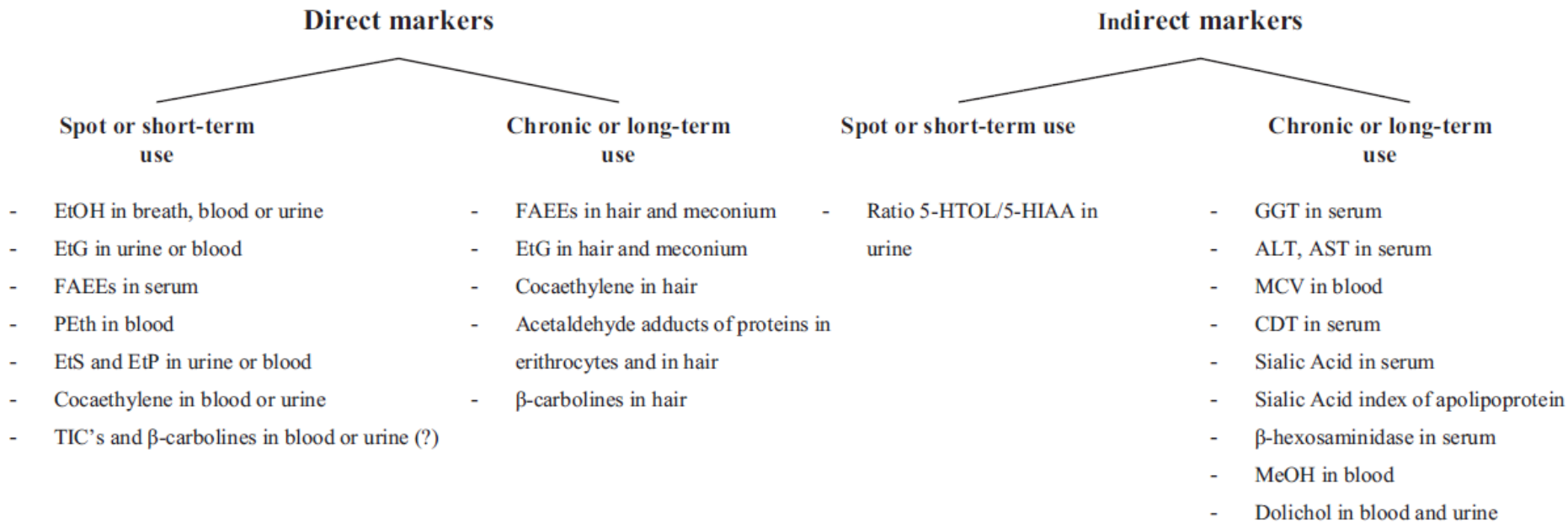


Figure 4. Direct and indirect markers of alcohol consumption. Adapted from (Pragst and Yegles, 2007).

Alcohol biomarkers, and their interpretation, will be discussed in this chapter except for EtG, which will be discussed extensively in the second chapter.

2. Indirect biomarkers of alcohol consumption

2.1. Gamma–glutamyltransferase

Description & normal values

The gamma–glutamyltransferase (GGT) is a glycoprotein enzyme found in endothelial cell membranes of various organs. It is involved in the transfer of the gamma-glutamyl moiety of glutathione, linked through glutamate gamma carboxylic acid, to various peptide acceptors and in the renal reabsorption of amino acids. Although it is produced in many tissues, only the hepatic isoform is detectable in serum.

Values of this enzymatic activity that are considered as "normal" depends on the analytical technique used. The usual serum concentrations vary consequently between 5 and 36 IU/L. Elevated serum GGT level remains the most widely used marker of alcohol abuse *(Anton et al., 2002; Conigrave et al., 2003; Hietala et al., 2005)*. Levels typically rise after continuous, rather than episodic, heavy drinking (> 40 g/d) *(Hietala et al., 2005)*. However, some studies in large populations have shown that even moderate drinkers display significantly higher levels of GGT than abstainers, specifically men *(Gjerde et al., 1988; Hietala et al., 2005)*. Within 2–6 weeks of abstinence, levels generally decrease back to normal reference range *(Hietala et al., 2006)*, with the half–life of GGT being 14–26 days. Persistently abnormal values in the absence of continuing ethanol exposure suggest liver disease, typically when GGT is initially 8–10 times higher than the normal value and if the elevation persists after 6–8 weeks of abstention from alcohol.

Sensitivity, specificity, and context of use

Although several studies have reported a positive correlation between ethanol intake and serum GGT activity, the sensitivities and specificities in clinical materials have, however, shown notable variability.

The sensitivity of this marker for the detection of chronic alcoholism varies from 34 to 85% depending on the considered study and the quantities of consumed alcohol *(Chrostek et al., 2006; Niemelä, 2007; Sillanaukee et al., 2000)*. This low sensitivity means that there are large numbers of false-negatives with this biomarker, which means that a heavy and chronic consumption of ethanol may exist without necessarily an impact on the liver and can therefore exist without an increase in the activity of GGT. Brenner *et al*., (1997) observed that only 22.5% of construction workers who drank an average of 50–99 g/d had elevated GGT values, and even among those consuming >100 g/d, only 36.5% displayed high GGT levels *(Brenner et al., 1997)*. Furthermore, sensitivity of GGT seemed to be poor among young adults (< 30 years) *(Conigrave et al., 2002; Sillanaukee et al., 1998)*, even when they have alcohol dependence *(Bisson and Milford-Ward, 1994)*. However, in most studies, GGT sensitivities have exceeded those of other commonly used markers. In a collaborative project on markers of alcoholism, the WHO and the International Society for Biomedical Research on Alcoholism (ISBRA) indicated elevated serum GGT concentrations in 52% of alcohol-dependent subjects, as compared to sensitivities of 39% for both CDT and AST *(Helander and Tabakoff, 1997)*. It should also be noted that the sensitivity of GGT as an alcohol marker has usually shown to be higher for men than for women *(Anton and Moak, 1994; Mundle et al., 2000; Yersin et al., 1995)*.

Elevations of GGT levels are not specific for excessive alcohol drinking, since increases also occur in liver diseases and with certain drugs. Obesity is also an important factor, which can increase serum GGT *(Daeppen et al., 1998; Lam and Mobarhan, 2004; Lawlor et al., 2005; Puukka et al., 2006a)*. GGT induction has also been linked with the formation of reactive oxygen species and it may therefore be considered as a marker of oxidative stress *(Furukawa et al., 2004; Lee et al., 2004)*. GGT levels may also rise in patients as a result of many other non-alcoholic diseases, such as non-alcoholic fatty liver diseases, hepatobiliary disorders, diabetes, pancreatitis and cholestasis, dyslipidemias, certain cardiovascular pathologies (hypertension), and severe traumas. Besides, numerous drugs, such as certain antiepileptics, barbiturates, oral anticoagulants or antibiotics, are also known to provoke a rise of this marker levels. The specificity of GGT in the demonstration of chronic alcoholism is therefore low.

Furthermore, age seems to affect the GGT activity. In general, GGT increases with age *(Conigrave et al., 2002; Daeppen et al., 1998; Puukka et al., 2006b; Sillanaukee et al., 1998),* which means a decrease of the specificity in older individuals. Despite this lack of specificity, various studies indicate that 50 to 72% increases in serum GGT can be explained by excessive consumption of ethanol *(Kristenson et al., 1980; Penn and Worthington, 1981).*

Based on the above statements, it appears that the clinical value of GGT in the assessment of excessive ethanol intake should be carefully interpreted. It should be noted that the interpretation of both GGT and CDT is usually the basis of the detection of excessive consumption of ethanol, since the risk that the concerned person is a chronic alcoholic is generally upper to 90 % when these two markers are simultaneously perturbed *(Hietala et al., 2006).*

As the laboratory tests for the determination of GGT are inexpensive and readily available, its determination in serum remains up to date widely used as a marker of *ante-mortem* ingestion of alcohol. However, it presents only little interest in forensic (especially *post-mortem*) toxicology.

2.2. Aminotransferases

Description & normal values

The aminotransferases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), are also often considered as markers of heavy drinking, and their elevation is frequent in alcoholic patients *(Niemelä, 2002; Rosman and Lieber, 1994)*. Their elimination rate is up to 3 weeks. These enzymes catalyze the reversible transformation of alpha-keto acids into amino acids by transferring amino groups. The liver contains abundant amounts of these enzymes and, consequently, it may be assumed that they reflect hepatocyte injury rather than alcohol consumption *per se (Salaspuro, 1987).*

Sensitivity, specificity, and context of use

The sensitivity of AST as a marker of alcoholism has been estimated at 35-47% and that of ALT is considered to be even lower *(Helander and Tabakoff, 1997; Hietala et al., 2006; Prytz*

and Melin, 1993). They are also increased in abstinent alcoholics with a chronic liver disease. A selective increase of AST is generally revealing a mitochondrial damage in hepatic tissue, skeletal muscle (alcoholic myopathy) or cardiac muscle (alcoholic cardiomyopathy). However, the ratio AST/ALT seems to provide meaningful information *(Reichling and Kaplan, 1988)*. A cut-off value of the ratio > 2 is usually assumed to reflect an alcoholic etiology of the liver disease, while most patients with non-alcoholic liver disease exhibit a ratio < 1 *(Matloff et al., 1980; Niemelä, 2002; Prytz and Melin, 1993; Rosman and Lieber, 1994)*.

As for GGT, AST and ALT are not increased after a single episode of excessive drinking. They are poor indicators of abusive drinking due to their low sensitivity and specificity; they have therefore a limited utility in the diagnosis of alcoholism.

2.3. Mean corpuscular volume

Description & normal values

The Mean Corpuscular Volume (MCV) is an indicator of the size of erythrocytes and represents the average volume of a red blood cell, which is expressed in femtoliter (fL, or 10^{-15} liter). It is measured by the ratio of the hematocrit to the number of erythrocytes per liter of blood. The reference range for MCV is 80-96 fL/red cell in adult *(Vajpayee et al., 2011)*; reference ranges may vary depending on the individual laboratory and patient's age. MCV shows rather strong correlations with reported drinking and there seems to be a distinct dose-dependent response between erythrocyte cell volume and the intensity of ethanol intake *(Koivisto et al., 2006)*. It has been known that the mean volume of erythrocytes is increasing (> 100 fL) with excessive alcohol consumption, giving rise to macrocytosis *(Seppä et al., 1991; Wu et al., 1974)*. The origin of this macrocytosis, observed in chronic alcoholic patients, is poorly understood and probably related to a nutritional deficiency that alters the permeability of the red cell membrane. However, many studies have supported a direct hematotoxic role of ethanol and of its metabolite, acetaldehyde *(Latvala et al., 2001; Niemelä and Parkkila, 2004; Tyulina et al., 2006).*

Sensitivity, specificity, and context of use

Studies in heavy drinkers have indicated over 40% sensitivity for this marker, the sensitivity being typically somewhat higher for women *(Morgan et al., 1981)*. Further, it should be noted that MCV has limited specificity and, consequently, presents a significant risk of "false-positives". An increase in MCV has been observed in patients with vitamin B_{12} or folic acid deficiency, liver diseases, hematological diseases, hypothyroidism, or reticulocytosis. Such increase is also observed in smokers and patients treated with certain antiepileptic drugs *(Savage et al., 2000)*. Consequently, the measurement of MCV has limited value as a single marker of alcoholism *(Meerkerk et al., 1999)*. It is regularly used as an element for the monitoring of abstinence, because it presents a slow normalization (half-life 2 to 4 months) after withdrawal *(Morgan et al., 1981)*. Moreover, the testing methodology is easy and inexpensive.

As its sensitivity and specificity are rather low, MCV clinical value should be carefully interpreted. It is generally used in combination with other markers of alcohol consumption. The measurement of the MCV has no interest in forensic toxicology especially in *post-mortem* cases, because of the phenomena of hemolysis and dilution occurring after the death.

2.4. Carbohydrate–deficient transferrin

Description & normal values

Transferrin, a negatively-charged glycoprotein, is synthesized and secreted by the liver and circulates in the bloodstream. It is involved in iron transport in the body and has a half-life of 7-10 days. It contains two carbohydrate residues and two *N*–linked glycans *(MacGillivray et al., 1983)*. Six sialic acid moieties may be attached. With heavy alcohol intake, these moieties can lose carbohydrate content, hence the term “carbohydrate–deficient” transferrin (CDT) *(Stibler and Borg, 1988)*. The predominant transferrin isoform, accounting for over 80% of the total transferrin in both healthy and alcoholic populations, is tetrasialotransferrin *(Legros et al., 2002; Mårtensson et al., 1997)*. The diagnostically most interesting isoforms are nevertheless desialylated isoforms (asialo-, monosialo-, and disialo-transferrins); they all

increase in alcohol abusers. Moreover, the asialo form seems to be absent in samples from abstainers or moderate drinkers *(Arndt, 2003; Legros et al., 2002)*, which could make it the most specific isoform. The mechanisms by which altered sialylation is brought about remain largely unknown *(Sillanaukee et al., 2001; Xin et al., 1995)*. Excessive and chronic ethanol consumption may impair the synthesis (incorporation of sialic acid moieties), secretion, and membrane assembly of glycoproteins by inhibition of sialyl-transferases in the Golgi apparatus and by activation of hepatocyte membrane sialidase enzyme. By these different mechanisms, it leads to remove the carbohydrate groups from transferrin, which results in increasing the CDT while the concentration of total transferrin does not change *(Stibler, 1991)*. Disturbed function of various liver cell receptors could possibly also influence the serum concentrations of the desialylated fractions.

Cut-off of this marker depends on the method used to determine CDT. Currently, several methods for its measurement are available. CDT can be analysed by electrophoretic, chromatographic, and immunometric methods, as well as more recently by mass spectrometric methods [215,216]. Previously, the concentration of CDT was expressed as simple units (U/L), whereas in more recent methods the results are given as a percentage of total transferrin (%CDT). The main advantage of the latter approach is that it takes into account the natural variability in serum transferrin *(Helander, 1999; Keating et al., 1998)*. This has been especially important in assays of women with a high prevalence of iron deficiency and in patients with liver diseases *(Helander et al., 2005)*.

Sensitivity, specificity, and context of use

Several studies have reported a high sensitivity and an excellent specificity for CDT *(Kwoh-Gain et al., 1990; Stowell et al., 1997; Walter et al., 2001)*. Few cases of "false-positives" have been reported in patients with non-alcoholic cirrhosis, chronic hepatitis C, hepatoma, and in patients presenting genetic variants of the polypeptide chain, or a congenital metabolic disease affecting transferrin. Because of its high specificity (up to 90%), CDT is used to support diagnostic decisions in both clinical and forensic (*ante-mortem*) settings; *e.g.* it has become one of the fundamental parameters to evaluate, in driving license re-granting

programs, the physical fitness of individuals whose driving licenses were confiscated for drunk driving *(Bianchi et al., 2010; Maenhout et al., 2012)*. However, this biomarker presents also a risk of "false-negatives". Helander and Tabakoff, in the WHO/ISBRA collaborative study, found a sensitivity of only 39% for alcohol-dependent subjects *(Helander and Tabakoff, 1997)*. Another previous study on 200 alcohol abusers with no apparent liver disease also showed that the sensitivity of CDT was only 34% in problem drinkers who reported a mean of approximately 100 g of ethanol per day, as compared to the sensitivities of 47% and 34% for GGT and MCV, respectively *(Niemelä et al., 1995)*. In patients drinking a mean of over 150 g of ethanol per day, the sensitivity increased to 64%, as compared to 55% for GGT and 39% for MCV.

There is currently no uniform opinion about the amount and pattern of drinking needed to increase serum CDT. It is believed that the levels increase once alcohol consumption exceeds 50–80 g/day for 2 to 3 weeks, and they normalize with a mean half–life of 2–4 weeks of abstinence *(Lesch et al., 1996; Stibler, 1991)*. However, it has been suggested elsewhere that the amount of 60 g, or even 80 g, of alcohol per day is not sufficient to increase CDT values over the reference limit in the general population *(Lesch et al., 1996; Salmela et al., 1994)*. Thus, even if CDT may not be sensitive enough for screening alcohol consumption, it can be used to detect changes in alcohol intake in chronic alcohol abusers *(Burke et al., 1998)*. One of its advantages over many other markers is that it may not be, or very poorly, influenced by the presence of a liver disease *(Nalpas et al., 1997)* and that it is not affected by medications *(Fleming et al., 2004)*.

The clinical performance of CDT may vary depending on gender, although the reasons underlying such findings are still unclear. CDT is more sensitive in men than in women *(Huseby et al., 1997; Mundle et al., 2000)*. It has been suggested that women express higher CDT levels under physiological conditions, but may produce less CDT in response to heavy drinking *(Anton and Moak, 1994)*. Pregnancy seems to increase CDT levels, even in the absence of alcohol abuse *(Kenan et al., 2011; Stauber et al., 1996)*. Consequently, the diagnostic accuracy of CDT for detecting alcohol abuse may be limited in pregnant women and should be carefully assessed in relation to alcohol consumption. One has also to keep in

mind that there is a significant inter-ethnic variability for this marker (*e.g.* Asian and black African populations present CDT concentrations often much higher than those of Caucasian populations) *(Kim et al., 2007)*.

Based on the above, CDT is a useful tool for the detection of chronic alcoholism. However, it still has some limitations due to the lack of methodological standardization and definitive cut-off values. In addition, analytical techniques are still relatively expensive. Finally, the interpretation of results obtained from serum or vitreous humor in *post-mortem* cases is complex and is not well developed, which limits its use in forensic toxicology.

2.5. GGT–CDT combinations

Since no single marker of alcoholism offers perfect validity to document heavy drinking, considerable research has been undertaken to evaluate the use of a combination of markers. The most popular of these are combinations of GGT, MCV, AST, and CDT *(Rinck et al., 2007; Watson et al., 1986)*. Such combinations raise screening sensitivity, but result obviously in a loss of specificity and are cost-consuming. Some studies have proposed a mathematically-formulated equation from GGT and CDT [0.8*ln(GGT)+ 1.3*ln(CDT)]. The score would be elevated in a higher percentage of alcohol abusers than the level of either GGT or CDT alone *(Anttila et al., 2003; Hietala et al., 2006; Sillanaukee and Olsson, 2001)*. This approach, when the latter component of the equation is replaced by the results from %CDT assay, was found to further improve the method *(Hietala et al., 2006; Sillanaukee and Olsson, 2001)*.

GGT–CDT (abbreviated also as γ-CDT) values appear to increase after a daily ethanol consumption exceeding a threshold of 40 g. The diagnosis sensitivity of the combination was more than 20% higher than that achieved by the use of each marker individually. GGT–CDT values also show a strong correlation with the amounts of consumed ethanol *(Hietala et al., 2006)*. This combination can be used for following-up abstinence (it indeed shows a rather consistent decline during supervised abstinence, with a mean normalization occurring after 2–3 weeks). Since this approach is also cost-effective and easy to manage in hospital laboratories, it could be suitable for routine clinical, but not for forensic, applications.

3. Direct biomarkers of alcohol consumption

Before addressing direct markers, a brief reminder of the metabolism of ethanol is needed. Ethanol is a small water soluble molecule that can cross cell membranes. After ethanol has been ingested, 10-20% will be absorbed by the stomach; however, the majority (80%) will be absorbed by the small intestine and enter the bloodstream *(Paton, 2005)*. A small proportion (2-10%) of absorbed ethanol is excreted unchanged in the urines, exhaled breath and sweat. More than 90% is metabolised. Ethanol metabolism can occur in various organs and tissues, such as kidney, muscle, lung, stomach, small intestine, and even brain, but is primarily carried out by the liver *(Pawan, 1972; Zakhari, 2006)*. 90-95% of absorbed ethanol is metabolised by oxidation reactions with nicotinamide adenine dinucleotide (NAD), catalyzed by alcohol- and aldehyde-dehydrogenases (ADH and ALDH) to produce acetaldehyde (CH3CHO) and, then, acetic acid (CH3COOH) (Fig. 5). A secondary oxidation pathway for ethanol metabolism is the microsomal alcohol oxidizing system (MEOS; involving microsomal cytochrome P450 (CYP) 2E1), which accounts for around 10% of total ethanol clearance by the liver *(Fraser, 1997; Pawan, 1972)*. A third oxidative pathway *via* the heme enzyme catalase also exists, which converts a small proportion of ethanol (0-2%) to acetaldehyde and water *(Mumenthaler et al., 1999; Pawan, 1972)* (Fig. 5).

Non-oxidative metabolism of ethanol is minimal, but its products may have pathological and diagnostic relevance. Ethanol is non-oxidatively metabolised by many pathways. One leads to the formation of molecules called fatty acid ethyl esters (FAEEs) from the reaction of ethanol with fatty acids using the FAEE synthase and the acylCoA-Ethanol-O-acyltransferase enzymes *(Kinnunen and Lange, 1984)*. A second non-oxidative pathway results in the formation of a type of fat molecule containing phosphorus (i.e. phospholipid) known as phosphatidylethanol (PEth). This pathway requires the phospholipase D (PLD) enzyme *(Laposata, 1999)*, which breaks down phospholipids (primarily phosphatidylcholine) to generate phosphatidic acid (PA).

The other non-oxidative metabolites of ethanol are ethylglucuronide (EtG) and ethylsulfate (EtS). Only a very small fraction of the ethanol ingested (<0.1%) undergoes these enzymatic reactions. EtG is mainly formed by hepatic glucuronidation *via* conjugation of ethanol with an activated glucuronic acid *(Goll et al., 2002)*; this phase II reaction is catalyzed by UDP-glucuronosyltransferase (UGT) enzymes. EtS is also a minor phase II metabolite formed by sulfoconjugation of ethanol *via* sulfotransferase (SULT) enzymes *(Schneider and Glatt, 2004)* (Fig. 5).

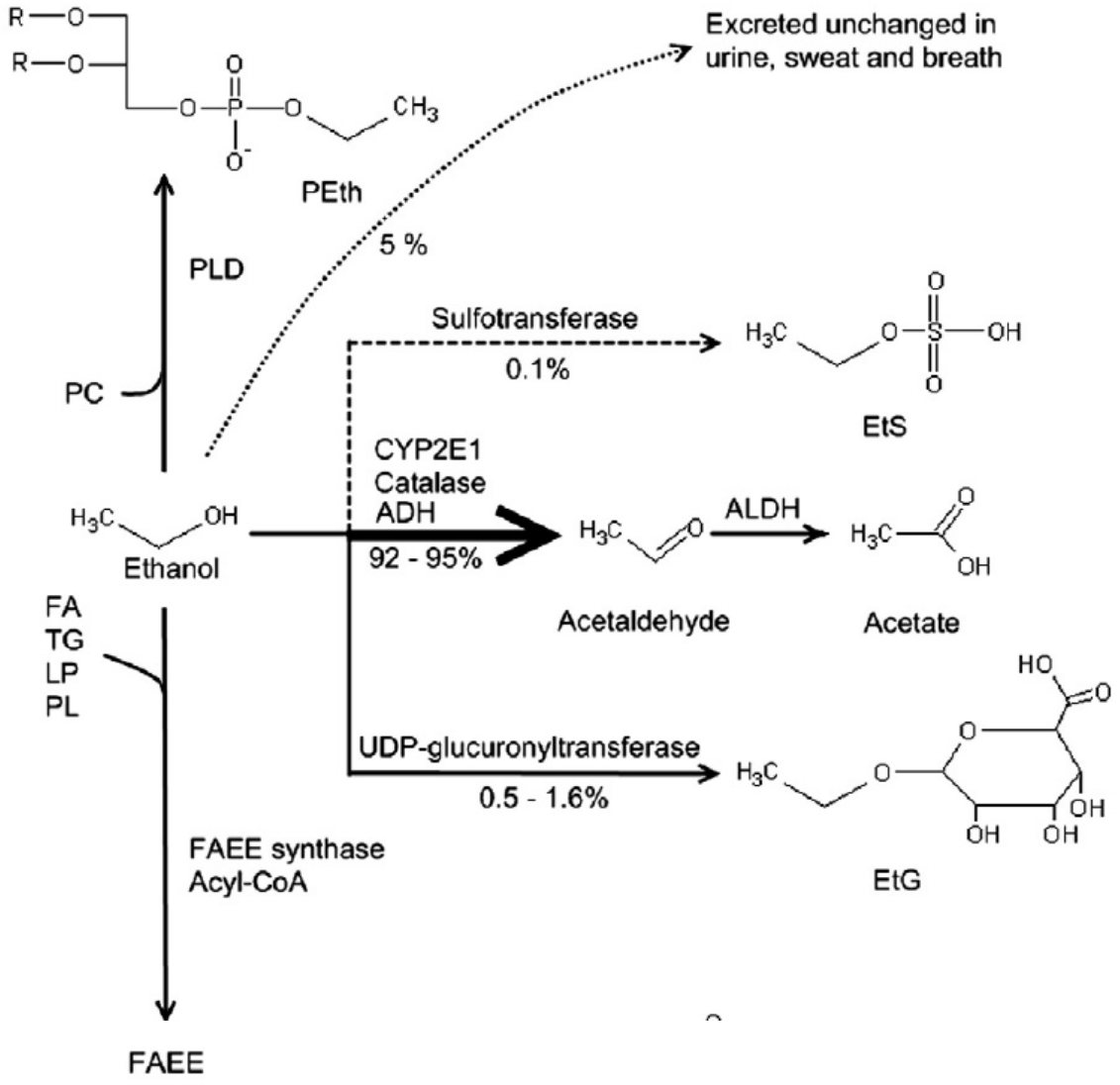


Figure 5. Ethanol metabolism and formation of oxidative and non-oxidative ethanol metabolites. FA, fatty acids; TG, triglycerides; LP, lipoproteins; PL, phospholipids; PC, phosphatidylcholine; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; UDP, uridine diphosphate. Adapted from (Maenhout *et al.*, 2013).

3.1. Ethanol

The presence of ethanol in body fluids can be easily determined and is one of the first parameters considered as a marker of alcohol consumption. Measurements of ethanol can be performed on a number of different matrices, including blood, breath, urine, saliva, vitreous humor, bile, liver, spleen, etc. By using ethanol as a marker to assess intake, false-positive results can be eliminated. It has a very high specificity for excessive alcohol intake, but the time window for positivity is relatively short (breath 4-6 h, blood 8-10 h, urine 18-24 h) after alcohol intake, with a maximum blood concentration obtained 30 min to 1 h after ingestion and an average rate of elimination from blood of 0.10 to 0.2 g/L/h in occasional drinkers and of 0.25 to 0.35 g/L/h in chronic drinkers *(Jones, 2010)*. Thus, ethanol elimination rate is influenced by previous drinking practices, *e.g.* heavy drinkers typically show 1.5-times faster rates than non-drinkers.

Context of use

Unfortunately, ethanol short half-life limits a wider use of this marker. Because of its rapid elimination, ethanol measurements may be of limited value in assessment of chronic heavy drinking during days or weeks preceding sampling. However, ethanol is still an interesting marker and is widely used for the detection of acute and recent alcohol consumption, both in living and *post-mortem* situations. In forensic situations, it is common to adjust the determined ethanol concentration in matrices other than blood in order to obtain a value that can be compared to blood alcohol concentration; the specimen-to-blood ethanol concentrations varies depending on the water content of the matrix *(Levine, 2006)*. One example is the analysis of serum as opposed to whole blood; the measured ethanol concentration will be higher for serum because it has 12% to 20% higher water content than whole blood *(Levine, 2006)*.

Confounding factors and precaution

Correct interpretation of ethanol concentration in forensic autopsy samples can be difficult due to the possibility of *post-mortem* production of ethanol *(O'Neal and Poklis, 1996)*, which may occur both in the body before sample collection and in the sample after collection. Preserving the specimens with fluoride after autopsy inhibits ethanol formation *(Blackmore, 1968; Jones et al., 1999; Lewis et al., 2004)*, but production in the body before sample collection might constitute a problem. Ethanol may also arise as a putrefactive product formed by a wide range of microorganisms, which may penetrate from the skin or intestine. Ethanol production can be prevented by refrigeration of the body within 4 h following death *(Clark and Jones, 1982)*, but this is not a common procedure. The levels of ethanol produced after death have been reported as low (below 0.5 g/L) in the majority of cases *(O'Neal and Poklis, 1996)*, but may occasionally reach significant concentrations (above 1.5 g/L) *(Canfield et al., 1993; Kuhlman et al., 1991)*, if the conditions for formation are optimal. To determine whether detected ethanol is of *ante-* or *post-mortem* origin, the case history is important, but criteria, such as the degree of putrefaction, ethanol level and the distribution of ethanol between different body fluids, may be useful *(O'Neal and Poklis, 1996)*. The presence of ethanol in urine or vitreous humor was previously suggested as a criterion for an exogenous exposure of ethanol *(Zumwalt et al., 1982)*. However, numerous cases of *post-mortem* production of ethanol in urine *(Gilliland and Bost, 1993)*, and even in vitreous humor *(de Lima and Midio, 1999; Gilliland and Bost, 1993)*, have been reported.

As a conclusion, sensitivity and specificity values for this marker in detecting alcohol consumption > 50 g/d have been reported as being 88% and 92%, respectively *(Olsen et al., 1989)*. The detection and/or dosage of ethanol are routinely performed by immunochemical techniques or gas chromatography-flame ionization detection (GC-FID). This latter approach is rapid, relatively simple, inexpensive, sensitive, accurate, and capable of quantifying ethanol levels as low as 0.05 g/L.

3.2. Fatty acid ethyl esters

Description

FAEEs are formed by the esterification of ethanol with free endogenous fatty acids and acyl-CoA/fatty acids. The esterification process is facilitated by two enzymes: FAEE synthase and acyl-CoA/ethanol O-acyl-transferase *(Kaphalia and Ansari, 2001; Laposata and Lange, 1986)*. In humans, FAEE synthases are found in almost all tissues, mainly in the liver and pancreas *(Laposata and Lange, 1986)*. The FAEE group consists of more than 20 different compounds, such as ethyl laurate (E12), ethyl myristate (E14), ethyl palmitate (E16), ethyl palmitoleate (E16:1), ethyl stearate (E18), ethyl oleate (E18:1), ethyl linoleate (E18:2), ethyl linoleneate (E18:3), ethyl arachidonate (E20:4), and ethyl docosahexanoate (E22:6). FAEEs have the ability to accumulate in some organs, such as the brain, pancreas, and myocardium. Furthermore, FAEEs accumulate in the adipose tissue and hair *(Kulaga et al., 2009)*.

More than 15 FAEEs were identified in hair and it seems that the concentrations of 4 of them are interesting for the diagnosis and/or the follow-up of a chronic alcoholism: ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate. These 4 FAEEs should be quantified and the sum of their concentrations should be used for interpretation. A cut-off for the sum of these 4 esters in hair, to assess chronic excessive alcohol consumption behavior, is proposed by the Society of Hair Testing (SoHT) at 0.5 ng/mg scalp hair measured in the 0-3 cm proximal segment. If the proximal 0-6 cm segment is used, the proposed cut-off is then 1.0 ng/mg scalp hair. If samples less than 3 cm are used, the results should be interpreted with caution. For teetotalers and weak social drinkers, concentrations lower than 0.4 ng/mg hair were found *(Yegles et al., 2004)*.

Context of use

The plasma elimination of FAEEs has a two-phase kinetic profile with a primary half-life of about 3 h and a terminal half-life of about 11-16 h, followed by distribution into tissues or hydrolysis under the action of FAEE hydrolases *(Doyle et al., 1994)*. Because of the longer secondary elimination phase, FAEEs persist in blood at least 24 h after the last ethanol intake *(Doyle et al., 1996)*. Thus, their detection in blood could be interesting to confirm acute or

recent alcohol consumption. In heavy drinkers, FAEEs could be detected up to 99 h after drinking cessation *(Borucki et al., 2007)*. Moreover, as FAEEs accumulate in hair, they could be interesting to assess a chronic consumption. Determination of FAEEs in maternal hair is proposed as the best option for the evaluation of chronic alcohol use during pregnancy *(Pragst and Yegles, 2008)*. However, Morini *et al.,* (2010) reported that monitoring the levels of FAEEs in hair of pregnant women is not useful in detecting ethanol intake lower than 30 g/day *(Morini et al., 2010)*. Moreover, a low concentration of FAEEs, 0.06–0.37 ng/mg (mean 0.17 ng/mg, n = 17), has been detected in hair of strict teetotalers *(Auwärter et al., 2004, 2001)*.

Confounding factors and precaution

The detected levels of FAEE should be interpreted with caution for many reasons.

First of all, it should be noted that FAEEs are sensitive to cosmetic treatment. Some studies have shown that daily use of certain hair treatments, especially shampoos, hair sprays, and lotions, could affect the observed results and lead to "false-positives" *(De Giovanni et al., 2008; Hartwig et al., 2003)*. Furthermore, regular use of products with ethanol content as low as 10% can potentially elevate FAEE results above the recommended cut-off to determine heavy drinking behavior *(Gareri et al., 2011)*. Secondly, FAEE levels may vary depending on gender, although the reasons underlying such findings are still unclear. In serum and plasma, males have a peak serum FAEE concentration approximately 2-fold higher than that for females. It could be due to the fact that women have a reduced activity of enzymes required for the synthesis of FAEEs or an increased activity of enzymes involved in the degradation of FAEEs *(Soderberg et al., 1999)*. Finally, there is an artifactual FAEE formation *in vitro* when serum samples are stored at room temperature for at least 1 day, due to production from white blood cells or platelets, known to have FAEE synthase activity. Storage of the sample at +4 °C or −80 °C for up to 2 days does not alter the FAEE concentrations *(Soderberg et al., 1999)*. Headspace solid phase microextraction in combination with gas chromatography–mass spectrometry and use of deuterated FAEEs, as internal standards, is a suitable technique for determination of FAEE in hair. This analytical technique is considered as expensive and therefore unsuitable for routine use.

3.3. Phosphatidylethanol

Description

Phosphatidylethanol (PEth) is a unique phospholipid which is formed in cell membranes only in the presence of ethanol. It was discovered for the first time in mammalians in 1983 *(Alling et al., 1983)*, and then described in humans by Gustavsson in 1994 *(Gustavsson, 1995)*. The formation of PEth is catalyzed by phospholipase D (PLD), an ubiquitary enzyme *(Gustavsson et al., 1991; Kobayashi and Kanfer, 1987)* normally devoted to the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA). Short-chain alcohols compete with phospholipids as a substrate for phospholipase D, resulting in the formation of the corresponding phosphatidylalcohol in tissues *(Gustavsson, 1995)*. PLD has a high affinity for ethanol (100–1000-fold higher than for water). In the presence of ethanol, it promotes a transphosphatidylation reaction, with the production of PEth *(Kobayashi and Kanfer, 1987)* (Fig. 5).

PEth is not a single molecule, but a group of glycerophospholipid homologues with a common phosphoethanol head group onto which two long carboxylic acid side chains, typically containing from 14 to 22 carbon atoms with different grades of insaturation (0–6 double bonds), are attached *(Helander and Zheng, 2009; Holbrook et al., 1992)*. The numerous combinations of chain length and double bonds enable the formation of a very large theoretical number of different PEth species and so far 48 homologues have been identified in human blood *(Gnann et al., 2010)*.

Context of use

In blood, PEth resides mainly in erythrocytes having a half-life of approximately 4 days *(Aradottir et al., 2006)*. Zheng *et al.* reported an estimated half-life for PEth in whole blood of 7 days *(Zheng et al., 2011)*.

Because PEth is specifically formed in the presence of ethanol and it has a long half-life due to its slow degradation rate *(Aradottir et al., 2006; Varga et al., 1998; Zheng et al., 2011)*, it

has been suggested that PEth could potentially be used as a marker of chronic alcohol consumption. In clinical studies conducted on chronic heavy drinkers, PEth was found to be detectable in blood up to 28 days after sobriety *(Varga et al., 1998; Wurst et al., 2010)*, and a significant correlation between reported ethanol intake and measured PEth level was reported *(Aradottir et al., 2006)*.

Currently, the international scientific community has not yet established a cut-off value for PEth concentration in blood to be used for differentiating an acceptable social ethanol intake from an at-risk alcohol use (40-60 g/d) and chronic excessive drinking behavior (> 60 g/d). Consequently, a very interesting question, still unsanswered, is the quantity of ethanol that has to be consumed for a certain time-period to give a positive blood PEth assay. It has been observed that a single ethanol dose of 30–47 g did not produce any measurable amounts of PEth in blood *(Varga et al., 1998)*. The threshold of total ethanol intake leading to positive PEth values was estimated at around 1000 g in three weeks, with a daily consumption of at least 50 g/d *(Varga et al., 1998; Wurst et al., 2010)*. A recent drinking experiment, which employed a sensitive LC-MS/MS method for the quantification, showed that the formation of PEth began immediately after the first intake of alcohol (0.5–8 h) *(Gnann et al., 2012)*. This result can give novel potential applications of PEth in the diagnosis of excessive alcohol drinking episodes and/or "binge drinking" behaviors. However, study of large populations of social drinkers and teetotalers is needed in order to determine whether an efficient cut-off value can be established for differentiating teetotalers from social/moderate drinkers and "binge drinkers".

The mean values and confidence intervals of total PEth concentrations in blood of heavy (DAI > 60 g/d) and social drinkers (DAI ≤ 60 g/d) showed a significant statistical difference *(Viel et al., 2012)*. These findings demonstrate a good clinical efficiency of PEth for detecting heavy drinking. Differing from the traditional indirect biomarkers used for diagnosing a chronic excessive drinking behavior (MCV, AST, ALT, GGT, and CDT), blood PEth concentration seems not to be influenced by age, gender, other ingested substances or non-alcohol-associated diseases, such as hypertension, kidney and/or liver diseases *(Wurst et al.,*

2010). These points give PEth some advantages compared to aforementioned traditional biomarkers.

Confounding factors and precaution

Human red blood cells seem to be peculiar in forming PEth *in vitro* in the presence of ethanol *(Aradóttir et al., 2004a; Aradottir et al., 2002)*. This characteristic represents an important drawback of PEth as a marker of chronic alcohol abuse: samples collected when the blood ethanol concentration (BAC) is higher than 0.1 g/L can generate false-positive results due to the neo-formation of PEth *in vitro* (in the post-sampling period), which can occur at room temperature (16–20 °C), but also at −20 °C, and being slower at +4 °C *(Aradóttir et al., 2004a)*. Ethylene-diamine tetra-acetic acid (EDTA)-anticoagulated or heparinized blood samples can be stored for up to 3 weeks at +4 °C *(Aradóttir et al., 2004a; Zheng et al., 2011)*, or frozen in liquid nitrogen and stored at −80 °C without affecting PEth levels *(Aradóttir et al., 2004a, 2004b; Aradottir et al., 2002)*, the latter being not practical in a routine setting. Precautions have to be taken into consideration to avoid potential false-positive PEth levels, because of *in vitro* formation of PEth at −20 °C and at room temperature in blood samples containing ethanol *(Aradóttir et al., 2004b)*.

3.4. Ethylglucuronide & Ethylsulfate

Description

Ethylglucuronide (EtG) will be discussed extensively in the second chapter. Next to EtG, ethylsulfate (EtS), a minor metabolite of ethanol, appears to be a good candidate as a biomarker for recent alcohol consumption.

Until 2004, EtS was only demonstrated as an ethanol metabolite in animal models *(Helander and Beck, 2004)*. Helander and Beck found that sulfate conjugation is also a metabolic pathway for ethanol in humans and that EtS is present in urines after alcohol intake. EtS is formed by the transfer of a sulphuric group from 3'-phosphoadenosine- 5'-phosphosulfate (PAPS) to ethanol accomplished by the superfamily of cytosolic sulfotransferases (SULTs;

Fig. 5) *(Wurst et al., 2006)*. Only a very small fraction (around 0.1%) of the ethanol ingested undergoes sulfate conjugation in humans. About 0.010–0.016% of the ethanol dose is excreted as EtS in urines.

Context of use

EtS provides a profile of urinary excretion relatively comparable to that of EtG, with an appearance in urines occurring about 1 hour after ingestion of alcoholic beverages, and a peak about 4h after that of ethanol in the blood. The detection window is about 30-36 hours *(Wurst et al., 2006)*. In the blood, the average time difference between the maximal concentrations of ethanol and its metabolites is approximately 2-3h and 1-2h for EtG and EtS, respectively. They are both still detected in blood up to 4-8 h after ethanol elimination *(Halter et al., 2008; Schmitt et al., 1995)*. Thus, a positive finding of EtG and/or EtS is considered as a reliable indicator of recent intake of alcohol, even when ethanol is no longer detectable.

As FAEEs, both EtG and EtS accumulate in hair and can be used to assess chronic alcohol consumption. Their determination in maternal and/or neonatal matrices (meconium) is proposed as a good choice to assess gestational ethanol exposure *(Morini et al., 2010; Pragst and Yegles, 2008)*.

Confounding factors and precaution

False-negative and false-positive results may occur if the samples are not stored properly prior to analysis *(Helander et al., 2009b, 2007)*.

For best interpretation of EtS concentrations, some precautions should be taken into account:

- Stability of EtS in urine stored at +4°C is comparable to that of EtG (*i.e.* more than 3 weeks) *(Wurst et al., 2006)*. However, it has been found to be biodegradable under high bacterial density conditions *(Baranowski et al., 2008)*.

- The urinary EtS concentration can be markedly lowered by the consumption of high amounts of water or fluids prior testing. Expressing urinary EtS as a ratio to creatininuria may be therefore recommended to compensate for (intentional) urine dilution *(Helander and Beck, 2004)*. Moreover, in patients with decreased renal function, detection times were found to be significantly longer compared to those in healthy volunteers *(Høiseth et al., 2012)*.

- Unintentional ethanol intake due to ethanol-based mouth-wash *(Høiseth et al., 2010a; Reisfield et al., 2011)* and hand sanitizers can yield positive urinary EtS levels, even when applying a very low analytical cut-off limit.
- False-positive results can also occur due to the consumption of EtS-containing beverages, like alcohol-free wine (which could contain up to around 40 mg/L of EtS) or grape juice *(Høiseth et al., 2010a; Musshoff et al., 2010)*. Therefore, the use of wine could result in higher relative concentrations of EtS *(Halter et al., 2008; Høiseth et al., 2010a)*.
- Finally, genetic polymorphisms in sulfotransferases might influence the metabolism and excretion patterns of EtS, thus inter-individual differences can be expected *(Halter et al., 2008; Wurst et al., 2006)*.

Several studies dealt with the kinetics and detection windows of EtS in urine. Nevertheless, there is currently no consensual cut-off value for EtS in urine. In the context of abstinence tests, Albermann *et al.* showed that 0.05 mg/L (EtS) could be a potential cut-off to exclude the repeated intake of alcohol *(Albermann et al., 2012)*. Using a cut-off of 0.025 mg/L, Stewart *et al.*, (2013) determined a sensitivity of 82% and a specificity of 86% for urine EtS to identify recent drinking in liver disease patients *(Stewart et al., 2013)*. However, EtS concentrations within such mentioned cut-offs can be found after unintentional consumption of alcohol. Other studies suggested EtS cut-offs at 0.1 mg/L *(Dahl et al., 2011)* or even at 0.5 mg/L *(Reisfield et al., 2011)*. Further research is required to validate these cut-offs.

At the analytical level, different methods have been developed to determine EtS, including LC–MS/MS with negative electrospray ionization (ESI) *(Wurst et al., 2006)* and capillary zone electrophoresis (CZE) with indirect UV detection. For confirmation analysis, MS detection rather than indirect UV absorption is required *(Caslavska et al., 2011)*. It is to note that there is no immunochemical assay for EtS determination.

4. Other biomarkers of alcohol consumption

4.1. Acetaldehyde & acetaldehyde-protein adducts

Acetaldehyde is the main product of oxidative ethanol metabolism; it has also been used as a marker of recent drinking. Since acetaldehyde is a highly reactive molecule, it can readily

react with the nucleophilic groups of proteins, resulting in stable adducts with several proteins, such as hemoglobin, albumin, lipoproteins, tubulin, CYP2E1, and red blood cell membrane proteins *(Eriksson and Fukunaga, 1993; Nicholls et al., 1992; Stevens et al., 1981)*. Hemoglobin–acetaldehyde adducts (HAAs) have received more attention *(Sillanaukee et al., 1991; Stevens et al., 1981)*. Adduct levels in blood or in urine indicate drinking behavior and have been proposed as potential markers of alcohol abuse. Acetaldehyde-protein adducts (APAs) have a longer half-life time than free acetaldehyde and remain high in blood for approximately a month after alcohol intake *(Halvorson et al., 1993)*.

APAs have been measured in whole blood, serum, plasma, and other tissues, using either immunological or HPLC techniques; although no routine applications have been developed so far. Very little is known about sources of false-positive results for APAs, except that diabetics have levels of HAAs twice as high as alcoholics *(Sillanaukee et al., 1991)*. Sensitivity and specificity values of this potential marker among heavy–drinking males have been reported as 65-70% and 93%, respectively, with values in females of 53% and 87% *(Worrall et al., 1991)*. On the other hand, Hazelett *et al.* (1998) did not find gender differences in the performance of HAA adducts and reported sensitivity and specificity values of 67% and 77%, respectively *(Hazelett et al., 1998)*.

Neither acetaldehyde nor APAs have been sufficiently examined for their value in detecting and monitoring alcohol consumption. Accumulating evidence has indicated that adduct formation leads to a breakdown of immunological tolerance *in vivo* and induction of autoantibodies towards the resulting neo-antigens *(Latvala et al., 2005; Niemelä, 2001)*. Such autoantibodies have been found in alcoholic patients, and they may be useful in the differential diagnosis of alcoholic *versus* non-alcoholic liver diseases *(Latvala et al., 2005)*. The possible utility of APAs as a marker of alcohol abuse during pregnancy has also been investigated. HAAs were detected in the blood of pregnant drinkers with the highest concentrations in mothers who subsequently delivered infants with FASD *(Niemelä et al., 1991)*.

4.2. 5-hydroxytryptophol

Serotonin (5-hydroxytryptamine, 5-HT) is produced enzymatically from tryptophan by hydroxylation and decarboxylation. 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole–3–acetic acid (5-HIAA) are normal end-products in the metabolism of serotonin, with 5-HIAA being the major urinary metabolite. 5-HTOL is excreted in urine predominantly as 5-hydroxytryptophol glucuronide (GTOL) *(Beck and Helander, 2003)*. The formation of 5-HTOL is increased dramatically after alcohol intake, and then remained for about 5–15 hours after ethanol has been eliminated *(Beck and Helander, 2003)*. This biochemical effect can be used in the detection of recent alcohol intake.

In routine clinical use, urinary 5-HTOL is reported as a ratio to 5-HIAA instead of creatininuria, because this practice compensates for interferences from variations in urine dilution, as well as serotonin turnover (due to dietary intake of serotonin) *(Helander et al., 1992)*. The detection of elevated 5-HTOL/5-HIAA ratio depends on the dose of ethanol: higher doses of alcohol producing higher values. Voltaire *et al.*, (1992) proposed a 5–HTOL/5–HIAA ratio > 20 pmol/nmol as an indicator of recent alcohol consumption *(Voltaire et al., 1992)*.

The urinary 5-HTOL/5-HIAA ratio has been applied as a sensitive, specific, and reliable marker for recent alcohol intake in both clinical and forensic contexts *(Beck and Helander, 2003; Carlsson et al., 1993; Helander et al., 1992)*, particularly binge-type ethanol use, and as a potential method to rule out artifactual ethanol formation in *post-mortem* cases *(Helander et al., 1992)*. 5-HTOL is also effective for monitoring lapses into drinking during out-patient treatment and for objective evaluation of treatment efforts *(Carlsson et al., 1993)*. As the test measures very recent alcohol intake, its clinical use for monitoring a patient's intake requires frequent urine collections. As compared to other direct markers (EtG and EtS) the 5-HTOL/5-HIAA ratio is found equally sensitive, but with a much shorter window of detection for the surveillance of abstinence relapses *(Høiseth et al., 2008a)*.

4.3. Total serum sialic acid

Sialic acid (SA) refers to a group of *N*–acetylated derivatives of neuraminic acid in biological fluids and in cell membranes. They are attached to non-reducing terminal residues of the carbohydrate chains of glycoproteins and glycolipids. SA may have a variety of biological functions *in vivo*, such as stabilization of the conformation of glycoproteins and cellular membranes, cell-to-cell interactions, membrane transport, membrane receptor functions, and regulation of the permeability of the basement membranes in glomeruli *(Crook et al., 1996)*. The range of normal serum values of SA is 1.58-2.22 mmol/L. Many studies have further shown that SA concentration in serum, as well as in saliva, may be increased in alcoholic subjects *(Pönniö et al., 1999; Sillanaukee et al., 1999b)*.

Sillanaukee *et al.,* (1999) reported that SA was elevated by 37% ($n = 26$, $P < 0.001$) and 33% ($n = 23$, $P < 0.001$), respectively, among female and male alcohol-dependent patients, as compared with social drinkers, demonstrating sensitivity and specificity values, respectively, of 58 % and 96 % for women and 48 % and 81 % for men *(Sillanaukee et al., 1999b)*. Another study suggests that TSA levels are significantly increased even after a short period of heavy drinking and may be a potential marker for relapse *(Pönniö et al., 2002)*.

Interestingly, a positive correlation was found between the amount of alcohol consumption and TSA level *(Idiz et al., 2004; Sillanaukee et al., 1999a)*. However, neither the dose of alcohol needed to increase SA nor the mechanism underlying its increase has been defined. The half-life time of SA has not been reported, but it seems to be longer than that of CDT and GGT *(Sillanaukee et al., 1999b)*.

It appears that certain disorders may cause interference with this test. Certain forms of cancer *(Sillanaukee et al., 1999a)*, cardiovascular diseases *(Lindberg et al., 1992)*, and diabetes *(Ozben et al., 1995)* indeed result in the elevation of SA residues in serum. TSA levels in serum have also been shown to vary with body mass index, age, and blood pressure among both men and women *(Pönniö et al., 1999)*.

TSA can be measured by two approaches. The first is to hydrolyze conjugated SA residues from serum proteins with strong acid or the enzyme TSA neuraminidase. The second is to hydrolyze proteins conjugated to SA residues in serum samples, to purify SA, and then to quantify it using HPLC with an anion-exchange column and pulsed amperometric detection *(Romppanen et al., 2002)*. Whereas these methods are quite easy and could be performed in most research laboratories, they are not commonly available throughout all clinical laboratories. Even if TSA could be a useful marker for alcohol consumption, more studies are still necessary to assess its usefulness and relevance.

4.4. Plasma sialic acid index of apolipoprotein J

Apolipoprotein J (Apo J) is a glycoprotein of 70 kDa that is found in high-density lipoprotein complexes (HDL2 and HDL3). The physiological significance of Apo J is not well understood, but it is thought to be involved in the exchange of lipids, especially cholesterol, between different lipoproteins *(Burkey et al., 1991)*. The term sialic acid index of Apo J (SIJ) has been coined to express the molecular ratio of sialic acid to Apo J protein. SIJ decreases after chronic alcohol drinking and it increases with the cessation of drinking. SIJ returns to normal levels over a period of weeks, with an approximate half-life of 4 to 5 weeks *(Ghosh et al., 2001, 1999)*. Even if the SIJ might hold potential as a promising biomarker for heavy ethanol intake, further comprehensive studies should be performed to characterize potential variability due to age, gender, level of drinking, other drug use, and other pathologies.

4.5. Beta-Hexosaminidase

Beta hexosaminidase (β-HEX; also called *N*-acetyl beta glucosaminidase) is a lysosomal hydrolase that exists in most cells and tissues and is involved in the metabolism of carbohydrates and gangliosides in liver cells. The β-HEX molecule is composed of combinations of two polypeptide chains, termed alpha and beta, which results in the existence

of several isoforms of β-HEX (A, B, I, P, and S) *(Isaksson and Hultberg, 1989)*. Isoforms B, I and P are heat stable, while isoforms A and S are heat labile *(Price and Dance, 1972)*. The heat stable isoforms of β-HEX (B, I, P), collectively called β-HEX B, has been shown to be elevated in serum and urine from alcoholics and healthy volunteers, after heavy alcohol consumption (> 60 g/d for at least 10 days) *(Hultberg et al., 1980; Kärkkäinen et al., 1990)*, because of the damage of lysosomes and the subsequent cellular release of the enzyme into the blood. Serum β-HEX levels return to normal after 7–10 days of abstinence *(Hultberg et al., 1980)*, whereas urine β-HEX normalizes after about 4 weeks of abstinence *(Martines et al., 1989)*.

Kärkkäinen *et al.*, (1990) reported sensitivity of 69 % and 81 % for serum and urine β-HEX, respectively *(Kärkkäinen et al., 1990)*. However, β-HEX appears not to be as effective in identifying less excessive (<60 g/d), but still harmful levels of drinking in unselected populations *(Nyström et al., 1991)*. Moreover, reduced serum β-HEX levels have been seen in association with chronic renal failure *(Kärkkäinen et al., 1990)*.

Although a high specificity (approximately 90% in both matrices) have been reported for β-HEX *(Hultberg et al., 1995; Kärkkäinen et al., 1990)*, serum levels of β-HEX have been noted to be increased in hypertension, diabetes mellitus, cirrhosis, thyrotoxicosis, pregnancy, in users of oral contraception, cerebral infarction, myocardial infarction, and also slightly with age *(Hultberg and Isaksson, 1983; Hultberg et al., 1996; Pitkänen et al., 1980)*. β-HEX is considered as an inexpensive test, it can be measured using standard laboratory techniques as spectrophotometry and fluorimetry.

4.6. Other markers

Other potential markers of excessive alcohol intake include lipid profile, monoamine oxidase-B (MAO-B), dopamine receptor D2 (DRD2), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), hyaluronic acid, certain cytokines, plasma α-aminobutyric acid,

leucine ratio, urinary salsolinol, and urinary dolichols *(Helander, 2003; Niemelä, 2007; Ferguson and Goldberg, 1997)*. Some of them may be useful in determining a genetic predisposition for alcohol abuse or dependence *(Niemelä, 2007; Ferguson and Goldberg, 1997)*. All require further clinical evaluation and some can require complex measurement techniques. They are of less utility in assessing a specific exposure incident.

Main data characteristic of ethanol markers are summarized in Table 2.

Regarding all the aforementioned data, it is obvious that no laboratory test is totally reliable enough on its own to support a diagnosis of alcoholism; laboratory tests need to be part of a diagnostic process that includes a detailed clinical history, examination and the use of questionnaires. Furthermore, the sensitivity and specificity of the different laboratory markers vary considerably and depend on the population studied (Table 2).

Moreover, since none of these biomarkers offer perfect validity, as a reflection of alcohol consumption, attempts have been made to improve the sensitivity of single laboratory markers by combining them. Although most of the combinations have shown enhanced sensitivity (*e.g.* CDT plus GGT, CDT plus MCV), none of them is currently accepted.

Table 2. Main characteristics of biomarkers of alcohol consumption.

Marker	Matrix	Detection Window	Type of drinking characterized	Cut-off	Cost	Sensitivity[d]	Specificity[d]
GGT	serum	2-6 weeks	>50 g/d for several weeks	50 UI/L	Low	Moderate	Moderate
AST	serum	2-3 weeks	chronic heavy drinking	Depend on method	Low	Low	Low
ALT	serum	2-3 weeks	chronic heavy drinking	Depend on method	Low	Low	Low
MCV	serum	Unknown but half–life ~ 2 months	chronic heavy drinking	100 fL	Low	Low	Moderate
CDT	serum	2-4 weeks	>60 g/d for ~ 2-3 weeks	Depend on method	Medium	Moderate	High
Ethanol	breath blood urine	4-6 hours ~ 8 hours 18-24 hours	acute and/or recent consumption (even low-levels) limited in chronic drinking	0.1 g/L	Low	88%	100% except in *post-mortem* situations
FAEE	blood hair meconium	~ 24 hours months 3-6 months	recent alcohol consumption chronic alcohol consumption chronic alcohol consumption	Unknown 0.5 ng/mg[a] 0.5 ng/g	Relatively high	High	High
PEth	blood	up to 4 weeks	chronic alcohol consumption	Unknown	Relatively high	moderate	High
EtS	serum urine hair meconium	~ 12-16 hours 30-36 hours months months	recent alcohol consumption recent alcohol consumption chronic alcohol consumption chronic alcohol consumption	Unknown 0.1 mg/L Unknown	Relatively high	High	High
EtG	serum urine hair meconium	up to 17 hours up to 5 days abstinence months months	recent alcohol consumption recent alcohol consumption abstinence chronic alcohol consumption chronic alcohol consumption	Unknown 0.5 mg/L < 7 pg/mg[b] > 30 pg/mg[b] 2 nmol/g	Relatively high Depend on method Relatively high	High	High
APAs	blood	up to 3 weeks	chronic heavy alcohol consumption	Unknown	Low	moderate	High
5-HTOL	urine	~ 5-15 hours	recent alcohol consumption	> 20 pmol/nmol[c]	Relatively high	High	High
TSA	serum	Unknown	heavy alcohol consumption	Unknown	Relatively high	moderate	High
SIJ	serum	>5 weeks	heavy alcohol consumption	Unknown	medium	High	High
β-HEX	serum urine	7-10 days 4 weeks	chronic heavy alcohol consumption	Unknown Unknown	Low	moderate High	High

[a]Measured in the 0–3 cm proximal scalp hair segment, [b]Measured in the 0-3 up to 0-6 cm proximal scalp hair segment, [c]5-HTOL/5-HIAA ratio, [d]Low: < 40%, moderate: 40 to 80 %, high: > 80%.

Part 2: Ethylglucuronide and research methodology

1. Generality

The glucuronidation of ethanol was first described in the beginning of the 20th century *(Neubauer, 1901)* who reported qualitative detection of an ethanol conjugate in the urine of dogs and rabbits; it was subsequently detected in human urine for the first time in 1967 *(Jaakonmaki et al., 1967).*

The Ethyl-β-D-Glucuronide (EtG; molar mass = 222.19 g/mol,) is a nonvolatile, acidic, water-soluble, direct and minor metabolite of ethanol. It is formed by conjugation of ethanol with uridine 5'-diphosphate glucuronic acid (UDP-GA) *via* the action of UDP-glucuronosyl transferases (UGT), and is therefore exclusively produced *in vivo* following ethanol exposure. It accounts for about 0.5% of total ethanol elimination. EtG is mostly found in the blood, urine, liver, and bile. It can also be found in lesser amount in the cerebrospinal fluid, bone marrow, muscle, adipose tissue, brain, and hair *(Høiseth et al., 2007b; Schloegl et al., 2006b; Wurst et al., 1999b)* (Table 3).

Table 3. Ethyglucuronide concentrations measured in different tissues (µg/g) and biological fluids (mg/L) compared to blood alcohol concentrations (BAC).

Biological fluid	**BAC: 0.1–0.6 g/L**	**1–1.5 g/L**	**>2 g/L**
Blood	0.1–4.9	0.5–56	1.2–40
Urine	3.8–80	15	33–509
Bile	1.1–7.0	2.8	6.3–42
Tissue	**BAC: 0.1–0.6 g/L**	**1–1.5 g/L**	**>2 g/L**
Liver	7.9–13	6.7	43–77
Bone marrow	0.5–1.0	0.8	1.0–9.4
Skeletal muscle	0.1–0.6	0.3	0.6–1.8
Adipose tissue	0.0–0.4	0.2	0.4–1.2

2. Interest of ethylglucuronide

2.1. Characteristics

Since EtG is a direct metabolite of ethanol that is exclusively produced *in vivo* following alcohol consumption, it is a highly specific biomarker for ethanol consumption. As a biomarker, EtG has also a very high sensitivity, which exceeds that seen with standard ethanol determinations and it is capable of detecting exposure to minute quantities of ethanol. One of its most important advantages as a biomarker of alcohol consumption is that it can be detected in various body fluids (whole blood, serum, urine, vitreous humor, and bile), tissues, hair, and meconium. This advantage widens the scope of this biomarker.

2.2. Stability

EtG has been shown to be a stable marker. Studies have shown that urine samples stored at 4 °C for five weeks have no change in EtG concentration *(Schloegl et al., 2006a)*. During the study, there was no evidence of decomposition in urine. Furthermore, no *post-mortem* formation was found indicating that a positive result proves alcohol consumption prior to death *(Schloegl et al., 2006a)*.

2.3. Detection window

- **In plasma:**

EtG appears in the blood within 45 min after ethanol consumption (even after a small intake of around 7-10 g). After ingestion of alcohol, the average time difference between maximum concentrations of ethanol in blood and those of EtG in plasma is approximately 3-5.5 h; it is detected up to 17 h after consumption of ethanol *(Halter et al., 2008; Høiseth et al., 2007a; Schmitt et al., 1997)*.

- **In urine:**

The elimination of EtG is primarily renal. The amount of EtG excreted in the urine represents less than 0.1% of the dose of ingested ethanol. EtG appears in the urine within one hour after

consumption of ethanol. Its maximum concentration is reached after about 5.5 h *(Dahl et al., 2002; Halter et al., 2008; Høiseth et al., 2007a)*. However, it is important to note that the maximum concentration and the detection window of EtG depend on the ethanol intake, as well as on the metabolism of each individual.

There have been numerous studies characterizing the time window during which EtG remains detectable in urines following alcohol intake in healthy volunteers *(Dahl et al., 2002; Halter et al., 2008; Høiseth et al., 2007a)*. These studies involved doses of ethanol ranging from 0.1 to 0.85 g/kg body weight and the detection window was 24-48 h. Other studies showed that for a larger dose of ethanol (>1 g/kg), EtG could remain detectable for five days or more *(Alt et al., 1997; Borucki et al., 2005; Helander et al., 2009a)*. Accordingly, it is known that a positive finding of EtG in blood and/or urine (even when ethanol is no longer detectable) provides a strong indication that the person was recently drinking alcohol; therefore it has the major advantage to increase the detection window observed with ethanol. Fig. (6) represents the detection of ethanol in blood and that of EtG in serum and urine following acute ethanol consumption.

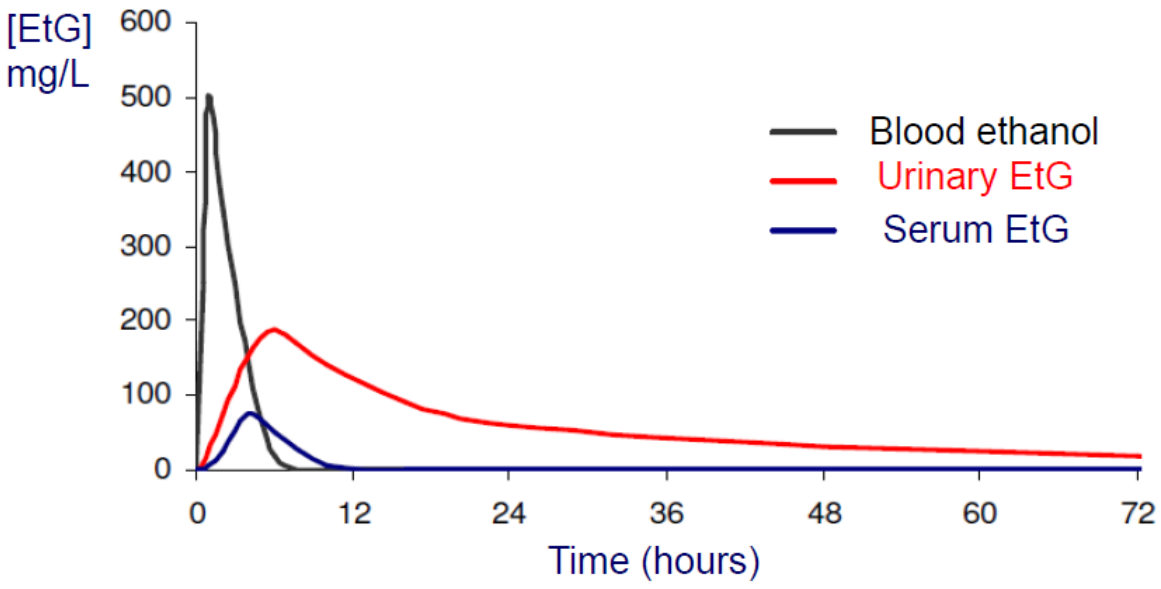


Figure 6. Graphic representation comparing the detection window of ethanol in blood and EtG in serum and urine, following acute ethanol consumption.

- **In hair:**

The accumulation of EtG in the hair has been also demonstrated *(Sachs, 1997)*. The advantages of hair analysis include a significant increase of the detection window and the potential establishment of longer-term drinking history, which makes this an attractive approach. EtG can be detected several weeks to several months, depending on hair length, and therefore can be used for the monitoring of chronic alcohol consumption. Furthermore, segmental analysis may provide insight into drinking patterns over preceding months. As EtG is a non-volatile metabolite, it is unlikely to be present in hair, as a result of environmental contamination (although external contamination through sweat is possible).

- **In meconium:**

The determination of EtG in meconium was proposed for the first time in 2008, as a biomarker of maternal ethanol consumption *(Morini et al., 2008)*, to differentiate heavy maternal alcohol consumption during pregnancy from occasional use or no use at all. This analysis allows a detection window corresponding to approximately the last 20 weeks of gestation.

2.4. Cut-off values

2.4.1. Blood and urine

To date, there have been insufficient clinical studies on plasma EtG to allow a reliable determination of the most appropriate cut-off values. In a volunteer study, a maximum plasma EtG concentration of 360 µg/L (range 280-410 µg/L) was found in samples taken 1.5 to 24 h after the intake of a 0.5 g/kg dose of ethanol and 1060 µg/L (range 800-1220 µg/L) after a dose of 1.0 g/kg dose of alcohol *(Høiseth et al., 2010b)*.

It is important to note that no correlation was observed between the concentrations of EtG measured in blood or in urine and ethanol concentrations in blood *(Alt et al., 1997; Schmitt et al., 1997)*. This suggests that high concentrations of EtG in blood or urine do not necessarily indicate a high consumption of ethanol. However, the measurement of EtG in blood has been suggested as a complement to the analysis of ethanol to determine or approximate the time of

ingestion *(Høiseth et al., 2007a)*. A blood ethanol:EtG ratio higher than 1 is suggestive of an ethanol ingestion within 3.5 h before sample collection, whereas a ratio lower than 1 suggests that ethanol was ingested more than 3.5 h before sampling *(Høiseth et al., 2007a)*. Assessment of drinking time may be even more valid when results from two samples collected 30 to 60 min apart are used. The authors concluded that two successive increasing blood EtG concentrations and a high ethanol:EtG ratio supports recent drinking *(Høiseth et al., 2007a)*.

Concerning urine, similarly to blood, no uniformly accepted cut-off value for EtG concentration has been defined by commercial laboratories. Although occasionally touted as an "80-hour ethanol test", 100% sensitivity for ethanol consumption was demonstrated only up to 39.3 h and it was found to rapidly declined with urine EtG concentrations falling below the cut-off of 0.1 mg/L *(Borucki et al., 2005)*. Studies in healthy volunteers who were given alcohol at doses between 0.1 and 0.8 g/kg body weight have consistently shown that applying cut-off values of 0.1-0.2 mg/L for urine EtG successfully detected the intake of ethanol *(Dahl et al., 2002; Halter et al., 2008; Helander et al., 2009a)*. In another recent study, and in order to establish the optimum EtG cut-off for urinary abstinence tests of healthy persons after drinking small, but realistic amounts of alcohol (one or two glasses of beer or white wine), Albermann *et al*., showed that 24 h after the experiment, 75% (9/12) of the urine samples were tested negative for EtG using a cut-off of 0.5 mg/L *(Albermann et al., 2012)*. In half of their samples, concentrations below 0.1 mg/L EtG were detected. They concluded that urinary cut-offs for EtG of 0.5 mg/L or higher are not suitable for testing abstinence, and that even 0.1 mg/L is not effective to detect the intake of small amounts of alcohol in the context of abstinence tests *(Albermann et al., 2012)*.

However, since external factors may be associated with changes in urine EtG concentrations *(Wurst et al., 2004)*, and since selection of an appropriate cut-off is critical in circumstances where freedom or livelihood may be adversely affected by a positive result, cut-offs as high as 0.5 mg/L have been used (in routine clinical applications) to reduce the risk of false-positive results *(Helander et al., 2009a)*.

2.4.2. Hair

A significant correlation between EtG concentration in hair and the amount of alcohol intake has been demonstrated *(Appenzeller et al., 2007)*, and the levels of hair EtG seemed to reflect the average daily amount of alcohol ingested *(Appenzeller et al., 2007; Kharbouche et al., 2010)*.

On June 28th 2012, the use of EtG in hair was adopted by the Society of Hair Testing (SoHT), as the first choice marker for abstinence assessment (SoHT report, 2012). In their consensus, they concluded that a concentration ≥ 7 pg/mg EtG in the 0-3 up to 0-6 cm proximal scalp hair segment strongly suggests repeated ethanol consumption, and that a lower concentration is not in contradiction to the self-reported abstinence of a person during the corresponding time period before sampling. For this purpose, either gas or liquid chromatography coupled to (tandem) mass spectrometry should be used with a LOQ ≤ 3 pg/mg (SoHT report, 2012).

They also mentioned that in doubtful cases and for exclusion of false-positive and false-negative results, the determination of both EtG and FAEEs in hair can be useful. However, a negative FAEE result cannot overrule an EtG result ≥ 7 pg/mg.

In order to establish chronic excessive alcohol consumption, another consensus of the SoHT was adopted in 2009 and then revised in 2011 *(Kintz, 2010)*. In this consensus, the cut-off for EtG in hair to strongly suggest chronic excessive alcohol consumption is proposed at 30 pg/mg scalp hair measured in the 0–3 up to 0–6 cm proximal segment. If samples less than 3 cm are used the results should be interpreted with caution.

In coherence with these cut-offs, Kharbouche *et al*., (2012) revealed that the hair EtG diagnostic performance was significantly better (P<0.05) than any of the traditional biomarkers alone *(Kharbouche et al., 2012)*, and that EtG, as a single biomarker, yielded a stronger or similar diagnostic performance in detecting at-risk or heavy drinkers, respectively, than the best combination of traditional biomarkers (CDT and GGT). They concluded that the combination of EtG with traditional biomarkers did not improve the diagnostic performance of EtG alone *(Kharbouche et al., 2012)*.

According to the SoHT consensus, hair EtG is considered as the only biomarker that provides an accurate and reliable diagnostic test for assessment of abstinence, as well as for identifying chronic and excessive alcohol consumption, whereas the traditional biomarkers failed to do so.

3. Context of EtG determination: clinical & forensic applications

The measurement of EtG has been carried out in a variety of clinical and forensic settings.

3.1. In clinical settings:

- Alcohol misuse can be implicated in a significant proportion of subjects admitted in hospital emergency departments. Two studies addressing the use of EtG measurements in emergency room settings have been carried out, one using urine and another using plasma samples *(Kip et al., 2008; Neumann et al., 2008)*. In these studies, the authors showed that 25-38% of subjects, either tested negative for blood ethanol or with only low blood ethanol concentrations, were positive for EtG.
- Monitoring and documentation of abstinence in subjects undergoing alcohol detoxification programs is important, but can be difficult using blood or urine ethanol measurements due to the relatively short period of time these markers remain positive following alcohol ingestion. This application is considered as one of the most wide-spread use of EtG, most often in cases involving professionals with history of alcohol misuse who are submitted to abstinence and a monitoring program as a condition for continued licensure and/or employment. Many studies have documented this application: in a group of 139 detoxified alcohol-dependent patients followed up for 12 weeks after discharge from inpatient treatment, Junghanns *et al.,* (2009) showed that 28% of subjects denying relapse were tested positive for EtG measured by LC-MS/MS *(Junghanns et al., 2009)*. Similarly, four out of 30 patients, for whom neither clinical assessment nor routine laboratory testing suggested relapse, were tested positive for EtG in urine with concentrations ranging from 4.2 to 196.6 mg/L *(Wurst et al., 1999a)*.
- In alcoholic liver disease (ALD) patients, abstinence is required to remain on the transplant list, but is difficult to assess as traditional markers are affected by liver diseases *per se* and

patients usually refrain from drinking in the 24–36 h period prior to testing by breath, blood or urine alcohol measurements. EtG determination could play an important role in this context. In a study involving 18 liver transplant candidates who denied alcohol consumption, nine had positive EtG results in 24 (49%) of 49 urine samples. Only one of the 127 breath alcohol tests performed was positive *(Erim et al., 2007)*. In another study in 109 patients undergoing assessment for liver transplantation, 20% of subjects with alcohol-related liver disease had positive urine EtG results, but only 4% self-reported alcohol use *(Webzell et al., 2011)*. These results, as well as those of other studies, highlight the superiority of EtG as a biomarker of abstinence before liver transplantation.

- Fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorder (FASD) are recognized as causes of congenital abnormalities, cognitive dysfunction, and developmental delay. However, diagnosis after birth can be difficult. In this context, EtG is proposed as a promising biomarker of prenatal exposure to ethanol. In a study conducted by Pichini *et al.*, (2009), EtG was detectable in over 80% of meconium samples collected from the infants of 177 randomly selected women from Italy and Spain *(Pichini et al., 2009)*. In this study, a cut-off of 2 nmol/g (444 ng/g) was found to have 100% sensitivity and specificity to distinguish heavy maternal ethanol consumption during pregnancy from occasional (or no) use (assessed by a questionnaire, as well as meconium FAEE measurement) *(Pichini et al., 2009)*. A similar study on 602 meconium samples from a maternal health evaluation showed that only 16.3% of cases had detectable EtG. In none of the 602 cases did the mothers report serious alcohol consumption. Moreover, two outliers (EtG at 10200 and 82000 ng/g) suggested heavy alcohol consumption that was not reported by the mothers *(Bakdash et al., 2010)*. Only one study has been published on the measurement of EtG in urine and hair of pregnant women *(Wurst et al., 2008)*. In this study, twenty-six women out of 103 (25.2%) were identified as possible alcohol consumers and seven subjects had hair EtG concentrations highly suspicious of heavy drinking, although only one was positive according to the AUDIT questionnaire.

3.2. In forensic settings:

Other uses of EtG include investigation of road accidents, monitoring of motorists with alcohol abuse history who have abstinence as a condition for continued driving privileges, and people on probation for alcohol violence. Additionally, use of EtG assessment has been studied in *post-mortem* investigations and cases of criminal liability *(Høiseth et al., 2007a, 2007b; Wurst et al., 1999a)*. In *post-mortem* investigations, blood EtG determination seemed to be a reliable marker of *ante-mortem* ingestion of alcohol, and it could be considered in forensic autopsy cases when *post-mortem* formation of ethanol is questioned *(Høiseth et al., 2007b).*

4. Analytical methods for measurement of EtG

The first methods for the determination of EtG in blood and urine were described in the mid-1990s and were based on gas chromatography–mass spectrometry (GC–MS) *(Schmitt et al., 1995).* Since this period, numerous analytical methods have been developed for EtG analysis in biological matrices, such as nuclear magnetic resonance (NMR), capillary zone electrophoresis (CZE), GC-MS, liquid chromatography–mass spectrometry (LC-MS), liquid chromatography with pulsed electrochemical detection, and immunochemical test. Each method has inherent strengths and weaknesses in terms of specificity, sensitivity, assay complexity, cycle-time and instrumentation cost and/or availability.

Gas chromatographic methods for EtG analysis typically require pre-analytical derivatization to achieve acceptable chromatographic results. This results in increased turnaround time for assay results. Liquid chromatography is thus the preferred separation method with mass (or tandem mass) spectrometry employed as a highly sensitive and specific method of detection and quantitation. This technology has been used to determine EtG in urine *(Junghanns et al., 2009; Wurst et al., 1999a),* serum *(Wurst et al., 2000),* whole blood *(Høiseth et al., 2007b), post-mortem* body fluids and tissues *(Wurst et al., 2000, 1999b),* meconium *(Morini et al., 2008),* saliva *(Hegstad et al., 2009),* and hair *(Janda et al., 2002).*

A comparison of five LC-MS methods for the measurement of urinary EtG recommended solid-phase extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as the reference method, because it has the best selectivity and sensitivity *(Helander et al., 2010)*.

However, since LC–MS/MS is a relatively expensive technique for screening alcohol use in routine clinical practice and is only available in specialized laboratories, the widespread use of EtG has been hampered. In 2006, a commercially available enzyme immunoassay (EIA) method based on a monoclonal antibody (DRI Ethyl Glucuronide Enzyme Immunoassay; DRI-EtG EIA) was developed for the analysis of EtG in urine. This method showed a good correlation ($r^2 = 0.93$) with a well-established LC–MS method indicating low cross-reactivity of the EtG antibody with other urinary constituents *(Böttcher et al., 2008)*. Moreover, Rainio *et al.* has recently tested the same immunological approach to measure EtG in vitreous humor (VH) and serum from 58 individuals representing a forensic autopsy population *(Rainio et al., 2013)*. They concluded that, with a cut-off of 0.3 mg/L for VH-EtG, the immunoassay correctly identified 92% of the cases with a history of excessive alcohol use, whereas the BAC was positive (cut-off 0.1 g/L) in only 68% of the cases. They obtained a lower sensitivity and specificity for serum EtG as compared to VH-EtG. They also showed that EtG immunoassay has a strong correlation with the LC–MS/MS reference method ($r = 0.94$, $P < 0.001$) and there was 100% agreement in the frequency of positive and negative findings between the immunoassay results and the LC–MS/MS analysis *(Rainio et al., 2013)*.

This result indicates that the immunoassay of VH-EtG could be a useful forensic tool for screening of *ante-mortem* alcohol use. However, despite that immunological EtG measurement may provide a convenient, specific, and cost-effective tool, this method is still considered as a screening test (for qualitative and semi-quantitative measurement) and EtG-positive samples should always be analysed by LC-MS/MS for confirmation and quantitation.

5. Interpretation, precautions, and confounding factors

1. Studies have shown that EtG is found at higher concentrations in serum than whole blood with a median serum-to-blood ratio of 1.69 *(Høiseth et al., 2009b)*. Therefore, it is, important to be aware of the matrix used when interpreting or comparing results between publications.

2. The elimination rate of EtG does not appear to differ between healthy individuals and heavy drinkers during alcohol detoxification *(Høiseth et al., 2009a)*. However, a decreased elimination rate and higher blood concentrations have been reported in patients with renal diseases *(Høiseth et al., 2009a)*, which would delay excretion of this metabolite.

3. The effect of water diuresis has been published. While it is possible to lower EtG concentration by drinking large volumes of water, expressing EtG as a ratio to creatinine overcomes this problem of urinary dilution *(Dahl et al., 2002; Goll et al., 2002)*. However, Helander *et al*. reported wide interindividual variations in detection times even after adjusting concentrations for urine dilution *(Helander et al., 2009a)*.

4. Kharbouche *et al*. showed that EtG was not significantly influenced by gender, or by age *(Kharbouche et al., 2012)*. In another study, Wurst *et al.* showed that race, smoking, body mass index, liver cirrhosis, age at which subjects began drinking regularly, and total body water had no significant influence on EtG concentrations in urine *(Wurst et al., 2004)*. However, the same study revealed that EtG concentrations were influenced by age, gender, cannabis use, kidney disease, and the amont (in grams) of ethanol ingested during the previous month. Thus, it is important to consider these factors for result interpretations.

5. Stability: Despite the satisfactory stability of EtG in urine samples when stored at + 4 °C *(Schloegl et al., 2006a)*, it has been documented that both false-positive and -negative results can arise from bacterial contamination of urine *(Helander and Dahl, 2005)*. Glucuronide conjugates are cleaved by bacterial β-glucuronidase enzymes. On one hand, studies have shown that EtG, but not EtS, is sensitive to bacterial hydrolysis when samples are infected with *Escherichia coli*, *E. cloacae*, *K. pneumonia*, or *Clostridium sordelli (Alt et al., 1997;*

Helander and Dahl, 2005). Hydrolysis was faster with *E. coli* contamination with 60% to 100% of EtG hydrolyzed over a 5-day observation period *(Helander and Dahl, 2005)*. Since *E. coli* is the most common pathogen in urinary tract infections, there is a risk of falsely-low EtG results. On the other hand, Helander *et al.*, (2007) showed that EtG can be formed in urine specimens after collection, if the sample is infected with *E. coli* and if ethanol is present or produced during storage *(Helander et al., 2007)*. The formation of ethanol in unpreserved specimens can occur from microbial contamination and fermentation. Formation of post-collection EtG is not always prevented by addition of sodium fluoride preservatives or storage at + 4°C *(Helander et al., 2007)*, and, therefore, caution is advised when interpreting results. Interestingly, EtS concentrations were shown to be stable, suggesting a lack of EtS formation in contaminated samples *(Alt et al., 1997; Helander and Dahl, 2005)*. Since EtS is unaffected by contamination, it is recommended that urine EtG measurement is combined with EtS analysis to avoid both false-positive and -negative results. In *post-mortem* context, blood EtG results must be carefully interpreted in cases in which heavy putrefaction is present. As part of a large stability study, Hoiseth and coworkers assayed blood EtG in 39 autopsy cases, in which ethanol was thought to possibly be present as a result of *post-mortem* production *(Høiseth et al., 2008b)*. In 19 cases, EtG was present, whereas it was not detected in blood from the remaining 20 cases. They concluded that the absence of EtG in these cases might represent a false-negative result due to *post-mortem* degradation of EtG *(Høiseth et al., 2008b)*. Therefore, although a positive EtG finding is likely to represent ethanol ingestion, a negative result must be interpreted with caution, especially in heavily-putrefied bodies.

6. When stored at room temperature in ventilated vials, EtG concentration can increase due to water evaporation *(Schloegl et al., 2006a)*. On the other hand, EtG-positive tissue material, allowed to slowly decompose at room temperature, exhibited a decrease in EtG concentration over time; this decrease could have resulted from the degradation of the analyte *(Schloegl et al., 2006a)*.

7. As it is a direct conjugative metabolite of ethanol, EtG is essentially 100% specific for ethanol. However, EtG assay is incapable of determining ethanol source (environmental, hygienic, cosmetic, or incidental dietary exposure). Unintentional ethanol intake from ethanol-based mouthwash *(Costantino et al., 2006)* and hand sanitizers *(Rohrig et al., 2006)* may generate positive results. Moreover, the drinking of non-alcoholic beer, which contain around 0.4 % ethanol vol/vol, as well as the consumption of foods made with baker's yeast, sugar and water, can lead to ethanol formation by fermentation and, consequently, to increased EtG concentrations in urine *(Thierauf et al., 2010a, 2010b)*. These observations highlight the need for careful interpretation of low-level of EtG in urine.

8. Treatment of hair with ethanol-containing hair lotions does not significantly affect the EtG concentration *(Martins Ferreira et al., 2012)*. However, determination of EtG in hair appears to perform slightly worse in subjects who dye, bleach, or perm their hair, as well as in subjects who wash their hair more frequently *(Morini et al., 2009)*.

6. UDP-glucuronosyltransferases & interindividual variability

6.1. The glucuronidation reaction

Glucuronidation is the most important and common biotransformation reaction of the phase II metabolism of xenobiotics. It is catalyzed by different isoenzymes of the UGT superfamily. More than 26 different isoenzymes have been described in humans, each regulated in a tissue specific manner. Eighteen of them correspond to functional proteins and are encoded by two gene families, UGT1 and UGT2, that are, based on their sequence similarities, further divided into three subfamilies: UGT1A, UGT2A and UGT2B *(Guillemette, 2003; Kiang et al., 2005; Mackenzie et al., 1997)*.

UGTs are involved in the metabolism of endogenous compounds (e.g. bilirubine, thyroxine, hydroxy-steroids, and thyroid hormones), as well as xenobiotics (such as phytochemical compounds, polycyclic hydrocarbons, and a variety of drugs from all therapeutic classes) *(Kiang et al., 2005; Lépine et al., 2004)*. As illustrated in Fig. 7, the glucuronidation reaction

involves two molecules: the target substrate, also called aglycone, and the specific co-substrate (UDP-GA). During this reaction, a glucuronide group is transferred to the aglycone, on one of its functional sites (made most often of an atom of oxygen, nitrogen or sulfur). This transfer increases the polarity of the substrate and, consequently, its hydrophilicity, thereby promoting its excretion by the bile and urine.

6.1.1. Location and structure of UDP-glucuronosyltransferases (UGTs)

UGTs represent a superfamily of membrane bound glycoproteins, which are located in the endoplasmic reticulum (ER) of the cell. They consist of about 527-530 amino acid residues, and have a molecular weight of 50-57 kDa. They are classified as transmembrane proteins of the ER with a lumenal domain consisting of approximately 95% of the polypeptide and a cytoplasmic domain of about only 20 residues. These proteins are anchored to the membrane *via* a single C-terminal transmembrane domain followed by a short cytoplasmic tail *(Meech and Mackenzie, 1997)*. Fig. 7 presents schematically the structure of human UGT enzymes.

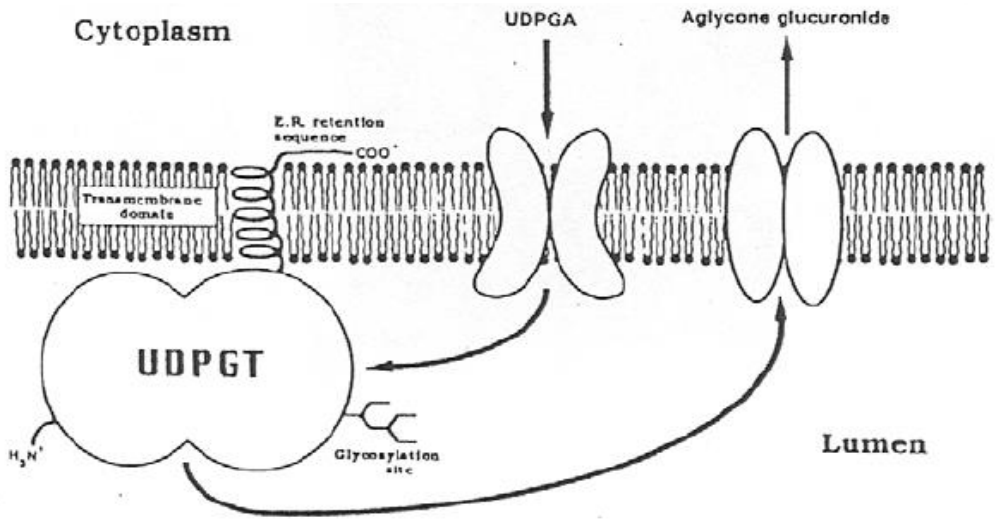


Figure 7. Schematic representation of human UDP-glucuronosyltransferase (UGT) topology, and proposed transporters in the endoplasmic reticulum (ER) membrane. UGTs consist of two domains and are predicted to function as dimers or higher oligomers. The amino-terminal domain binds to the aglycone and the carboxy-terminal domain to the UDPGA co-substrate; the catalytic site is placed between the two domains. Most of the enzyme mass is located on the lumenal side of the ER and the carboxy-terminal tail is on the cytosolic side of the membrane. UDPGA: uridine diphosphate glucuronic acid. From *(Meech and Mackenzie, 1997)*.

The UGT1A family members are all encoded by a single-gene locus located on human chromosome 2q37.1, while several genes, located on 4q13.2, define UGT2B and UGT2A3 enzymes *(Mackenzie et al., 2005)*. On the other hand, UGT2A1 and 2A2 proteins, similarly to UGT1A, arise from a single locus on chromosome 4q13.3 *(Court et al., 2008; Mackenzie et al., 2005).*

6.1.2. The human UGT1 family members

The entire UGT1 family is derived from a single gene locus (UGT1) spanning approximately 210 kb, which is mapped to chromosome 2 (2q37) and is composed of 17 exons *(Gong et al., 2001; Owens and Ritter, 1995; Ritter et al., 1992)*. To synthesize the final protein, only one of 13 different exon-1 sequences on the locus (at the 5'-end) is associated with four downstream exons (at the 3'-end), common to all UGT1A isoforms. The constant exons 2-5 encode a carboxy terminal portion of 280 amino acids identical for all UGT1A proteins. Each individual exon 1 is preceded by its own promoter and encodes a divergent amino terminal portion of approximately 250 amino acids. Of the 13 exon-1 sequences (leading to 13 possible mRNA transcripts), nine encode functionally-active proteins (UGT1A1, UGT1A3-1A10) and four correspond to pseudogenes (*UGT1A2p, UGT1A11p, UGT1A12p*, and *UGT1A13p*) *(Gong et al., 2001; Owens and Ritter, 1995; Ritter et al., 1992)* (Fig. 8/A). The exon-1 sequence of UGTs encodes the substrate- binding domain (N-terminal half of the protein), while the four common exons encode the co-substrate binding domain (C-terminal half of the protein) *(Meech and Mackenzie, 1997)*. The presence of different possible substrate-binding domains confers the large substrate specificity of UGT1A proteins.

While UGT1As are mostly expressed in the liver, many of them can be found in extrahepatic tissues *(Mackenzie et al., 2005; Ritter, 2007; Strassburg et al., 1998, 1997)*. As major contributors of the liver detoxification process, UGT1A1 is widely expressed throughout the human body, whereas UGT1A9 (Fig. 8/B) is mainly expressed in liver and kidney *(Strassburg et al., 1998, 1997; Tukey and Strassburg, 2000)*. UGT1A7, UGT1A8, and

UGT1A10 are only expressed in the gastrointestinal tract. UGT1A3, UGT1A4, and UGT1A6 are also found in the liver and the gastrointestinal tract. Some UGTs are also present in the brain, ovary, uterus, prostate, and breast *(Guillemette et al., 2004; Starlard-Davenport et al., 2008; Strassburg et al., 2008, 1997).*

6.1.3. The human UGT2 family members

The human UGT2 family members are encoded by unique or multiple genes of six exons, located on chromosome 4 (4q13) *(Mackenzie et al., 2005; Monaghan et al., 1994; Turgeon et al., 2000).* As illustrated in Fig. 8/A, and similarly to UGT1A family, the first exon encodes the substrate-binding domain, and the other coding exons encode the UDPGA-binding and transmembrane domains. Several UGT2Bs have been isolated from the liver, as well as from extrahepatic tissues *(Carrier et al., 2000; Lévesque et al., 1999, 1997; Monaghan et al., 1994; Riedy et al., 2000; Tukey and Strassburg, 2000; Turgeon et al., 2000)*. In addition, numerous pseudogenes were found *(Turgeon et al., 2000).* Similarly to the UGT1 family, members of the UGT2B subfamily share a high degree of similarity in the C-terminal portion of the protein and the highest degree of divergence in sequences encoded by exons 1. Three members of the UGT2A subfamily have also been characterized and mapped to chromosome 4q13. They share approximately 70% of identity with the UGT2Bs.

One of the most studied UGT2B enzymes is UGT2B7 (Fig. 8/C). It is abundantly expressed in the liver, the GI tract, but also in the breast, uterus, kidney, brain, and other tissues *(Turgeon et al., 2001).* UGT2B7 has the ability to glucuronidate several endogenous compounds, such as steroid hormones, retinoids, and fatty acids, but also exogenous molecules, such as morphine, zidovudine (AZT), efavirenz (EFV), nonsteroidal anti-inflammatory drugs (NSAIDs), and others. Although other UGT2B family members have been less studied, they have an important role, especially in the metabolism of endogenous compounds.

6.2. Genetic polymorphisms of UDP-glucuronosyltransferases

During the last decade, more than a hundred single-nucleotide polymorphisms (SNPs) on the majority of the UGT genes (e.g. *UGT1A1*, *UGT1A3*, *UGT1A4*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *UGT1A9*, *UGT2A1*, *UGT2B4*, *UGT2B7*, *UGT2B15*, and *UGT2B28*) were identified and characterized *(Guillemette, 2003; Miners et al., 2002)*. These polymorphisms were identified in various regions of each UGT gene, including the regulatory and coding sequences, but also in the introns and 5'- and 3'-untranslated regions. Among these SNPs, several present high variant allele frequencies. An international nomenclature committee compiles and updates the current nomenclature of the UGT supergene family and their genetic variants (http://www.pharmacogenomics.pha.ulaval.ca/cms/ugt_alleles/).

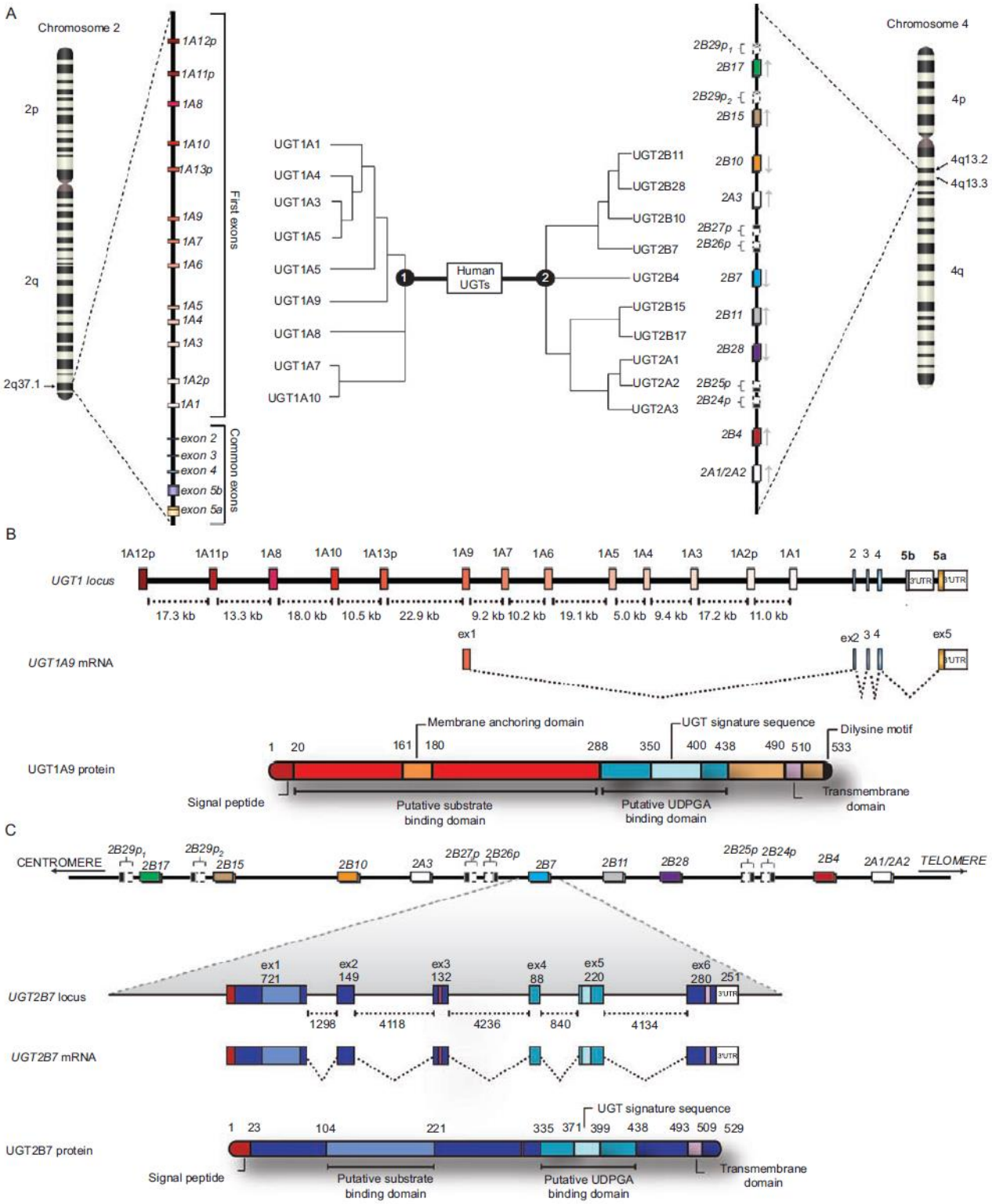


Figure 8. (A) Phylogram of the human UGT1A and UGT2B proteins and their corresponding cluster of genes on chromosomes 2 and 4, respectively. Schematic representation of the UGT1A9 (B) and UGT2B7 (C) protein primary structures derived from each locus. Similarities between the two classes of proteins are shown. From (Guillemette et al., 2010).

7. *In vitro* phenotyping of drug-metabolizing UGT enzymes

The importance of glucuronidation in the clearance of xenobiotics has stimulated research with *in vitro* approaches for the quantitative prediction of key pharmacokinetic parameters (e.g. hepatic clearance; CL_H) of new chemical entities eliminated by UGTs. Since the liver is considered as the primary organ of xenobiotic metabolism, research focused on hepatic glucuronidation. However, it is acknowledged that extra-hepatic UGTs, especially expressed in the gastrointestinal tract and kidneys, may significantly contribute to the metabolic clearance of some drugs *(Cubitt et al., 2009; Knights and Miners, 2010)*.

In the following sections, an overview of the different *in vitro* models and approaches to study human biotransformation is briefly given.

Main biological models and approaches for the *in vitro* study of metabolism:

The *in vitro* study of drug metabolism is mandatory during drug development. It is carried out to identify potential active and/or toxic metabolites and the specific enzymes responsible for the metabolism of a given molecule. It can also be useful for the prediction of potential drug–drug interactions at the metabolic level, and for the study of the impact of genetic polymorphisms on drug metabolism. Such study is also needed to estimate the contribution of a given isoform to the total clearance of a given drug *(Ekins et al., 2000; Williams et al., 2003)*. In order to conduct these studies, several models can be used, ranging from (recombinant) isolated enzymes to the intact perfused liver *(Brandon et al., 2003)*. These models and their major characteristics are listed in Fig. 9.

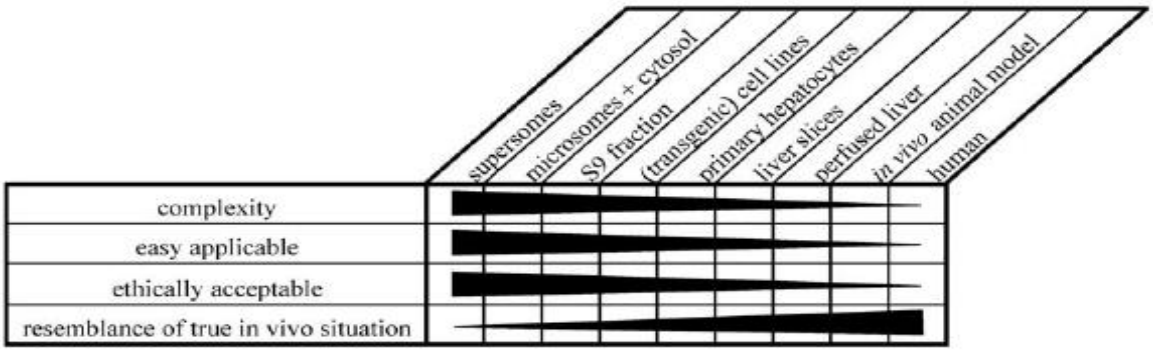


Figure 9. *In vitro* and *in vivo* models used in the development of new drugs, ranging from human to isolated enzymes. From (Brandon et al., 2003).

Enzyme reaction phenotyping variably employs human liver microsomes (HLMs), hepatocytes (which both express the full range of hepatically-expressed UGTs), and recombinant proteins, as the enzyme sources. Several approaches may be adopted for the reaction phenotyping of drug-glucuronidation reactions *(Court, 2004; Miners et al., 2006)*, most of which are underpinned by the availability of UGT-enzyme–selective inhibitors and substrate "probes". A combination of approaches/methods (inhibition test, correlation of microsomal activities test...) is often required to ensure the reliability of the results. The strategy used here for UGT phenotyping is based on well-established procedures for the CYPs *(Rodrigues, 1999; Venkatakrishnan et al., 2001)* with appropriate modifications.

This strategy is usually based on the following approaches:

1) Recombinant UGT enzymes (rUGTs)

Screening of glucuronidation activity using a battery of recombinant UGTs (rUGTs), followed by a comparison of enzyme kinetic parameters of each rUGTs with those obtained with HLM or hepatocytes, is regularly performed.

2) Isoform-selective glucuronidation activities (Correlation analyses)

Isoform-selective marker activities of UGT isoforms can be measured and, then, compared to the glucuronidation activities obtained with the candidate substrate. High correlation between the rates of glucuronidation of the test compound and that of a given UGT-enzyme selective substrate is a strong argument in favor of the implication of this UGT.

3) Isoform-selective inhibition

The use of selective chemical (or antibody) inhibitors of UGT isoenzymes can be useful to document and quantify the involvement of a given enzyme in a metabolic reaction. The extent in the reduction of the glucuronidation of the test compound reflects the contribution of that enzyme. However, few chemical (and no antibody) inhibitors have been described to be selective for the UGT enzymes.

These approaches usually allow the unambiguous identification of the UGT enzymes(s) responsible for the metabolism of a compound.

8. Quantitative *in vitro–in vivo* extrapolation (IV-IVE)

IV-IVE consists in the prediction of the intrinsic clearance (Cl_{int}) of a given substrate, and also in the prediction of the magnitude of inhibitory interactions. These extrapolations are considered as semi-quantitative, because of the difficulties of taking into consideration the physiological variability factors (e.g. hepatic blood flow, plasma protein binding, extra-hepatic metabolism...).

The study of biotransformation reactions requires the estimation of the enzyme kinetic parameters of metabolite production (apparent affinity, K_m and maximum activity, V_{max}). The intrinsic clearance (Cl_{int}) can then be estimated. It is considered as the first step of *in vivo* extrapolation. This determination requires a mathematical modeling of the rate (or velocity) of biotransformation (v), as a function of the concentration of substrate [S].

8.1. Determination of kinetic parameters

The Michaelis-Menten equation (equation 1), which corresponds to a hyperbolic function, (Fig. 10/A) allows most frequently the description of enzyme kinetic data.

$$v = \frac{V_{max} \times S}{K_m + S}$$

Equation 1

The Michaelis-Menten constant (K_m) describes the affinity of the enzyme (for recombinant enzymes), or the apparent affinity of microsomes, for the substrate. It corresponds to the concentration of substrate which leads to half of the maximum velocity rate (V_{max}). *In vivo*, therapeutic concentrations of a given drug are usually much lower than the *in vitro* observed K_m, and for values less than 10% of the K_m, the relationship between enzyme activity and substrate concentrations remains linear.

The fit to a model of Michaelis-Menten can be verified by the Eadie-Hofstee linearization (Fig. 10/A). In this graph, the velocity (v) is plotted as a function of the ratio between velocity and substrate concentration (v/S); the slope is equal to ($-K_m$).

However, particular kinetic profile may arise in the case of involvement of two or more enzymes in the studied metabolic pathway. If their affinities are sufficiently different, the relationship between v and S becomes "biphasic" and corresponds to the linear sum of two Michaelis functions (Equation 2) with components of high and low affinity. In this case, the graphical representation of the Eadie-Hofstee equation is no longer linear, but concave (Fig. 10/B).

$$v = \frac{V_{\max 1} \times S}{K_{m1} + S} + \frac{V_{\max 2} \times S}{K_{m2} + S}$$

Equation 2

Two other atypical kinetic models may occur in the following cases:

- **Substrate inhibition**

The case of substrate inhibition gives rise to a curve with a convex profile (Fig. 10/C), due to a rate of biotransformation which is not maintained at high substrate concentrations *(Houston and Kenworthy, 2000)*. This type of inhibition can be likened to a case of non-competitive inhibition and is described by the Equation 3 where K_{si} is the constant describing substrate inhibition. The graphical representation of the Eadie-Hofstee equation can provide a visual confirmation (Fig. 10/C).

$$v = \frac{V_{\max}}{(1 + (K_m / S) + (S / K_{si})}$$

Equation 3

- **Autoactivation**

The case of autoactivation leads to a sigmoidal kinetic profile (Fig. 10/D), which is modeled by the Hill equation (Equation 4). S_{50} corresponds to the apparent K_m and (n) corresponds to the Hill coefficient *(Clarke, 1998)*. The graphical representation of the Eadie-Hofstee equation is then characterized by a convex profile (Fig. 10/D).

$$v = \frac{V_{\max} \times S^n}{S_{50}{}^n + S^n}$$

Equation 4

In summary, the occurrence of atypical kinetics is generally evident from visual inspection of the Eadie-Hofstee plot (Fig. 10, left-hand panel), but goodness-of-fit parameters should be employed to identify the most appropriate model.

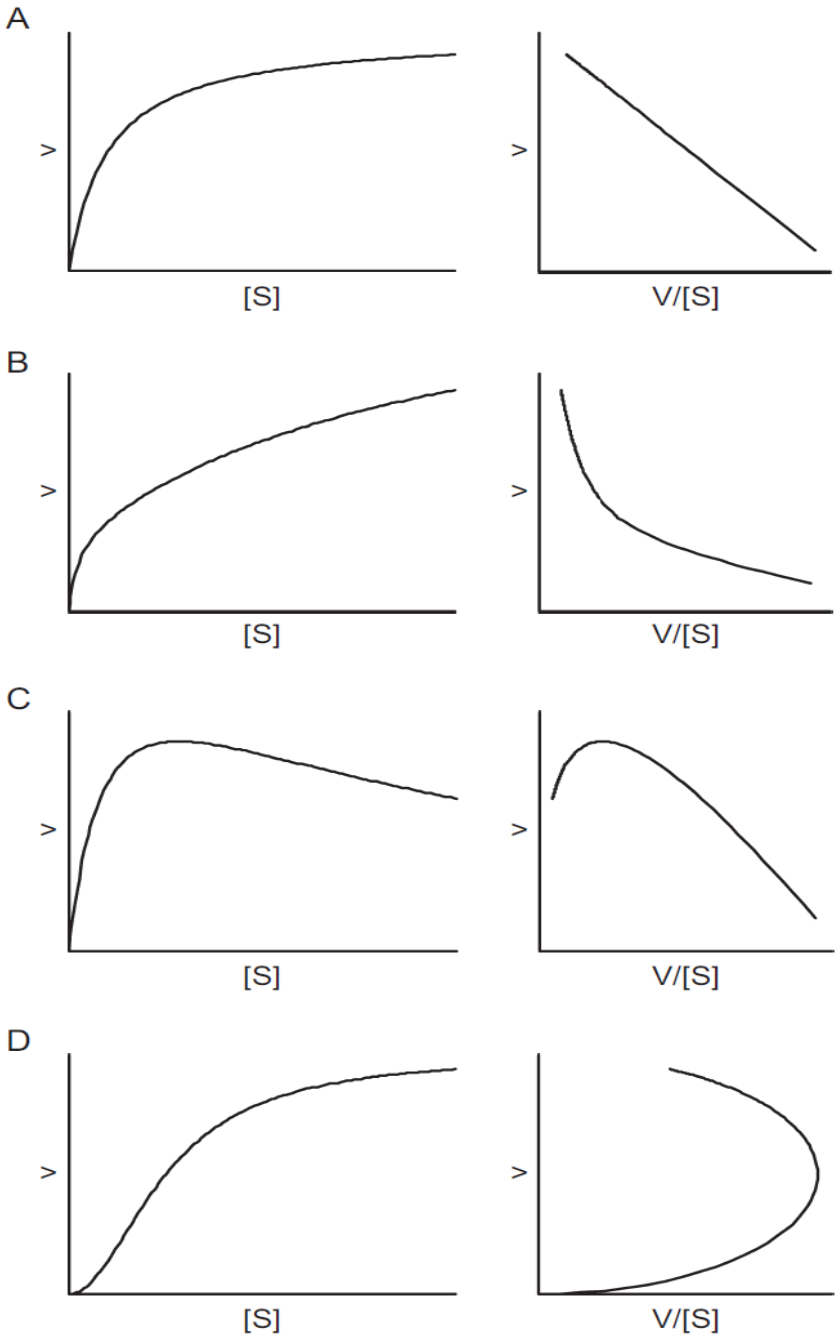


Figure 10. Rate of product formation (v) *versus* substrate concentration ([S]) (left-hand panel) and Eadie-Hofstee (right hand panel) plots for reactions following: (A) single-enzyme Michaelis-Menten kinetics; (B) two-enzyme Michaelis-Menten kinetics; (C) substrate-inhibition kinetics; and (D) autoactivation.

8.2. Interpretation and prediction of metabolic clearance

Intrinsic clearance (Cl_{int}) is an important parameter for the extrapolation of *in vitro* data to the *in vivo* situation *(Houston and Carlile, 1997; Houston, 1994)*. Cl_{int} is defined as a direct measurement of the enzymatic activity without the influence of the *in vivo* physiological parameters (blood perfusion of the organ, or plasma protein binding). It corresponds to a constant allowing the estimation of enzyme velocity rate at a given substrate concentration *(Shibata et al., 2000)*; Equation 5).

$$v = Cl_{int} \times S$$ **Equation 5**

If enzymatic kinetics are modeled by the Michaelis-Menten equation (Equation 1) and considering that usual *in vivo* concentrations of a substrate are well below the K_m, the equation can be simplified and the Cl_{int} parameter is then determined as the V_{max}/K_m ratio (Equation 6), or alternatively calculated directly from the concentration-time kinetics of substrate depletion at a single low concentration (if the tested concentration is much lower than the K_m) *(Jones and Houston, 2004)*. However, this calculation method is not very accurate and is less informative than the estimation made from a full kinetic.

$$Cl_{int} = \frac{V_{max}}{K_m}$$ **Equation 6**

Observation of atypical enzyme kinetics makes the use and the *in vivo* extrapolation of obtained results more difficult *(Houston and Kenworthy, 2000)*.

In the case of autoactivation, clearance reaches the maximum before the saturation of the enzyme. This parameter (Cl_{max}), estimated by the Equation 7 *(Houston and Kenworthy, 2000)*, represents a reliable equivalent of the Cl_{int} for such biotransformation reactions *(Witherow and Houston, 1999)*.

$$Cl_{max} = \frac{v}{S} = \frac{V_{max}}{S_{50}} \times \frac{(n-1)}{n(n-1)^{1/n}}$$ **Equation 7**

Cases of auto-inhibition, resulting from high concentrations of substrate, presumably correspond to *in vitro* artifact and have probably no existence *in vivo*. In these cases, V_{max} is delicate to consider and the estimation of the Cl_{int} from a single experimental point (lower than 10% of K_m value) is recommended.

Published values of microsomal protein content in hepatocytes and hepatocellularity per gram of liver are used to estimate whole-liver CL_{int} *(Barter et al., 2007)*, which can then be used to estimate the hepatic clearance using Equation 8, where Q_H is liver-blood flow and fu is the fraction of the unbound drug in blood.

$$CL_H = \frac{Q_H \times fu \times CL_{int}}{Q_H + (fu \times CL_{int})} \qquad \textbf{Equation 8}$$

9. Prediction of Drug-Drug Interactions

There are basically two mechanisms associated with enzyme interactions: inhibition and induction.

9.1. Enzyme inhibition

Inhibitors are compounds that decrease the rate of an enzyme-catalyzed reaction. Various drugs have been identified *in vitro* as inhibitors of UGT-mediated reactions. The screening of potential inhibitors typically involves the use of HLM, hepatocytes, or recombinant enzymes, as the enzyme source. The concentration of inhibited drug normally corresponds to its known K_m value, which simplifies estimation of the inhibitor constant (K_i) from the IC_{50}, thereby aiding the design of subsequent inhibition-kinetic studies.

The effect of an inhibitor on the enzyme can be described by one of three mechanisms: reversible, quasi-irreversible, and irreversible (suicide inhibition).

9.1.1. Irreversible & quasi-irreversible inhibition

Michaelis-Menten kinetics cannot be applied to irreversible inhibition. The inhibitor (or its metabolite) forms a covalent linkage with the enzyme which inactivates the enzyme. The

effect of an irreversible inhibitor depends on the rate at which the binding takes place. The restoration of normal enzyme activity requires the formation of new enzyme.

9.1.2. Reversible inhibition

Reversible inhibition is the most frequent mechanism. There are three important types of reversible inhibition (Fig. 11): competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition.

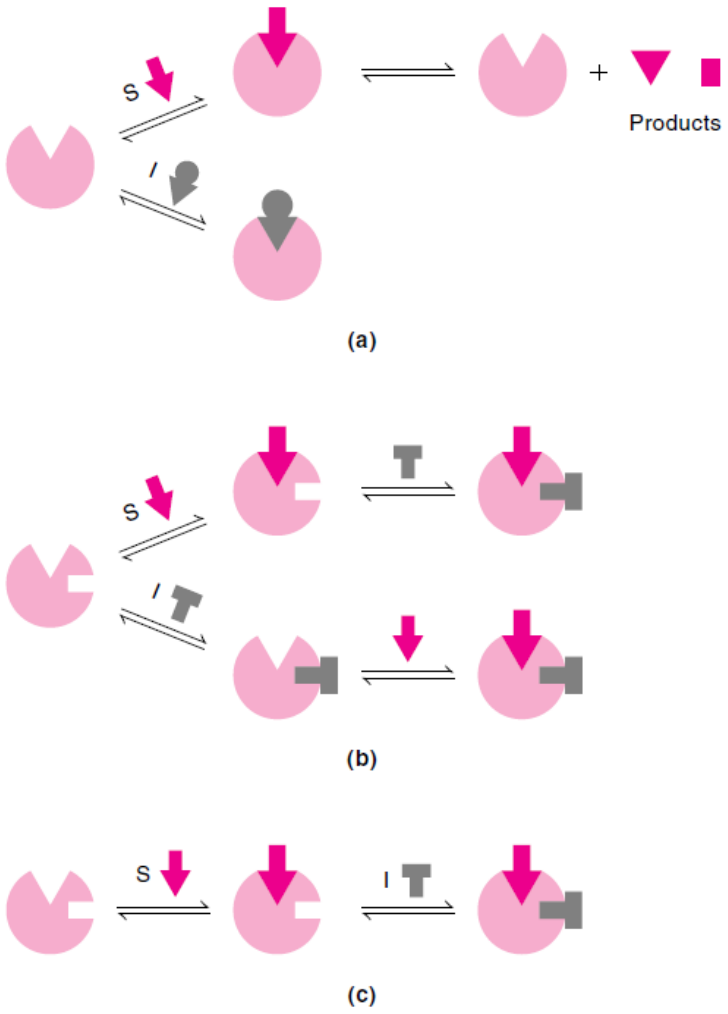
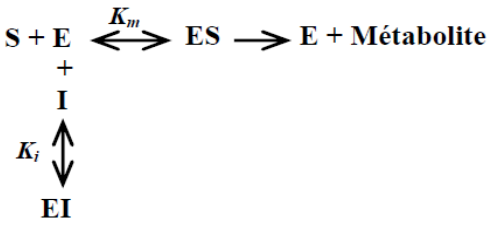
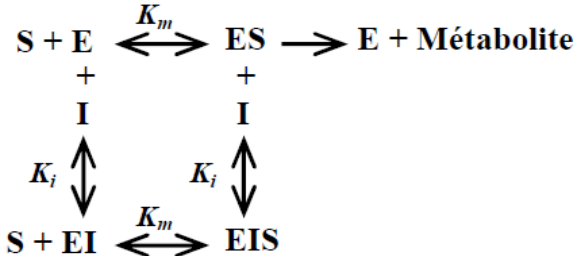


Figure 11. Three types of reversible inhibition. (a) Competitive inhibition: both the substrate and the inhibitor compete for the same active site; only the enzyme–substrate complex leads to product formation. (b) Noncompetitive inhibition: the inhibitor binds to a site other than the active site; the enzyme-inhibitor-substrate complex does not lead to product formation. (c) Uncompetitive inhibition: the inhibitor binds only to the ES complex; the ESI complex does not lead to product formation.

***9.1.2.1. Competitive inhibition*:** in this case, both the substrate (S) and the inhibitor (I) compete for the same active site of the enzyme (E). The reactions are:

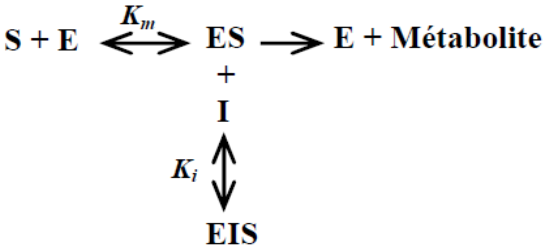


***9.1.2.2. Noncompetitive inhibition*:** a noncompetitive inhibitor binds to the enzyme at a site that is distinct from the substrate binding site; therefore, it can bind to both the free enzyme and the enzyme–substrate (ES) complex. The binding of the inhibitor has no effect on the substrate binding, and *vice versa*, but the complex enzyme-inhibitor-substrate (EIS) is inactive. The reactions are:



Neither EI nor ESI forms products. Because ‹ I › does not interfere with the formation of ES, noncompetitive inhibition cannot be reversed by increasing the substrate concentration.

9.1.2.3. Uncompetitive inhibition: an uncompetitive inhibitor does not bind to the free enzyme; instead, it binds reversibly to the enzyme–substrate complex to yield an inactive ESI complex. The reactions are:



The ESI complex does not form a product. Again, because ‹ I › does not interfere with the formation of ES, uncompetitive inhibition cannot be reversed by increasing the substrate concentration.

9.1.3. *In vitro* evaluation of enzyme inhibition

Two parameters are used to evaluate the inhibition potency of a molecule: 1) the concentration of inhibitor causing 50% of inhibition of the velocity rate (**IC_{50}**), and 2) the constant of inhibition **K_i** (i.e. the affinity of the inhibitor).

9.1.3.1. Calculation of IC_{50}

The calculation of the IC_{50} is achieved at fixed substrate concentration by evaluating the effect of increasing concentrations of the inhibitor on the velocity rate of the substrate metabolism. This determination requires neither the calculation of the kinetics of substrate biotransformation, nor the knowledge of the mechanism of inhibition. Depending on the substrate concentration, the isoforms involved in a metabolic pathway can vary. Therefore, the inhibition of a particular isoform of the global metabolism of a given substrate may vary depending on the incubated concentration. It is important to choose an appropriate concentration for this type of study; this concentration must be, in any case, less than the observed K_m.

9.1.3.2. Calculation of K_i

The mathematical model which describes the impact of a reversible inhibitor of the kinetics of an enzymatic reaction depends on the type of inhibition (competitive, noncompetitive, or uncompetitive; Equations 9, 10, and 11, respectively). $1/K_i$ represents the inhibitor potency.

$$v = \frac{V_{max} \times S}{K_m \times (1 + \frac{I}{K_i}) + S}$$

Equation 9

$$v = \frac{V_{max} \times S}{(K_m + S) \times (1 + \frac{I}{K_i})}$$

Equation 10

$$v = \frac{V_{max} \times S}{K_m + S \times (1 + \frac{I}{K_i})}$$

Equation 11

The linear transformation of enzyme kinetics (using the representation of Dixon or Lineweaver-Burk; Fig. 12) can be used for the choice of the model of inhibition and the calculation of K_i. However, linear transformations have been supplanted by a non-linear regression, allowing a more precise calculation of kinetic parameters.

Interaction by enzymatic inhibition can result, according to its mechanism, in either a decrease in the V_{max}, or an increase in the K_m. It results, whatever the mechanism of inhibition, in a decrease of the intrinsic clearance.

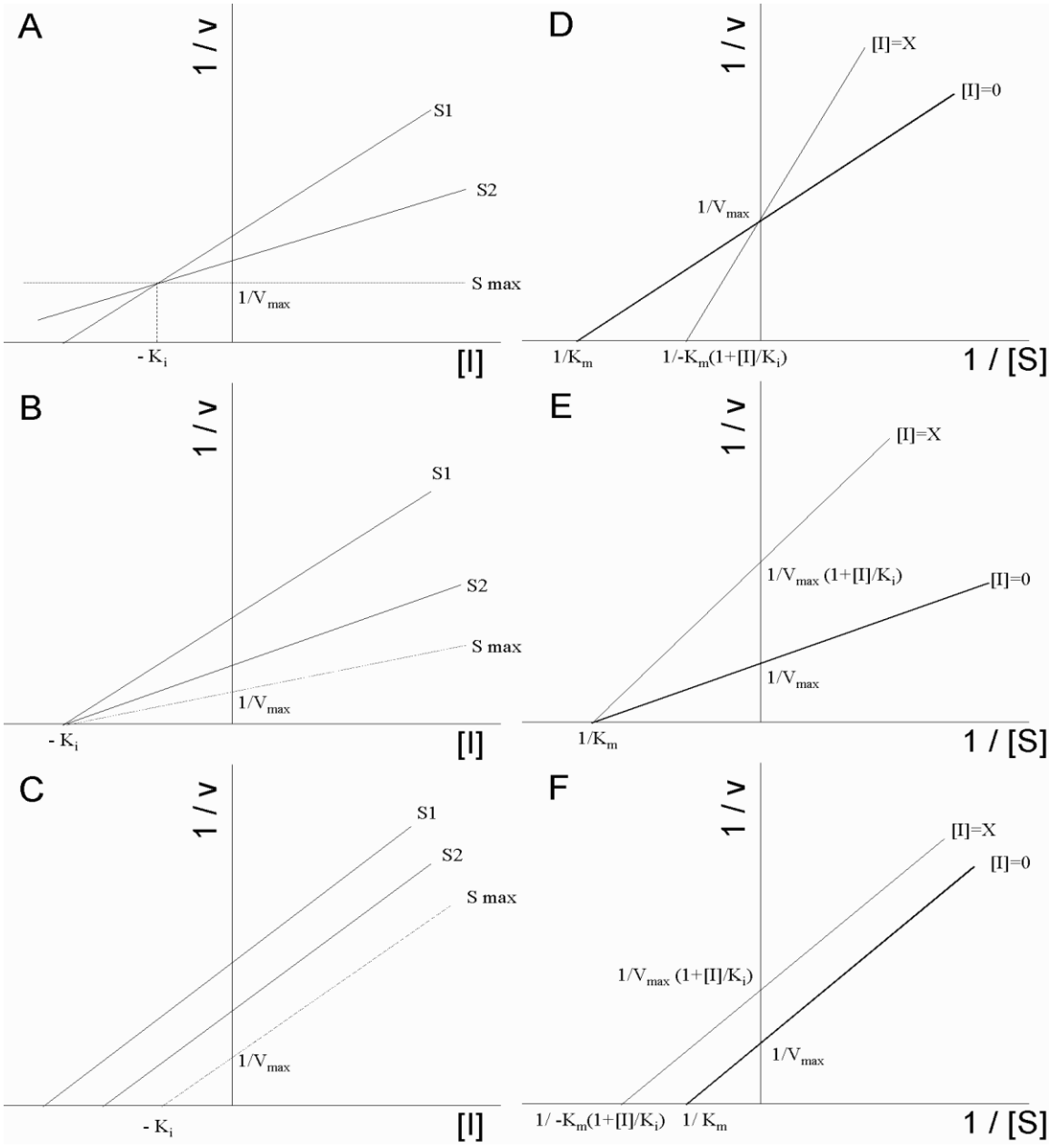


Figure 12. Dixon (A, B, C) and Lineweaver–Burk (D, E, F) plots: (A, D) competitive inhibition, (B, E) noncompetitive inhibition, and (C, F) uncompetitive inhibition.

9.2. Enzyme induction

The induction of enzymes follows a complex mechanism. Most often, the binding of the inducer to a nuclear receptor increases the transcription of the gene encoding the enzyme and, consequently, the protein synthesis. It can also involve a non-transcriptional mechanism (e.g., stabilization of the mRNA, or inhibition of the protein degradation process) *(Lin and Lu, 1998)*. Evaluation of enzyme induction is made with regard to a control, according to several criteria *(Kremers, 2002)*: measurement of enzyme activity, measurement of enzyme protein content using an immunochemical method, or measurement of mRNA by real-time quantitative PCR.

OBJECTIVES & CONTEXT

Although EtG is a relevant and promising marker in clinical and forensic toxicology, many factors appear to influence its concentrations in the body. Halter *et al.*, (2008) showed that the administration of a conventional dose of ethanol to 13 individuals resulted in highly variable (8-fold) maximum concentrations of serum EtG *(Halter et al., 2008)*, and that EtG concentrations did not correlate with BACs. The source of this variability remains to be identified.

Numerous factors could affect glucuronidation (e.g. genetic polymorphisms, inhibition or induction by xenobiotics, variations with age**…)** *(Kiang et al., 2005)* and might thus be involved in this marked interindividual variability of ethanol metabolism.

To date, no study has evaluated the impact of a functional difference in a given UGT isoform on the production of EtG. If one UGT enzyme is predominately responsible for ethanol glucuronidation in humans, the presence of functional genetic polymorphisms or potential interactions with other drugs may potentially complicate the interpretation of EtG concentrations. Understanding ethanol glucuronidation reaction and mechanisms of variability in drug glucuronidation will enhance our ability to explain observed variability in EtG concentrations and, consequently, to better interpret EtG results.

Cut-offs for the determination of EtG in urine and hair have been proposed *(SoHT report, 2012; Kintz, 2010; Rohrig et al., 2006)*. With these cut-offs, especially in hair, EtG can be used for the assessment of abstinence, as well as that of chronic alcohol consumption. However, considering all aforementioned factors that could affect glucuronidation, the relevance of these cut-offs in clinical and forensic practices can be questioned.

Our work was thus dedicated to:

1. The development of a GC-MS/MS method for the determination of ethylglucuronide in human biological matrices;

2. The identification and the study of the relative contribution of human UGT isoforms in the hepatic, renal and intestinal glucuronidation of ethanol, as well as the *in vitro* study of the impact of substances frequently used by consumers of ethanol on the hepatic production of EtG;

3. The study of the impact of functional genetic polymorphisms of UGT, which are most frequently found in the Caucasian population, on the hepatic production of EtG;

4. The study of the impact of *ante-mortem* consumption of cannabis and other illegal and/or medicinal drugs on EtG levels in *post-mortem* blood samples.

PERSONAL WORK & RESEARCH FINDINGS

Development and validation of a GC-MS/MS method for the determination of ethylglucuronide in human urine and serum (article 1)

Alaa Al Saabi, Gilles Tournel, Benjamin Hennart, Delphine Notebaert, and Delphine Allorge

Ann Toxicol Anal. 2011; 23(4): 183-191

The aim of this work was to develop and validate a gas chromatography negative chemical ionization-tandem mass spectrometry (GC-NCI-MS/MS) method to measure EtG concentrations in human urine and serum with both high sensitivity and specificity.

This analytical method has been validated according to the guidelines of the French Society of Analytical Toxicology (SFTA) and of the French Accreditation Committee (COFRAC; LAB GTA 04). The validation parameters were based on the following criteria: specificity, linearity, precision (repeatability and intermediate precision), reproducibility, limit of detection (LOD), and limit of quantification (LOQ).

The calibration curve for EtG (10–10 000 ng/mL for urine; 5–1 000 ng/mL for serum) were obtained with a good linearity and with an excellent correlation coefficient (r) > 0.996. Good intra- and inter-day precisions were observed with relative standard deviations (RSD%) lower than 20% over the calibration range. The LOD and LOQ were 5 and 10 ng/mL, respectively, for both matrices.

This work is the first report of the application of a GC-MS/MS method for the dosage of EtG in urine and serum. The obtained LOQ (10 ng/mL) appeared to be better than those previously reported in the literature with different analytical methods.

Abstract

Objectives: Ethyl-β-D-6-glucuronide (EtG) is a minor phase-II metabolite of ethanol. The aim of the work was to develop and validate a gas chromatography negative chemical ionization tandem mass spectrometry (GC-NCI-MS/MS) method to measure EtG levels in human urine and serum with both high sensitivity and specificity. **Methods:** EtG was extracted and purified from 1 mL urine or 0.5 mL serum by solid-phase extraction (SPE) using Mixed mode Anion-eXchange (Oasis® MAX) extraction cartridges, followed by derivatization with pentafluoropropionic anhydride (PFPA). The analysis was performed in the multiple reaction monitoring (MRM) mode using the transitions m/z 496→163 (for EtG quantification), m/z 347→163 and m/z 496→119 (for identification), and m/z 501→163 for the internal standard EtG-D5. The validation procedure was performed according to the guidelines of the French Society of Analytical Toxicology (SFTA) and the French Committee of Accreditation (COFRAC; LAB GTA 04). **Results:** Calibration curves were linear in the concentration range of 10 to 10000 ng/mL and 5 to 1000 ng/mL in urine and serum, respectively, with a coefficient of correlation (r) above 0.996. The LOD and LOQ values were 5 and 10 ng/mL, respectively, for both matrices. The intra- and inter-day precision (relative standard deviation RSD%) and relative bias were less than 20%. **Conclusion:** To our knowledge, this is the first report of the application of a GC-MS/MS method for EtG measurement in urine and serum. The achieved LOQ appears to be better than those reported in the literature using other validated analytical techniques. This method could be used routinely for EtG measurement in various clinical and forensic contexts.

Keywords: ethylglucuronide, gas chromatography-tandem mass spectrometry (GC-MS/MS), ethanol, serum, urine

Résumé :

Objectif : L'éthylglucuronide (EtG) est un métabolite mineur de l'éthanol, utilisé comme biomarqueur d'alcoolisation. Ce travail a consisté à développer une méthode simple et rapide de dosage de l'EtG dans l'urine et le sérum par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem (GC-MS/MS). **Méthodes** : L'EtG a été extrait et purifié à partir d'1 mL d'urine ou de 0,5 mL de sérum par extraction en phase solide, puis dérivé par l'anhydride pentafluoropropionique (PFPA). L'acquisition a été réalisée en mode MRM (multiple reaction monitoring) à l'aide des transitions 501→163 pour l'étalon interne EtG-D5, 347→163 et 496→119 pour l'identification de l'EtG et 496→163 pour la quantification de l'EtG. La validation de la méthode a été réalisée selon les recommandations de la SFTA et du COFRAC. **Résultats** : La linérarité de la méthode a été démontrée pour une gamme de concentrations de 10 à 10000 ng/mL dans l'urine et de 5 à 1000 ng/mL dans le sérum, avec un coefficient de corrélation (r) supérieur à 0,996. Les limites de détection et de quantification (LDQ) sont respectivement de 5 et 10 ng/mL dans les deux matrices. La répétabilité et la reproductibilité de la méthode ont été démontrées, avec un coefficient de variation inférieur à 20 %. **Conclusion** : Une méthode de dosage par GC-MS/MS de l'EtG dans l'urine et le sérum a été développée et validée. La LDQ de cette méthode apparaît être meilleure, que celles précédemment rapportées dans la littérature avec d'autres techniques. Cette méthode est applicable en routine pour le dosage de l'EtG dans un contexte médical ou médico-légal.

Mots clés : éthylglucuronide, éthanol, urine, sérum, GC-MS/MS.

1. INTRODUCTION

Alcohol abuse is one of the most frequent addictions worldwide and causes many serious social problems and pathologies [1]. The World Health Organization (WHO) has estimated that two billion people consume alcoholic beverages worldwide, with 76.3 million of those suffering diagnosable alcohol-use disorders [2]. For these reasons, a significant amount of research has focused on finding a useful and reliable marker of alcohol consumption. Such a marker would potentially allow clinicians, researchers and forensic experts to focus on groups with an increased risk of alcoholism, to monitor more efficiently curative treatment programs, and to determine the extent to which alcohol plays a role in the neurological impairment of drivers involved in accidents [3].

The known enzymatic and hematological alcohol markers (e.g. g-glutamyltransferase (GGT), aspartate and alanine aminotransaminases (AST, ALT) and mean corpuscular volume (MCV)) provide some information relating to alcohol use or abuse, but they cannot be considered to be satisfactory with regard to sensitivity and selectivity. Furthermore, these biomarkers can be influenced by age, gender, a variety of substances and non-alcohol-associated diseases, and they do not cover fully the time axis for alcohol intake [4].

Direct biomarkers, such as ethanol itself or some of its metabolites, have better specificity and reflect recent alcohol consumption. Ethanol itself is a good marker of alcohol consumption. However, its detection in body fluids is only possible during a relatively short time after alcohol intake, with a maximum blood concentration obtained 30 min to 1 h after ingestion and falling to normal in 8 to 10 h [5]. Its elimination from blood carries an average rate of 0.10 to 0.2 g/L/h in occasional drinkers and of 0.25 to 0.35 g/L/h in chronic drinkers [5]. Because of its relatively short half-life in the body, its use is limited in practice to the diagnosis of acute alcohol intake and not to the chronic alcohol abuse.

Fatty acid ethyl esters (FAEEs) are also direct alcohol markers containing the unchanged ethyl group of ethanol. After ethanol consumption, they are enzymatically formed in a side route of the ethanol metabolism in almost all tissues from free fatty acids or lipids. FAEEs are detectable in blood up to 24 h after the end of drinking and accumulate in fat tissues. They have proved to be interesting biomarkers of alcohol consumption [6].

Ethylglucuronide (ethyl-β-D-6 glucuronic acid, EtG) is formed by the conjugation of ethanol with activated glucuronic acid. It represents only 0.5% of complete alcohol elimination [7, 8], whereas more than 95% of ethanol is eliminated by oxidation, mainly in the liver [8]. EtG is a non-volatile acidic water-soluble and stable metabolite of ethanol that can be detected in various body fluids, tissues and hair. Compared to that of the conventional enzymatic markers, EtG analysis is much more sensitive and specific. Furthermore, it presents a particularly interesting detection window, as EtG can still be detected even after complete elimination of ethanol from the body [9, 10], specifically in urines where it can be detected up to 3 days [9]. All these aspects make EtG one of the most relevant biomarkers of alcohol consumption which can be applied for both clinical and forensic analyses.

Numerous analytical methods have been developed for EtG analysis in biological matrices, such as nuclear magnetic resonance (NMR) [11], capillary zone electrophoresis (CZE) [12, 13], gas chromatography–mass spectrometry (GC-MS) [14-16], liquid chromatography–mass spectrometry (LC-MS) [17-19], liquid chromatography with pulsed electrochemical detection [20] and immunochemical test [21, 22]. Each method has inherent strengths and weaknesses in terms of specificity, sensitivity, assay complexity, cycle-time and instrumentation cost and/or availability.

To our knowledge, a quantification of EtG by a GC-MS/MS method has not been published for analysis in urine and serum. The objective of the present work was to develop and validate an accurate and precise analytical method for EtG dosage in urine and serum with both high

sensitivity and specificity. For this reason, a solid-phase extraction (SPE) step followed by a gas chromatography tandem mass spectrometry (GC-MS/MS) analysis in a negative chemical ionization (NCI) mode was chosen. Following validation, the method was tested for the analysis of EtG in anonymous serum samples available in our laboratory.

2. MATERIALS AND METHODS

2.1. **Chemicals and reagents**

EtG (ref: EGL-332-10) and its deuterated analogue EtG-D5 (ref: EGL-780-10), used as an internal standard (IS), were obtained from Lipomed (Souffelweyersheim, France). Methanol (MeOH, ref: 20837.320) and formic acid (99-100%, ref: 20318-297) were obtained from VWR Prolabo (Fontenay-sous-Bois, France). Ammonium hydroxide solution (25%, ref: 1.05432.1000) and hexane (ref: HEO2212500) were purchased from Merck (Chibret, France) and Scharlau (France), respectively. The derivatization agent, pentafluoropropionic anhydride (PFPA 99%, ref: 206-604-2), was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). All chemicals were of the highest analytical grade. Solid phase extraction (SPE) Oasis® MAX cartridges (3 mL, 60 mg, ref: 86000368) and a SPE tank system working under vacuum were supplied by Waters (Saint-Quentin en Yvelines, France).

2.2. **Samples preparation**

Blank urine and serum samples were collected from five volunteers (who are not alcohol consumers or had stopped drinking alcohol for at least one week), here referred to as alcohol abstainers, and were analysed for the presence of EtG before the validation phase. Citrated tubes were used for blood sampling, whereas clean and dry containers were used for urine samples. Blood samples were centrifuged immediately to separate the serum. No additional preservative was used during the sampling. All samples were stored at - 20°C in order to maintain a good stability along the validation time [23].

2.2.1. Urine sample preparation

Appropriate volumes of standard EtG solutions were added to 1 mL of blank urine, resulting in final concentrations of 10, 100, 1000, 5000, 8000 and 10000 ng of EtG per mL of urine. Then, 25 µL of each urine sample was diluted by the addition of 975 µL of distilled water in the presence of 25 µL of EtG-D5 solution (1000 ng/mL in MeOH). The final mixture was vortexed and transferred onto an Oasis® MAX SPE cartridge.

2.2.2. Serum sample preparation

Calibration samples were prepared by adding suitable amounts of the EtG standard solutions to 0.5 mL of blank serum, resulting in final concentrations of 5, 10, 50, 100, 500 and 1000 ng/mL. 50 µL of EtG-D5 solution (1000 ng/mL in MeOH) was added to each sample. Then, these samples were applied for clean-up and extraction to an (Oasis® MAX) cartridge.

2.3. **Extraction procedure and derivatization**

The prepared samples were applied to an Oasis® MAX cartridge conditioned with 1 mL of MeOH and 1 mL of deionized water. Special care was taken to ensure that the columns did not dry out between the conditioning steps. To prevent the column from drying-out, which could reduce the extraction yield, once the conditioning has started, we maintained water in the SPE column by replacing water that drained through the column. The cartridge was then washed with 1 mL of ammonium hydroxide (NH_4OH, 2%) and, secondly, with 1 mL of MeOH. A strong vacuum was applied for 5 min to remove all residual liquid. EtG was eluted from the cartridge using 1 mL of a methanol/formic acid (98:2, v/v) solution. The eluate was evaporated to dryness under a stream of nitrogen using a heated metal block at 70°C. The residue was derivatized with 100 µL of pentafluoropropionic anhydride (PFPA) which had been previously shown to be the best agent for EtG derivatization with good stability up to 1 h of incubation at room temperature, as well as at 60°C [16]. The tubes were tightly closed,

mixed by vortexing (10 s), heated for 30 min at 70°C, then dried under N_2 and, finally, the residue was reconstituted in 50 µL of hexane. One µL of extract was injected into the GC-MS/MS system.

2.4. **Instruments and GC-MS/MS conditions**

Identification and quantification of EtG were performed in a GC-MS/MS system, which consists of a gas chromatograph (7890A series, Agilent, Massy, France) equipped with an automatic injector (7683B series, Agilent), coupled with a tandem mass spectrometer (Quattro MicroTM GC MICROMASS®, Waters). Chromatographic separation was achieved with a fused silica capillary column AT5-ms (ref: 15807, Alltech, Templemars, France) (30 m × 0.25 mm. × 0.25 µm).

The carrier gas was helium with a constant flow of 1 mL/min. One µL was injected in splitless mode at an injection temperature of 250°C. The initial oven temperature of 60°C was kept for 2 min, increased first at 35°C/min to 250°C, and then kept at this temperature for 8.43 min. The transfer line was held at 270°C. Retention times were of 6.53 min for EtG and 6.52 min for EtG-D5. Samples were ionized by NCI with methane, as the reagent gas, at a pressure of 0.2 mTorr. The ion source temperature was kept at 100°C.

Data acquisition and MS control were performed using the software Mass-Lynx version 4.1 (Waters). The GC-MS/MS was performed in multiple reaction monitoring (MRM) mode. The precursor ions m/z 496 and 347 for EtG and m/z 501 for EtG-D5 were selected in the first quadrupole. These precursor ions were chosen for further fragmentation according to their selectivity and abundance in the mass spectra. The resulting product ions m/z 119 and 163 for EtG and m/z 163 for EtG-D5 were selected in the second quadrupole after collision (in the collision cell) with argon, as the collision gas, at a pressure of 5 mTorr. The collision energy was maintained at 30, 10 and 8 eV for the transitions m/z 496→119, 347→163 and 496→163, respectively, and 10 eV for the transition m/z 501→163. Transition m/z 496→163

was retained for the EtG quantification, whereas transitions m/z 347→163 and m/z 496→119 were used for the EtG identification. The electron multiplier was set up at 550 V.

2.5. **Validation procedure**

The analytical method was validated according to the guidelines of the French Society of Analytical Toxicology (SFTA) [24] and of the COFRAC (LAB GTA 04) [25]. The validation parameters were based on the following criteria: specificity, linearity, precision (repeatability and intermediate precision), reproducibility, limit of detection (LOD), and limit of quantification (LOQ).

2.5.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the studied analyte in the presence of other possible interferences or endogenous components in the sample. In order to evaluate the selectivity of our method five blank samples of urine and serum (no analyte or IS added) obtained from alcohol abstainers and five "zero" samples (blanks with IS) were extracted by SPE, then derivatized and analysed by GC-MS/MS.

2.5.2. Linearity / calibration curves

The linearity of an analytical method corresponds to its ability, within a given range, to obtain results directly proportional to the analyte concentration in the sample. Calibration samples were prepared by adequately spiked blank samples with appropriate volumes of standard EtG solution. Five replicate measurements at six different spiked concentrations were analysed (10, 100, 1000, 5000, 8000 and 10000 ng/mL for urine and 5, 10, 50, 100, 500 and 1000 ng/mL for serum). Furthermore, the internal standard (EtG-D5) was used to eliminate injection error while maintaining a constant area ratio for concentration quantification. Calibration curve was established using the Internal Standard method; by plotting peak area ratios of EtG to IS against concentration of EtG. Six points, for both urine and serum, were calculated and fitted by a linear regression.

2.5.3. Lowest limit of detection and quantification (LOQ and LOD)

The limit of detection (LOD) of a compound analysed in a matrix is the lowest concentration of this compound that produces a detectable signal with a defined reliability, statistically different from that produced by a "blank" sample in the same conditions. It was also defined as the lowest concentration that gives a response of at least three times the average of the baseline noise (S/N ≥ 3, as determined by the Analyst software). The limit of quantification (LOQ) was defined as the lowest amount of analyte that can be quantitatively determined with an acceptable intra-day precision (RSD% less than 20%), corresponding to a signal/noise ratio (S/N) ≥ 10, as determined by the Mass Lynx software.

2.5.4. Accuracy and precision

Precision is the degree of scatter within a set of measurements and a measurement of the random errors. It was evaluated by calculating repeatability (intra-day precision) and intermediate precision (inter-day precision). Accordingly, intra-day assay or repeatability expresses the precision under the same operating conditions over a short-time interval and inter-day assay refers to the precision between runs and from day to day. Intra and inter-day assay precisions were expressed by the relative standard deviation (RSD). The acceptance criteria according to the SFTA guidelines are RSD% less than 20% over the calibration range. The RSD% was calculated using the following equation:

RSD% = (standard deviation / average) × 100

Five replicates for each calibration level were analysed on the first day of the validation procedure. Intra-day precision was calculated by analyzing all concentrations points. Inter-assay precision was assessed for each matrix at two different concentrations (100 and 5000 ng/mL for urine and 10 and 100 ng/mL for serum), following a typical 6-day validation procedure.

3. RESULTS

3.1. **Selectivity**

For each monitored transition, no chromatographic interference peak was observed around the retention times of EtG and IS in both urine and serum-free specimens, indicating that the method provides satisfactory selectivity for EtG analysis. In addition, no signal loss or increase (ion suppression) was observed at the retention time windows of EtG or EtG-D5. The RSD% values and the IS response for blank serum samples are shown in the Table I. The retention times of EtG and IS were 6.53 and 6.52 min, respectively. Representative mass chromatograms of one urine sample are shown in Figure 1.

3.2. **Linearity, LOD and LOQ**

Good linearity was observed for peak intensity within the specified EtG concentration range (10 to 10000 ng/mL for urine and 5 to 1000 ng/mL for serum). The correlation coefficients of the calibration curves were systematically > 0.996. A signal-to-noise ratio of 5 (LOD) at 5 ng/mL was reached for both urine and serum. Furthermore, the method exhibited a quantification limit of 10 ng/mL (Figure 2). This concentration was defined as LOQ since the standard deviation at this concentration was lower than 20% and the calibration line was still linear.

3.3. **Precision**

Good intra- and inter-day precision were observed with a RSD% lower than 20% over the calibration range. This result confirms the stability of the calibration curve along time (6 days, the period of validation strategy). The RSD% values that were used to estimate intra-day and inter-day precision are shown in the Table II.

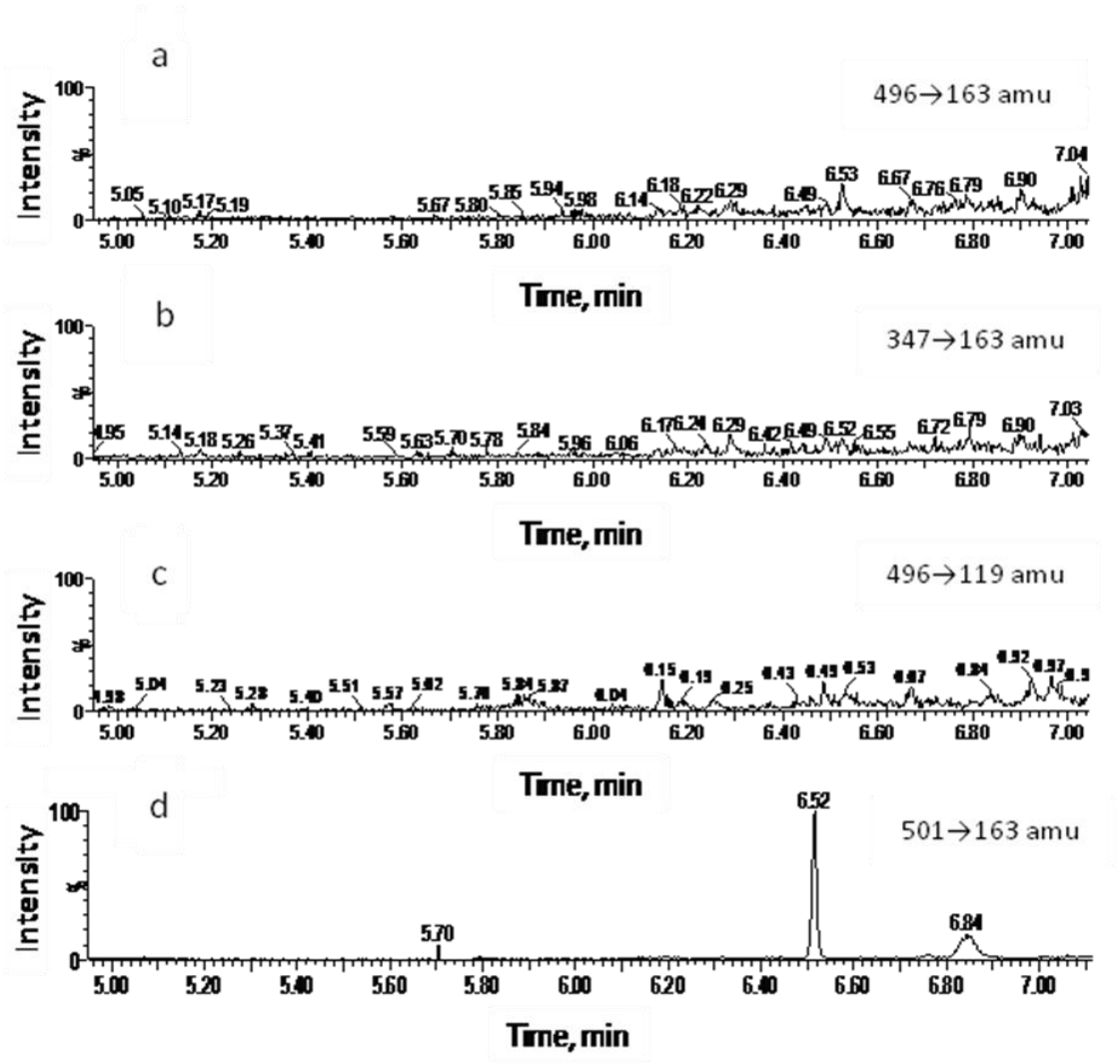
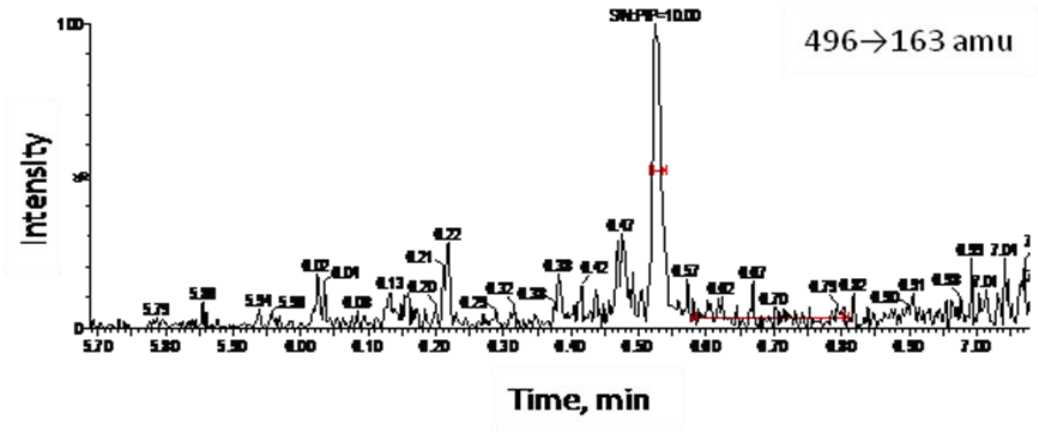


Figure 1. MRM chromatograms of blank urine samples (a, b, c) and a spiked sample with the internal standard at 50 ng/mL (d).

Table I. Relative standard deviation (RSD%) values and IS response for blank serum samples

Assay	EtG Peak Area	IS Peak Area	Area ratio	Average (n=5)	Standard Deviation (n=5)	RSD%
1	54	2959	0.0182			
2	72	3853	0.0194			
3	53	2870	0.0184	0.01846	0.001073	17.2
4	44	2434	0.0181			
5	60	3283	0.0182			

(a)



(b)

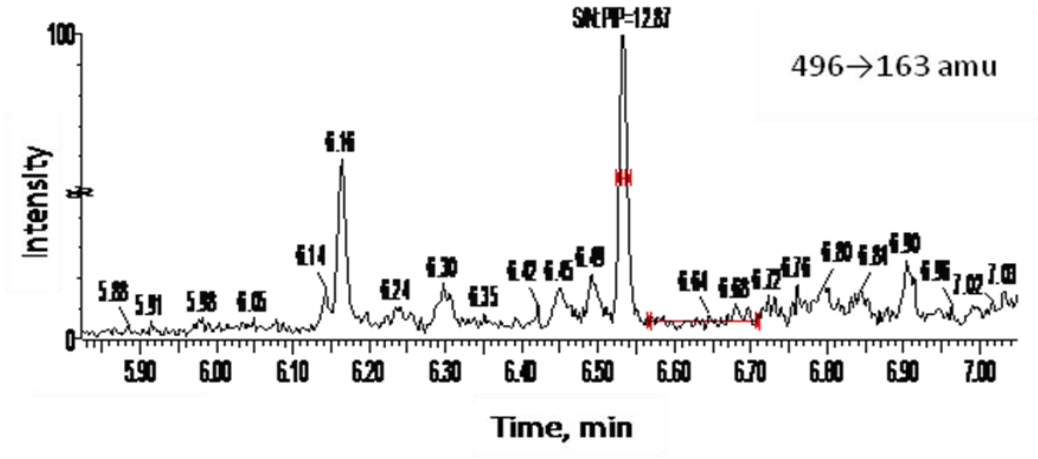


Figure 2. MRM chromatograms of serum (a) and urine (b) samples at 10ng/mL (estimated LOQ) show a S/N ratio ≥ 10.

Table II. Relative standard deviation (RSD%) values of the intra- and inter day precision for urine and serum matrices

	Urine						Serum					
concentration	**C_1**	**C_2**	**C_3**	**C_4**	**C_5**	**C_6**	**C_1**	**C_2**	**C_3**	**C_4**	**C_5**	**C_6**
average	0.03	0.117	1.139	5.931	10.137	12.637	0.078	0.128	0.675	1.357	9.364	19.695
standard deviation	0.002	0.003	0.093	0.367	1.001	0.772	0.006	0.011	0.082	0.13	0.607	1.052
RSD%[a]	6.66	2.56	8.16	6.18	9.87	6.1	7.69	8.59	12.14	9.57	6.48	5.34

	Urine		Serum	
Concentration	**$C_{2=100ng/mL}$**	**$C_{4=5000ng/mL}$**	**$C_{2=10ng/mL}$**	**$C_{4=100ng/mL}$**
average	0.109	6.195	0.185	0.889
standard deviation	0.006	0.596	0.016	0.04
RSD%[b]	5.5	9.62	8.64	4.49

[a] RSD% values of the intra-day precision; [b] RSD% values of the inter day precision

3.4. **Application of the method**

The developed and validated method of EtG in serum was used to analyse 22 anonymous and unrelated serum samples obtained from the Toxicology laboratory of the CHRU of Lille and compared with the serum ethanol concentrations determined by headspace GC. Table III lists the results together with blood alcohol levels. As depicted, even though some samples were negative for ethanol detection, it was possible to detect EtG. Some samples had to be diluted because of high concentration values. The mean concentration value for serum EtG was 1975 ng/mL (range 10 – 3941ng/mL), with some values under the LOQ. An authentic serum specimen at 55.7 ng/mL is shown in Figure 3. This result shows the usefulness of EtG detection and dosage compared to that of ethanol to assess ethanol consumption.

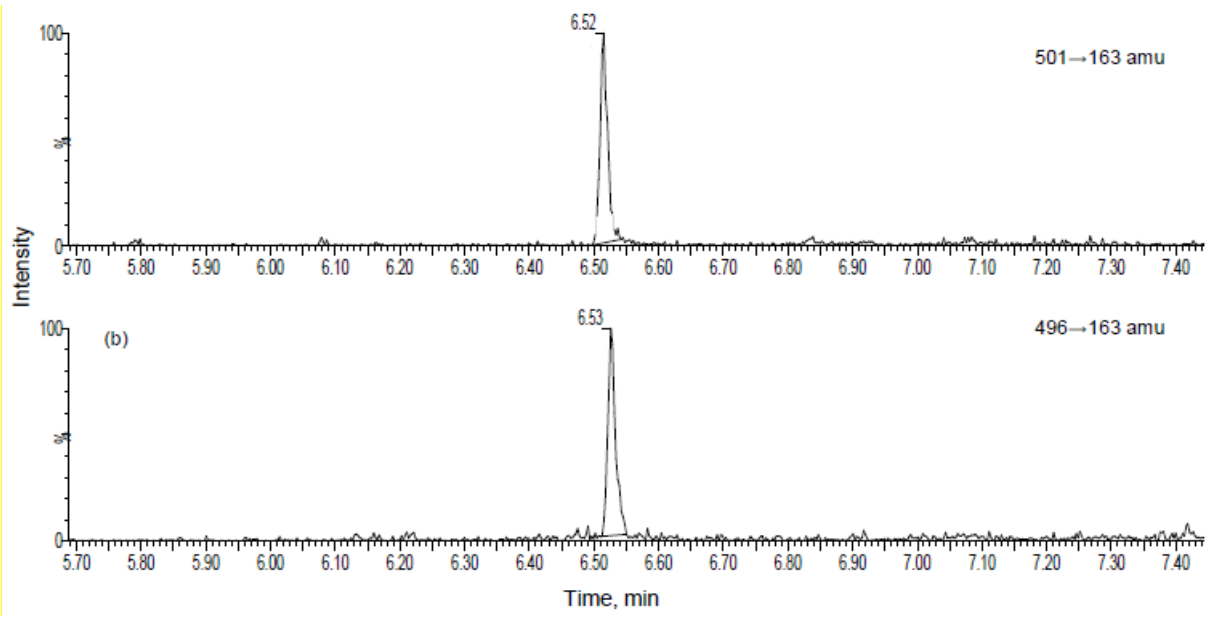


Figure 3. MRM chromatograms of an authentic serum specimen with both transitions (a) IS at 50 ng/mL, (b) authentic sample at 55.7 ng/mL.

Table III. Comparison of ethanol and EtG concentrations in 22 serum samples

Sample number	EtG (ng/mL)	Ethanol (g/L)
1	< LOQ	0
2	< LOQ	0
3	< LOQ	0
4	< LOQ	0
5	< LOQ	0
6	< LOQ	0
7	10	0
8	14.7	0
9	16	0
10	18.3	0
11	19.3	0
12	19.8	0
13	28.8	0
14	55.7	0
15	402	0.2
16	1215	0
17	1150	0.15
18	1068	1.3
19	1888	2.9
20	2478	2.5
21	3628	2.7
22	3941	1.9

< LOQ means below 10 ng/mL

4. DISCUSSION

EtG is a biomarker of alcohol used in clinical and medico-legal toxicology [26]. A sensitive, simple and reliable GC-MS/MS method has been developed and validated to determine EtG in human urine and serum according to the guidelines of the French Society of Analytical Toxicology (SFTA). GC-MS/MS could be a method of choice for EtG analyses as it combines the advantages of GC (high separation power) and tandem-mass spectrometry (high selectivity), minimizing or eliminating background interferences and, therefore, improving selectivity and sensibility. For the pre-analytical phase, a solid-phase extraction (SPE) was applied using Oasis® MAX cartridges. The principle of these cartridges combines anion exchange and reverse phase interactions. SPE could thus be a method of choice for EtG extraction because of its acidic properties which make it mainly retained by ionic interactions. This characteristic allows a washing step with methanol that removes interference compounds. Methanol elution of EtG in acidic conditions (formic acid /HCOOH/ 2%) enables fast evaporation of the solvent in comparison to other published methods where aqueous solutions have been used [27]. The calibration curve for EtG (10-10000 ng/mL for urine; 5-1000 ng/mL for serum) were obtained with a good linearity and with the correlation coefficient of $r>0.996$.

To our knowledge, this is the first report of the application of a GC-MS/MS method for EtG dosage in urine and serum. Furthermore, it is noteworthy that our achieved LOQ of EtG (10 ng/mL) in the studied biological matrices appeared to be better than those reported in the literature with different analytical methods (Table IV). In the case of urine, several publications list a LOQ between 50 and 560 ng/mL [14, 18, 20, 28-31]. For serum samples, the published LOQ range between 45 and 500 ng/mL [15, 29, 32-35].

In addition to the high sensitivity of our method, the calculated precision values fulfill the requirements of validation. The method has been successfully applied to the analysis of

anonymous serum samples obtained from our Toxicology Laboratory, and compared with the serum ethanol concentrations determined by headspace GC. The present GC-MS/MS procedure for EtG could be usefully applied for documentation of alcohol abstinence and for investigation of transportation accidents, monitoring of motorists with alcohol abuse histories who have abstinence as a condition for continued driving privileges.

5. CONCLUSION

In this study, a sensitive and reliable analytical GC-NCI-MS/MS method for the determination of EtG in urine and serum was developed and validated. The performance of this method is sufficient to measure low concentrations of EtG in both matrices, with a LOQ better than those obtained with other techniques. The method has been shown to be precise, and it has been successfully used in our laboratory. Therefore, this assay could be routinely used in the detection of EtG in various clinical and forensic contexts.

Table IV. Comparison of the LOD and LOQ of EtG in urine and serum between our GC-MS/MS method and other published methods

Urine	**Our results**	Favretto (2010) [30]	Shah (2006) [20]	Janda (2001) [29]	Freire (2008) [14]	Thierauf (2009) [28]	Beyer (2011) [31]
Method	GC-MS/MS	LC-MS	LC-PED	GC-MS	GC-MS	LC-MS/MS	LC-MS/MS
LOD (ng/mL)	**5**	50	80	168	5	5	100
LOQ (ng/mL)	**10**	100	370	560	100	50	100
Serum	**Our results**	Schmitt (1997) [15]	Nishikawa (1999) [33]	Janda (2001) [29]	Morini (2007) [34]	Halter (2008) [32]	Jung (2009) [35]
Method	GC-MS/MS	GC-MS	LC-MS	GC-MS	LC-MS/MS	LC-MS/MS	CZE
LOD (ng/mL)	**5**	100	30	37	9	-	-
LOQ (ng/mL)	**10**	-	100	173	45	100	500

GC, Gas Chromatography; LC, Liquid Chromatography; PED, Pulsed Electrochemical Detection; CZE, Capillary Zone Electrophoresis

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Involvement of UDP-Glucuronosyltransferases UGT1A9 and UGT2B7 in Ethanol Glucuronidation, and Interactions with Common Drugs of Abuse (article 2)

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The enzymology underlying the glucuronidation of ethanol has not received much attention to date. Foti and Fisher (2005) have shown that multiple UGT isoforms are likely responsible for the catalysis of EtG formation, with UGT1A1 and UGT2B7 being predominant *(Foti and Fisher, 2005).* The aim of this article was, first, to evaluate the specific contribution of UGT enzymes in *in vitro* EtG formation, and second, to study the impact of drugs frequently used by consumers of ethanol on the production of EtG.

The kinetics of ethanol glucuronidation was assessed *in vitro* using human liver (HLM), kidney (HKM), and intestinal (HIM) microsomes, as well as 12 major human recombinant UGTs. EtG was determined using liquid chromatography-tandem mass spectrometry. Interaction of morphine, codeine, lorazepam, oxazepam, nicotine, cotinine, cannabinol, and cannabidiol, with ethanol glucuronidation was assessed using pooled HLMs.

We showed that ethanol is primarily glucuronidated by the liver and that kidney and intestine tissues play only a minor role in this metabolic pathway. Multiple UGT isoforms seemed to be responsible for catalyzing the addition of glucuronic acid to ethanol, with UGT1A9 and 2B7 being the two most active isoforms (50% of the overall EtG formation). Only cannabinol (CBN) and cannabidiol (CBD) significantly affected ethanol glucuronidation. CBN significantly increases the glucuronidation of ethanol in a concentration-dependent manner, whereas CBD significantly inhibits EtG production by a noncompetitive mechanism.

These results add important information on EtG metabolism, which appears to be affected *in vitro* by cannabinoids, and highlight the importance to further study the impact of UGT1A9 and 2B7 genetic polymorphisms on the glucuronidation of ethanol.

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; Ki=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.

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-β-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 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: cannabinol (CBN) and cannabidiol (CBD)) on the production of EtG.

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 $MgCl_2$ 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 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®), 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 µL) contained 0.5 mg/mL microsomal proteins (microsomes or Supersomes®), 2 mM UDPGA, 10 mM $MgCl_2$ and 0.1 M Tris-HCl buffer (pH 7.4). Microsomes were first activated by incubation with the pore-forming peptide alamethicin (100 µg/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 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 uptisphere 100Aº, 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) 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® to HLM. CDCA (15 µM), TFP (40 µM), MEF (0.5 µM), and AZT (70 µM) 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 µL of ice-cold methanol containing glafenine (13.5 µM) 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® (both expressed in pmol/mg protein/min). The scaled velocities of UGT1A3, 1A4, 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 ($contribution_i$) was calculated using the following equation:

$$Contribution_i\ (\%) = (V_i \times RAF_i) / V_{pooled\ HLM}) \times 100$$

Chemical inhibition experiments

The glucuronidation of ethanol by HLM in the presence of niflumic acid (2.5 µM) 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 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 ± 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.

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.

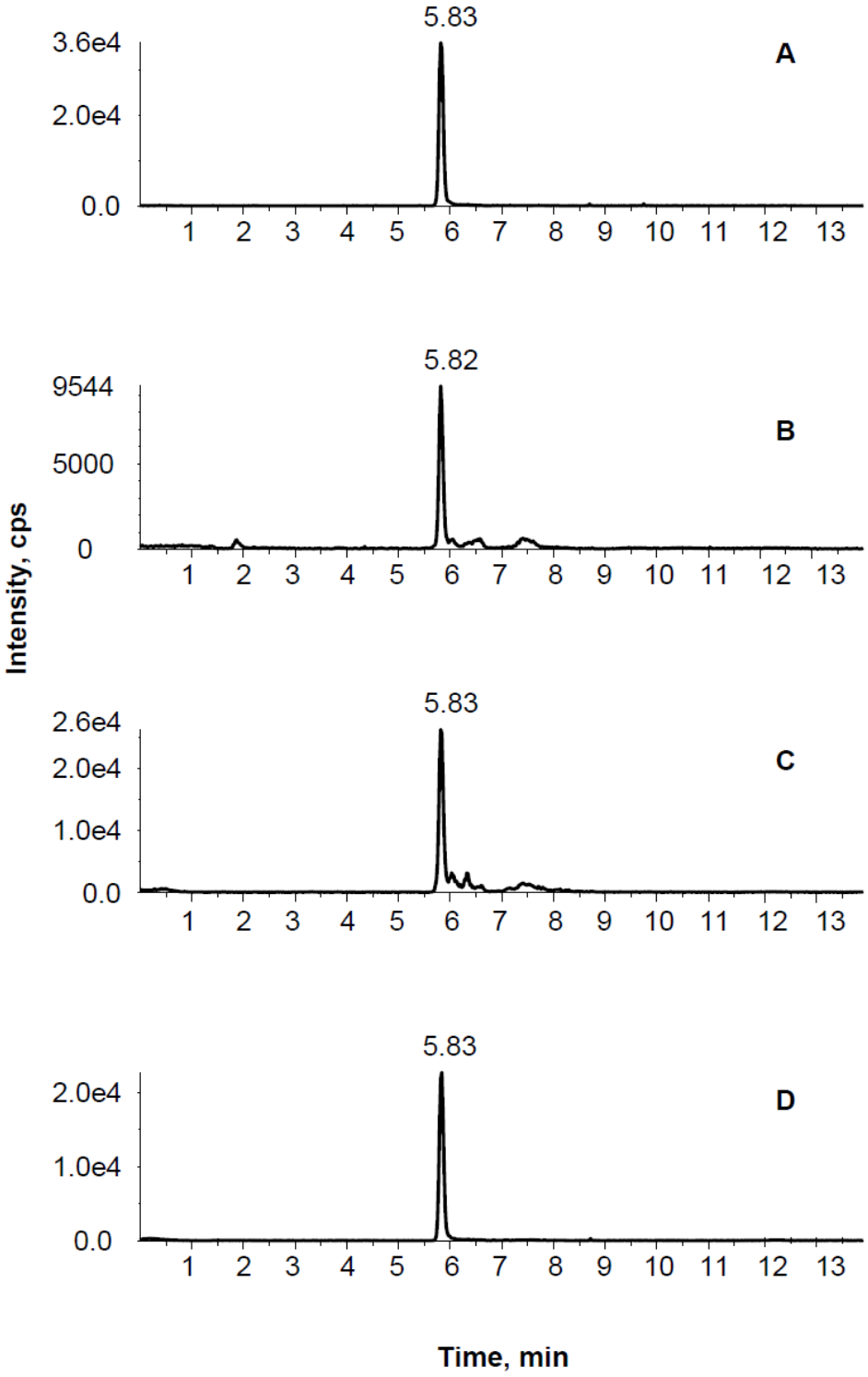

Figure 1: Multiple reaction monitoring (MRM) chromatograms of ethylglucuronide (EtG) after incubation of human liver microsomes with ethanol (100 mM). Transition of m/z 226→75 was used for IS (A), 221.0→112.9 and 221.0→85.0 were used for EtG identification (B, C), and transition of 221.0→75.0 was used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

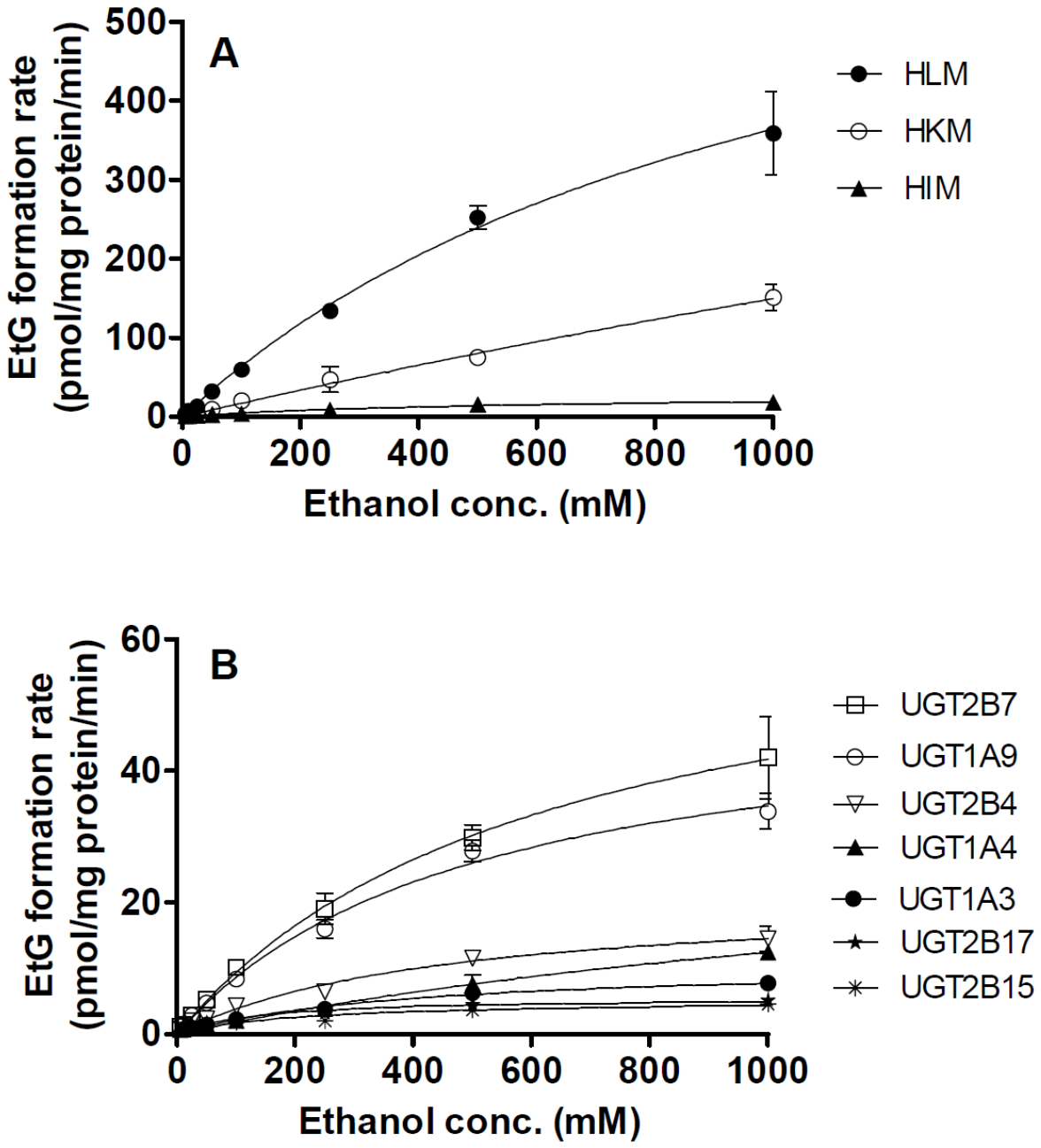

Figure 2: Enzyme kinetics of ethanol glucuronidation by HLM, HKM, and HIM (a); and recombinant hepatic UGT enzymes (b). Glucuronidation activities of microsomes and recombinant proteins were measured by incubating membrane fractions with increasing concentrations of ethanol at a constant concentration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

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. Km (and Clint) 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%.

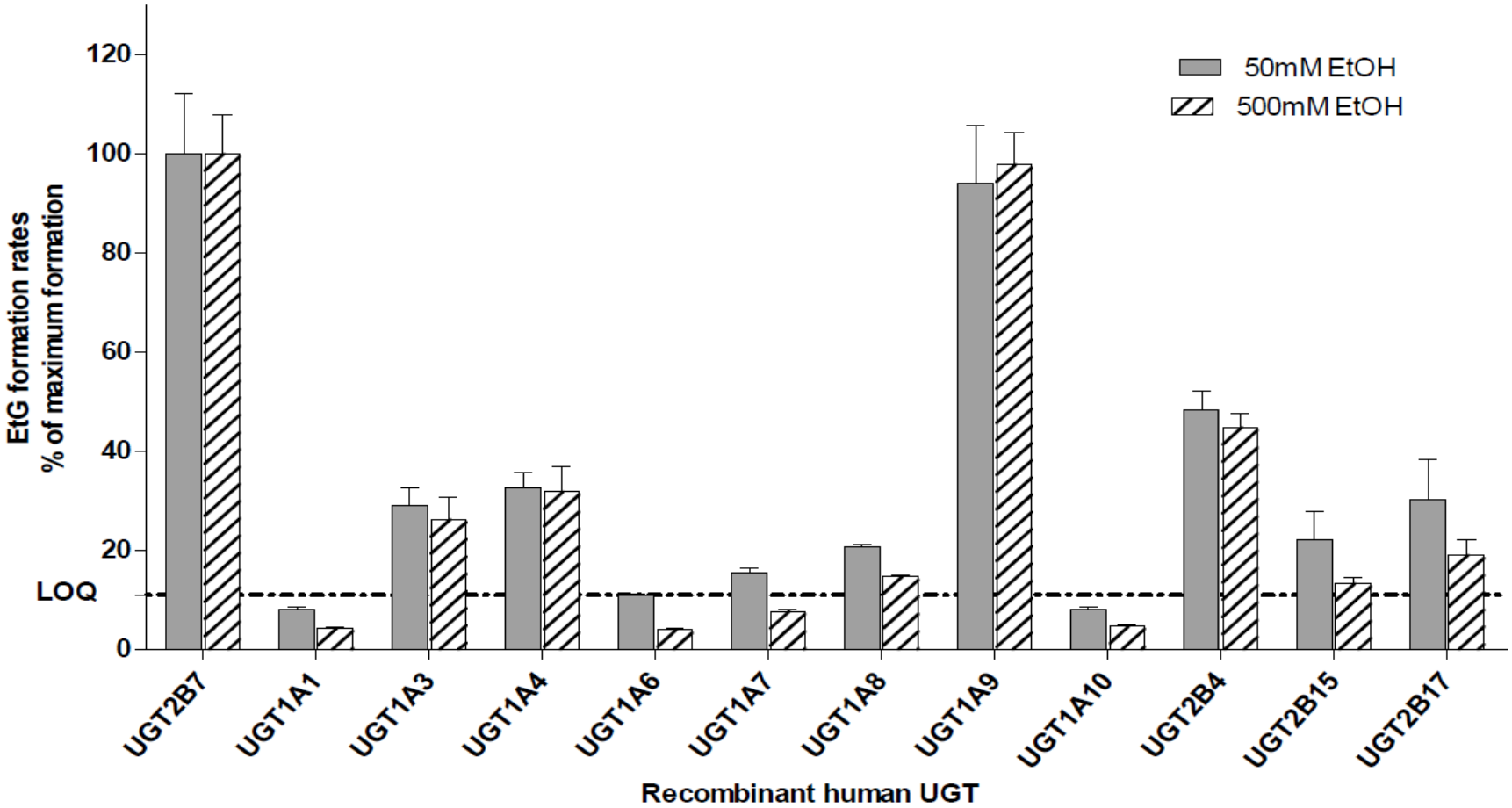

Figure 3: Ethylglucuronide (EtG) production following incubation of ethanol (50 mM and 500 mM) with 12 recombinant human UGTs (Supersomes). All tested isoforms, except UGT1A1, 1A6 and 1A10, produced EtG in detectable amounts (> limit of quantification; LOQ). Data (mean ± SD of triplicate experiments)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

Table 1 Kinetic parameters (± 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 ± 343.3	757.9 ± 150.1	0.7004	na[b]		
HKM	6224 ± 6843	1081 ± 1049	0.1736	na		
HIM	508.6 ± 102.7	28.34 ± 2.73	0.0550	na		
UGT1A3	385.1 ± 106.7	10.71 ± 1.4	0.0278	0.31	0.0086	1.2%
UGT1A4	1706 ± 699.1	33.64 ± 9.6	0.0197	0.67	0.0132	1.9%
UGT1A7	193 ± 74.88	3.05 ± 0.41	0.0155	nd[c]		
UGT1A8	312.1 ± 78.57	6.73 ± 0.68	0.0214	nd		
UGT1A9	501 ± 87.77	52.1 ± 4.43	0.1039	1.15	0.1196	17.1%
UGT2B7	619.7 ± 157.7	67.68 ± 8.97	0.1092	2.1	0.2293	32.7%
UGT2B4	442.1 ± 112.2	20.95 ± 2.41	0.0472	nd		
UGT2B15	203.1 ± 89.67	5.2 ± 0.81	0.0256	nd		
UGT2B17	132.9 ± 50.08	5.6 ± 0.66	0.0421	nd		

[a] Activities are not normalized to microsome or supersome UGT contents;

[b] na, not applicable; [c] nd, not determined.

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

Niflumic acid and fluconazole resulted in a limited inhibition of ethanol glucuronidation in HLM (9±5 % and 18±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; Kruskall-Wallis test: $p < 0.05$), whereas CBD significantly inhibited EtG production with an IC_{50} value of 1.17 mg/L (Fig. 4C; Kruskall-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±12% and -49±14%, respectively; $p = 0.05$). On the other hand, the activity of UGT2B7 was significantly increased by CBN (+429±112%; $p = 0.05$), whereas it was inhibited by CBD (-70±7%; $p = 0.05$; data not shown).

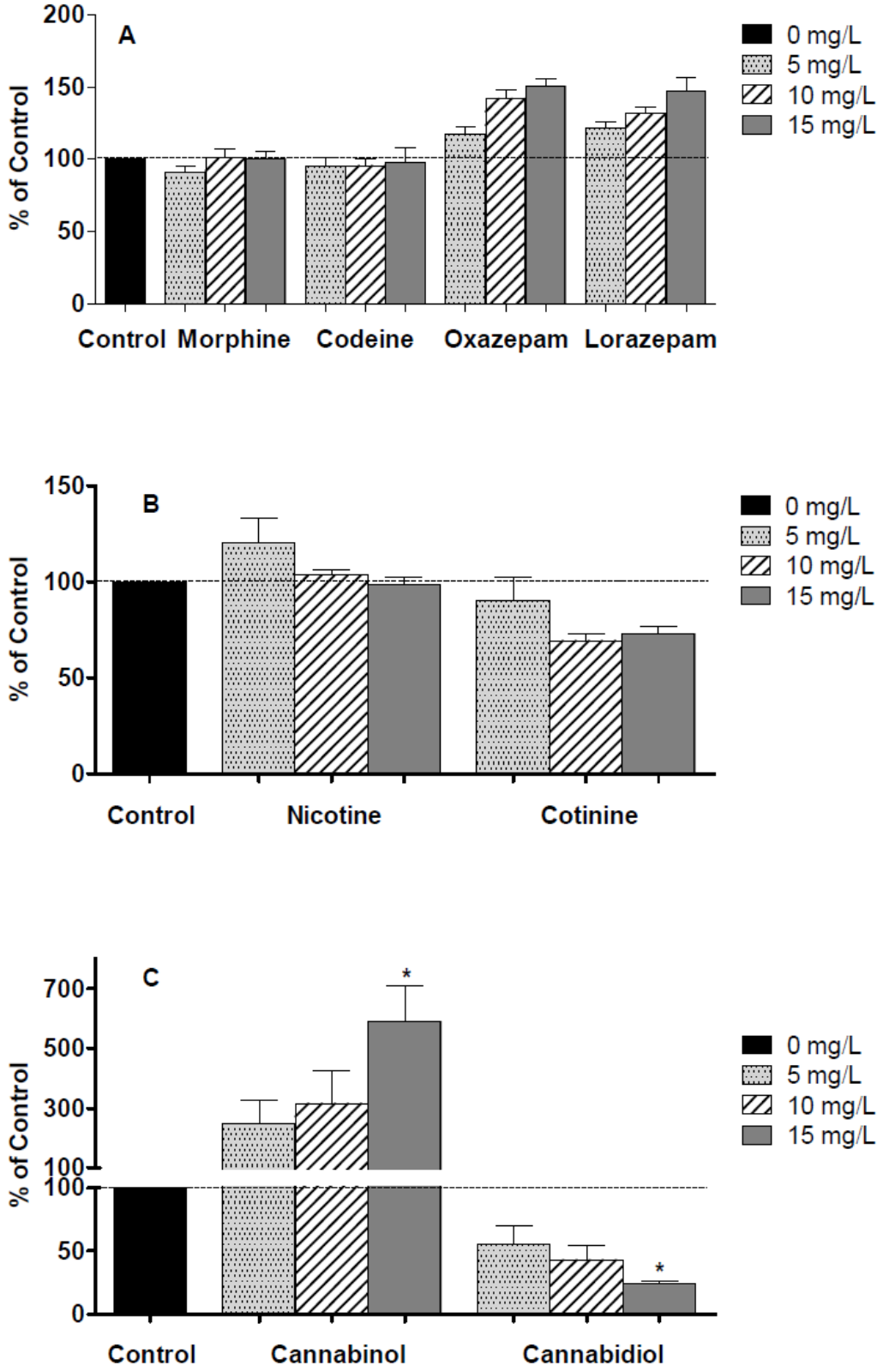

Figure 4: Effect of morphine, codeine, oxazepam, lorazepam (A), nicotine, cotinine (B), cannabinol and cannabidiol (C) on ethanol (25 mM) glucuronidation. Results are expressed as the mean (± SD) percentage of inhibition compared to control incubation (triplicate experiments). *, significantly different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

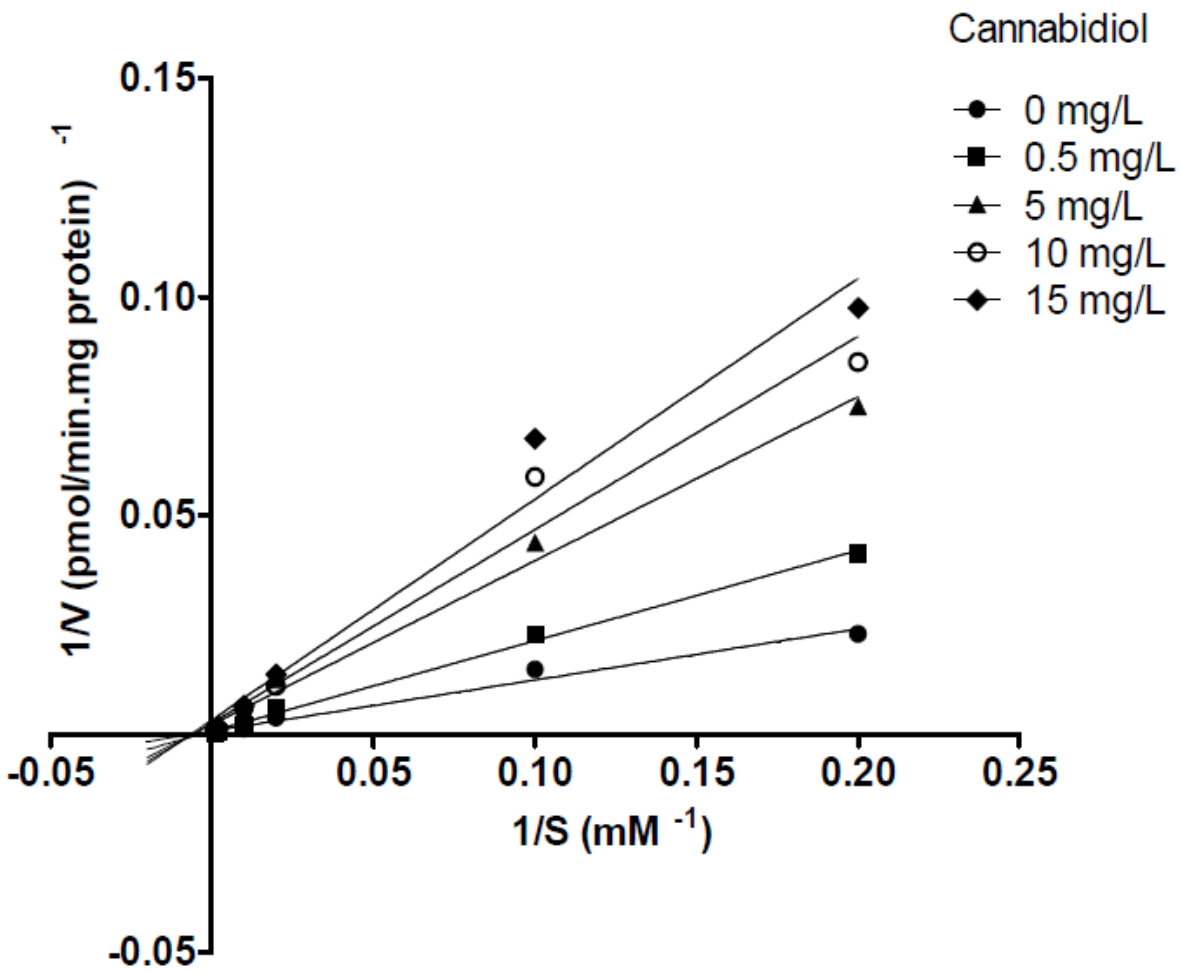

Figure 5: Lineweaver-Burk plots for the inhibition of ethanol glucuronidation by various concentrations of cannabidiol in human liver microsomes. Ethanol was incubated at 6 concentrations (range of 5 to 1000 mM). Each plot is derived from duplicate experiments.

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 ± 47.6 mM *vs* 1082 ± 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 ± 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 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; 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 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 (Kaivosaari 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 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.

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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Influence of UGT1A9 and UGT2B7 genetic polymorphisms on ethylglucuronide (EtG) production *in vitro* (article 3, in preparation)

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Introduction / Context

During the last decade, more than a hundred single nucleotide polymorphisms (SNPs) in UGT genes were identified and characterized *(Guillemette, 2003; Miners et al., 2002)*. These polymorphisms are found in various regions of the genes, including their coding sequences, but also their introns, and 5'-, and 3'-untranslated regions. Among these SNPs, several present variant allele frequencies up to 40-50% in the general population. Ethanol glucuronidation is thought to exhibit high interindividual variability, and no correlation between EtG and blood ethanol concentrations has been found *(Halter et al., 2008)*. As individuals exhibiting UGT allelic variants may have altered (enhanced or reduced) glucuronidation activity, the interindividual variability in EtG levels may be due to genetic polymorphisms in UGT-metabolizing enzymes. To date, no study has assessed the impact of UGT SNPs on the production rate of EtG.

In our previous work, we showed that although multiple UGT isoforms (UGT1A3, 1A4, 1A9, 2B4, 2B7, 2B15, and 2B17) are competent for EtG hepatic production, two of them (UGT1A9 and 2B7) are mainly involved in this pathway. The potential consequence of genetic polymorphisms, affecting one or both of these enzymes, on ethanol glucuronidation remained to be investigated. Numerous SNPs on UGT1A9 and 2B7 have been described, some of them exhibiting functional impact on their catalytic activity *in vitro (Bernard and Guillemette, 2004; Bernard et al., 2006; Court et al., 2003; Djebli et al., 2007; Duguay et al., 2004; Girard et al., 2006, 2004; Saeki et al., 2003)*.

The aim of the present work was 1) to document the variability in EtG liver production using a bank of HLMs; 2) to investigate the impact of specific genetic variants of *UGT1A9* (-275T>A, -440C>T, and IVS1+399T>C) and *UGT2B7* (-900G>A) on the *in vitro* production of EtG, and finally 3) to screen for additional variants in *UGT1A9* and *2B7* genes using direct sequencing in order to explain the variability in EtG hepatic production.

Materials and Methods

Chemicals and Reagents

EtG (reference EGL-332-10) and its deuterated analog EtG-D5 (reference EGL-780-10), which was used as an internal standard, were obtained from Lipomed (Souffelweyersheim, France). Uridine 5'-diphosphoglucuronic acid (UDPGA) and alamethicin were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ethyl alcohol (17 M, 99.8%) and $MgCl_2$ were purchased from Merck (Darmstadt, Germany), and Tris-HCl was obtained from Qbiogene (Illkirch, France). All reagents for DNA extraction and genotyping were of biological molecular grade; Milli-Q water and all other solvents were of high-performance liquid chromatography grade.

In vitro models

Human liver microsomes (HLM) were prepared from individual samples (n = 43; 12 women and 31 men, age range: 26 to 89 years old) derived from surgical specimens and obtained from Biopredic International (Rennes, France), as described previously *(Al Saabi et al., 2013)*. All samples were collected after donors had given their written informed consent, in accordance with the French bioethics laws. Approximately 10 mg of each liver sample was used for DNA extraction, and microsomes were then prepared from each sample by differential centrifugation, as described previously *(Picard et al., 2004)*. Protein concentration in each microsomal suspension was measured using the Bradford method using bovine serum albumin as a standard. Several pools, derived from these 43 individual preparations, were prepared according to their genotypes.

Incubation procedures

Incubations were performed using the incubation procedure described in our recently published paper *(Al Saabi et al., 2013)*. Briefly, the incubation mixture (100 mL) contained 0.5 mg/mL microsomal proteins, 2 mM UDPGA, 250 mM ethanol (for the study of ethanol hepatic glucuronidation distribution) or 5-1000 mM ethanol (for EtG production kinetics), 10 mM $MgCl_2$, and 0.1 M Tris-HCl buffer (pH 7.4). Microsomes (individual or pooled HLMs)

were first activated by incubation with the pore-forming peptide alamethicin (100 mg/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 (ethanol). After 45 min of incubation at 37°C, the reaction was stopped by addition of ice-cold acetonitrile (50 mL). After centrifugation (10 000 g for 5 min) to spin down proteins, the supernatant was stored at -20°C until analysis.

Genotyping of Human Liver

Genomic DNA was extracted from each human liver sample, as described previously *(Picard et al., 2007)* and genotyped for four SNPs: UGT1A9 -275T>A (rs6714486), -440 C>T (rs2741045), and IVS1+399T>C (rs2741049), and UGT2B7 -900G>A (rs7438135), using a validated TaqMan allelic discrimination assay on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France).

Sequencing of the UGT1A9 and 2B7 genes

The promoter and coding regions of *UGT1A9* and *UGT2B7* from 10 DNA samples (that were selected according to the rate of EtG production observed *in vitro*: low (<100 pmol/mg protein/min; n = 2), medium (160-200 pmol/mg protein/min; n = 5), and high (> 200 pmol/mg protein/min; n = 3) were sequenced. All primers were designed according to the nucleotide sequence of the *UGT1A9* and *2B7* genes reported in the GenBank database (accession number NT_ 021027.2 and 001074.2, respectively), and are listed in Table 1.

In a first step, nine PCRs were performed to generate two amplicons for *UGT1A9*, encompassing the promoter region and the first exon, and seven amplicons for *UGT2B7*, encompassing the promoter region and the six exons. These PCR reactions were carried out in a final volume of 25 µL in the presence of 20 mmol/L Tris-HCl buffer (pH 8) containing 2 mM $MgCl_2$, 200 ng of genomic DNA, 0.4 mM of each primer, 0.2 mM of each dNTP, and 0.3U Taq DNA polymerase (Invitrogen, Cergy-Pontoise, France), according to the manufacturer's instructions. The Mg^{2+} concentration and the annealing temperature were optimized for each primer set (Table 1). After an initial denaturation step at 94°C for 3 min, 33 cycles of 1 min at 94°C, 1 min at an optimized annealing temperature, and 1 to 2.5 min at

72°C, were performed, and a final extension period of 10 min was performed at 72°C. All PCR fragments were analysed on a 1% agarose gel and visualized using UV transillumination after EvaGreen-staining to check both the size and the specificity of the amplicons. Each fragment was directly sequenced on both strands using the BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and an automated 3130XL genetic analyzer (Applied Biosystems). Electrophoresis and analysis of DNA sequence reactions were performed with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Villepinte, France) and the SeqScape Software version 2.5.

Determination of EtG concentration

Samples were prepared by adding 20 µL of internal standard (EtG-D5) solution (2 mg/L in acetonitrile) to 150 µL of incubation supernatants. An aliquot (15 µL) of each sample was then injected in the LC-MS/MS system. EtG concentrations were analysed using our previously validated method *(Al Saabi et al., 2013).* The limit of quantification (LOQ) was 5 ng/mL. Good linearity was observed from the LOQ up to 500 ng/mL ($r > 0.999$).The within- and between-assay coefficients of variation and accuracy were less than 10% over the calibration range.

Data analysis

Distributions of the *UGT1A9* and *UGT2B7* alleles were compared to the Hardy–Weinberg theoretical distribution using the Fisher's exact test. All data points represent the mean of either duplicate or triplicate experiments. Enzyme kinetic parameters (apparent K_m and V_{max}) were estimated by fitting the Michaelis-Menten equation to the data using nonlinear regression analysis (Prism version 5.01; GraphPad Software Inc., La Jolla, CA) and are presented as a regression parameter estimate ± S.D. of the estimate. The fit of the model to the data was assessed from the Akaike information criterion. Differences between metabolic rates, as well as microsomal velocity rates, according to tested genotype groups, were considered significant when *P* value was less than 0.05. All statistical analyses were performed using nonparametric tests (Kruskall-Wallis or Mann-Whitney) using GraphPad Software.

Table 1. Details of the primers used for *UGT1A9* and *UGT2B7* amplification and sequencing

Amplified regions	Primers	Sequence (5'-3')	Size (bp)[a]	[Mg2+] (mM)[b]	Tm (°C)[c]
Primers for amplification the *UGT1A9* promoter (-2229 to +52)					
UGT1A9 Promoter	1A9-Prom.F	CAGAGTCGTGCTGTTTTGCC	2281	2	64
	1A9-Prom.R	GCAGCAGACACACACATAGAGG			
Primers for sequencing of the *UGT1A9* promoter					
UGT1A9 Promoter	1A9-Pro.1R	CACCAAGTGTACCTGTCTCTC			
	1A9-Pro.2F	TGGTTTTGCTGTTTCAGGG			
	1A9-Pro.2R	GTCAGGTAATATCTTGCCAG			
	1A9-Pro.3F	GCTAGGGGCATTGTCCAA			
	1A9-Pro.3R	TGCTGCTTCTCTTCTTCCA			
	1A9-Pro.4F	GGGTTGTCAGTCTCATTTCAGC			
Primers for amplification the *UGT1A9* Exon 1					
UGT1A9 Exon 1	1A9-Ex1.F	CCCACCTACTGTATCATAGG	1069	2	60
	1A9-Ex1.R	CGAGTACACGCATTGGCACA			
Primers for sequencing of the *UGT1A9* Exon 1					
UGT1A9 Exon 1	1A9-Ex1.1R	AGTGGCAAAGTATTCCCC			
	1A9-Ex1.1F	CGATCCTTTTGATAACTGTGG			
Primers for amplification the *UGT2B7* Promoter (-1098 to +7)					
UGT2B7 Promoter	2B7-Prom.F	CATCAGTGAGATCCTTCAGACCAG	1105	1.5	60
	2B7-Prom.R	CAGACATCCTGGTGCAATGC			
Primers for sequencing of the *UGT2B7* promoter					
UGT2B7 Promoter	2B7-Pro. 1R	GCATCTACCATAACAATCAG			
	2B7-Pro. 1F	GGCTAAGGACTATAGGGC			
Primers for amplification the *UGT2B7* Exon 1					
UGT2B7 Exon 1	2B7-Ex1.F	AAGGGTTACATTTTAACTTCTTGG	933	2	56
	2B7-Ex1.R	ATTCACTTACCAAAACCCCACT			
Primers for sequencing of the *UGT2B7* Exon 1					
UGT2B7 Exon 1	2B7-Ex1.1R	CCAGCAGCTCACTACAGG			
	2B7-Ex1.1F	GGAGAATTTCATCATGCAAC			
Primers for amplification and sequencing the *UGT2B7* Exons 2 to 6					
UGT2B7 Exon 2	2B7-Ex2.F	ACCTTTTTTTTTTCTATTCCTGT	204	2	52
	2B7-Ex2.R	CAAAATAAAACCAACAAAAGTATG			
UGT2B7 Exon 3	2B7-Ex.3 F	CCGCTGTGCTAATACTCTTT	207	1.5	55
	2B7-Ex.3 R	CCACACCAGTAAGGCACTT			
UGT2B7 Exon 4	2B7-Ex4.F	CTTTTGAATTCCACTCATG	177	1.5	52
	2B7-Ex4.R	GCTGTTACTAATATATTCAG			
UGT2B7 Exon 5	2B7-Ex5.F	ATTCCTATGAGTAATTTTGC	303	1.5	52
	2B7-Ex5.R	CTATCTGTAAATACCACC			
UGT2B7 Exon 6	2B7-Ex6.F	CTCTTCCTGCTACATTACTG	370	2	55
	2B7-Ex6.R	CTGAAGTAGTCTCACCTATC			

F: forward; R: reverse. [a]Size of amplified fragments. [b]Optimized Mg^{2+} concentration for each set of primers. [c]Optimized annealing temperature for each set of primers.

Results

Interindividual variability in ethanol hepatic glucuronidation

In order to assess the distribution of ethanol hepatic glucuronidation rates in humans, 43 HLM preparations were incubated individually with 250 mM ethanol. The results followed a normal distribution (Shapiro-Wilk test: *P*=0.661; m ± SD = 152.2 ± 39.32 pmol of EtG/mg microsomal protein/min) with a 5.4-fold difference between extreme values (minimum = 50.75; maximum = 274.27 pmol/mg/min; Fig. 1).

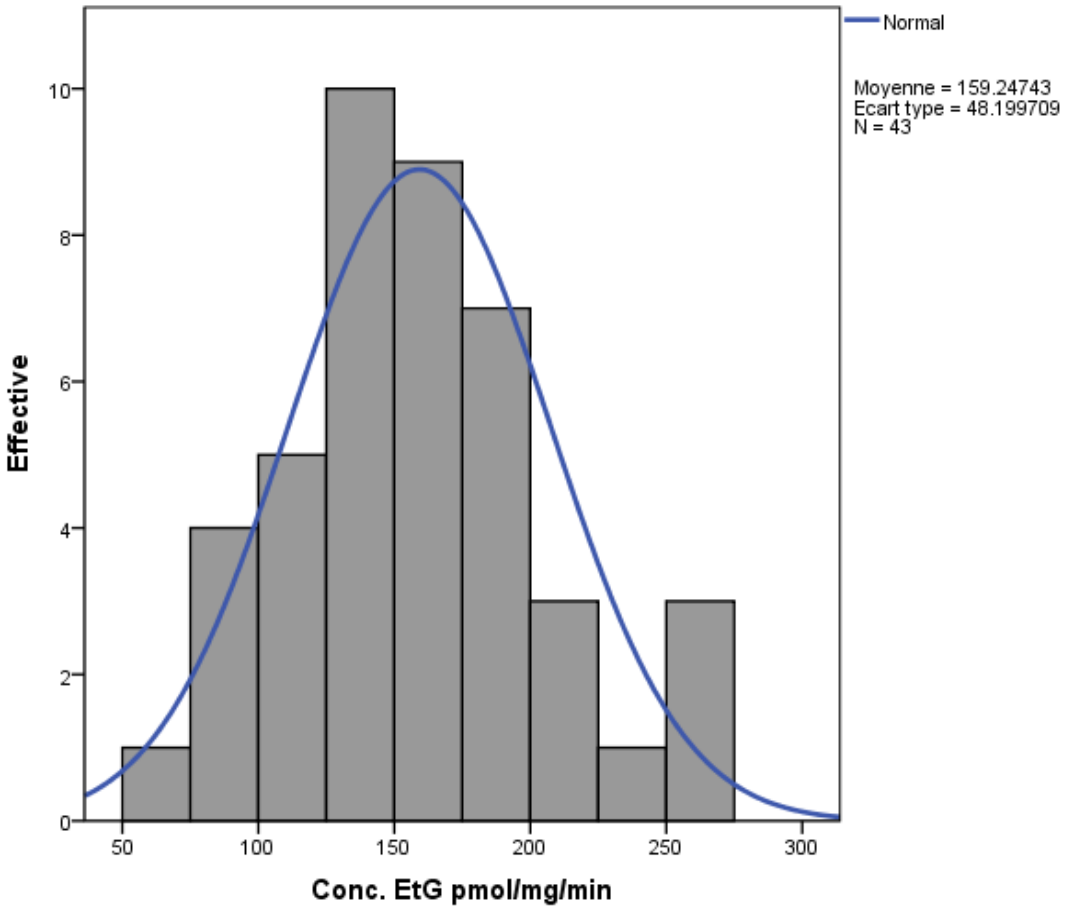

Figure 1. Distribution of ethylglucuronide (EtG) *in vitro* hepatic production after incubation of ethanol (250 mM) with 43 individual HLMs. Data are represented as the mean of duplicate experiments. EtG production rate is expressed as pmol of EtG/mg of microsomal protein/min.

Gender had no influence on EtG production by the 43 individual HLMs; the EtG metabolic rates for HLM derived from male individual samples (n = 31) and from female individual samples (n = 12) being 158.7 ± 9.14 and 160.7 ± 12.23 pmol/mg/min, respectively (*P*=0.860; Fig.2).

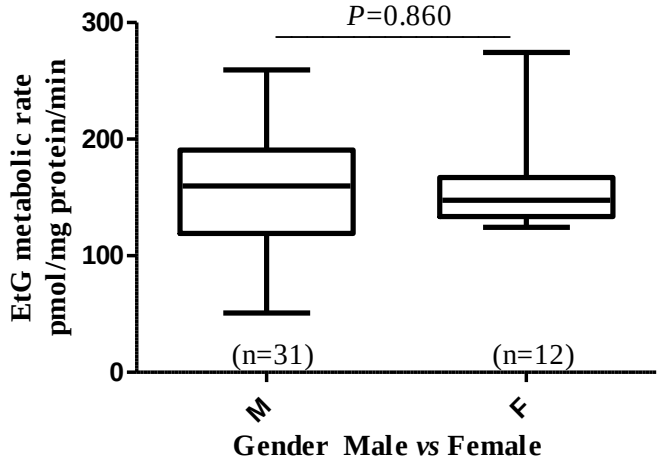

Figure 2. Box and whisker plot of the impact of gender on ethylglucuronide metabolic rate by 43 genotyped HLMs (middle lines represent the median, and the top and bottom extremities of the box represent the 25th and 75th percentiles).

No significant correlation was found between EtG formation rates and HLM donors' age (Spearman R test, $P > 0.05$, r = -0.1208). Age was thus not associated with the *in vitro* glucuronidation rate of ethanol (Fig. 3).

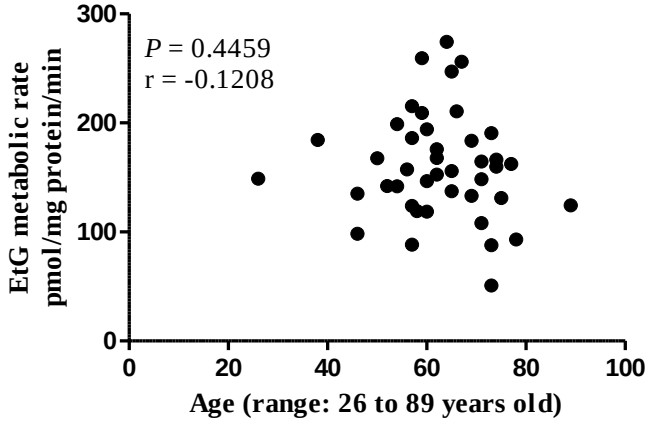

Figure 3. Absence of correlation between EtG formation rate and HLM donors'age (Spearman R test).

Identification of *UGT1A9* and *UGT2B7* genetic polymorphisms

To investigate potential sequence variations in *UGT1A9* and *UGT2B7* genes, a PCR-sequencing strategy was developed and applied to genomic DNAs from 10 unrelated individuals of Caucasian origin (selected according to their EtG production rate). Sequencing of the promoter region and the specific exon 1 of *UGT1A9* allowed the identification of a total of 9 and 1 SNP, respectively, which are all known polymorphisms (Table 2). Sequencing of the promoter region and the exons 1 to 6 of the UGT2B7 allowed the identification of a total of 13 SNPs, 4 of them being not previsouly described polymorphisms (Table 2). Polymorphisms were numbered according to the recommendations of Den Dunnen and Antonarakis *(den Dunnen and Antonarakis, 2001)*, with nucleotide +1 corresponding to the A of the ATG initiation codon on the mRNA sequence. Considering the high number of polymorphisms identified in a low number of DNA samples, the sequencing interpretation could not identify any SNP to be totally characteristic of samples with low or high rate of EtG production *in vitro*.

Individual genotypes for the 4 tested polymorphisms

Table 3 showed the results of the *UGT1A9* (-275T>A, -440C>T, and IVS1+399C>T) and *UGT2B7* -900G>A genotyping analyses in 43 human liver samples (used for the preparation of HLMs). *UGT1A9* and *UGT2B7* genotype frequencies for each SNP were consistent with the Hardy–Weinberg equilibrium. Variant allele frequencies were 0.07, 0.25, 0.34, and 0.52, respectively. The allele frequencies of these known polymorphisms were similar to those reported in the HapMap Caucasian population.

Table 2. Distribution of *UGT1A9* and *UGT2B7* polymorphisms in 10 individuals based on the sequence published in GenBank database

	Nucleotide changes	rs number	DNA1	DNA2	DNA3	DNA4	DNA5	DNA6	DNA7	DNA8	DNA9	DNA10
[EtG] pmol//mg/min			50.75	88.28	164.56	167.56	183.68	190.59	194.09	215.31	256.15	259.25
	c.-2152C>T	17868320	C/C	C/C	C/C	C/C	C/T	C/T	C/T	C/C	C/C	C/T
	c.-1887T>G	6731242	T/T	T/T	T/T	T/G	T/T	T/T	T/G	T/T	T/T	T/T
	c.-1819T>C	13418420	T/T	C/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T
	c.-1218G>A	17864684	G/G	G/G	G/G	G/A	G/G	G/G	G/A	G/G	G/G	G/G
***UGT1A9* Promoter**	c.-1213G>A	2741044	G/A	G/G	G/A	G/A	G/A	G/G	G/G	G/A	G/G	G/A
	c.-440C>T	2741045	C/T	C/C	C/T	C/T	C/T	C/C	C/C	C/C	C/T	C/C
	c.-331T>C	2741046	T/C	T/T	T/C	T/C	T/C	T/T	T/T	T/C	T/C	T/C
	c.-275T>A	6714486	T/T	T/T	T/T	T/T	T/A	T/A	T/A	T/T	T/T	T/A
	c.-127 -/T	35426722	delT	delT	delT	delT	delT	T/T	delT	T/T	delT	delT
UGT1A9 Exon 1	c.98 T>C	72551330	T/T	T/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T
	c.-900G>A	7438135	A/G	G/G	A/G	A/A	G/G	A/G	A/G	A/G	A/G	A/G
***UGT2B7* Promoter**	c.-327 A>G	7662029	A/G	G/G	A/G	A/A	A/G	A/G	A/G	A/G	A/A	A/G
	c.-161 T>C	7668258	C/T	C/C	C/T	T/T	C/T	C/T	C/T	C/T	T/T	C/T
***UGT2B7* Exon 1**	c.372A>G	28365063	A/G	A/A	A/A	A/G	A/A	A/A	A/A	A/G	A/A	A/G
	c.735A>G	28365062	A/A	A/A	A/G	A/G	A/A	A/G	A/G	A/A	A/A	A/A
***UGT2B7* Exon 2**	c.801A>T	7438284	A/T	A/T	A/T	T/T	A/T	A/T	A/T	A/T	A/T	A/T
	c.802T>C	7439366	T/C	T/T	T/C	C/C	T/T	T/C	T/C	T/C	T/C	T/C
***UGT2B7* Exon 3**	No polymorphism											
	1038 C>T	unknown	C/C	C/C	C/T	C/T	C/T	C/C	C/T	C/C	C/C	C/T
	1044 T>A	unknown	T/T	T/T	T/A	T/A	T/A	T/T	T/A	T/T	T/T	T/A
***UGT2B7* Exon 4**	c.1059C>G	4292394	C/G	C/C	G/G	G/G	C/G	G/G	G/G	C/G	C/G	G/G
	c.1062C>T	4348159	C/C	C/C	C/T	C/T	C/C	C/T	C/T	C/C	C/C	C/C
	1083 C>T	unknown	C/C	C/C	C/T	C/T	C/T	C/C	C/T	C/C	C/C	C/T
	1089 A>T	unknown	A/A	A/A	A/T	A/T	A/T	A/A	A/T	A/A	A/A	A/T
***UGT2B7* Exon 5**	No polymorphism											
***UGT2B7* Exon 6**	No polymorphism											

Impact of *UGT1A9* and *UGT2B7* SNPs on the *in vitro* production of EtG

Ethanol was incubated with each of the 43 microsomal preparations at a single concentration of 250 mM. The presence of the *UGT2B7* -900A allele (AA and GA genotypes; n = 35) produced a minor, but not significant, increase of the EtG formation rate compared with the GG genotype (*P*=0.16; Fig. 4/A).

Concerning *UGT1A9*, first of all, the -440C>T SNP had no influence on EtG production by the 43 HLM. EtG metabolic rates for the -440 CC, -440CT and -440TT genotypes being 163.0 ± 9.1; 147.4 ± 15.1, and 176.9 ± 11.1 pmol/mg protein/min, respectively (*P*=0.337; Fig. 4/B). HLM carrying the homozygous wild-type -275TT genotype (n = 37) showed a significantly lower EtG metabolic rate than HLM carrying the heterozygous genotype (n = 6) (mean ± SD: 150.3 ± 7.14 *versus* 214.4 ± 16.75 pmol/mg protein/min, respectively; *P*=0.0031; Fig. 4/C). Finally, concerning the *UGT1A9* intronic IVS1+399T>C SNP, the presence of the +399T allele (TT and TC genotypes; n = 24) was associated with a lower EtG metabolic rate in HLM compared with the CC genotype ($P < 0.05$). HLM carrying the homozygous mutated genotype +399CC (n = 19) showed a significantly higher mean EtG metabolic rate ($P < 0.05$) than HLM carrying the heterozygous T+399A genotype (n = 19), the latter being slightly (but not significantly) more active than HLM carrying the homozygous wild-type +399TT genotype (n = 5) (mean ± SD: 176.0 ± 11.22; 145.0 ± 10.42; and 135.2 ± 16.79 pmol/mg protein/min, respectively; Fig. 4/D).

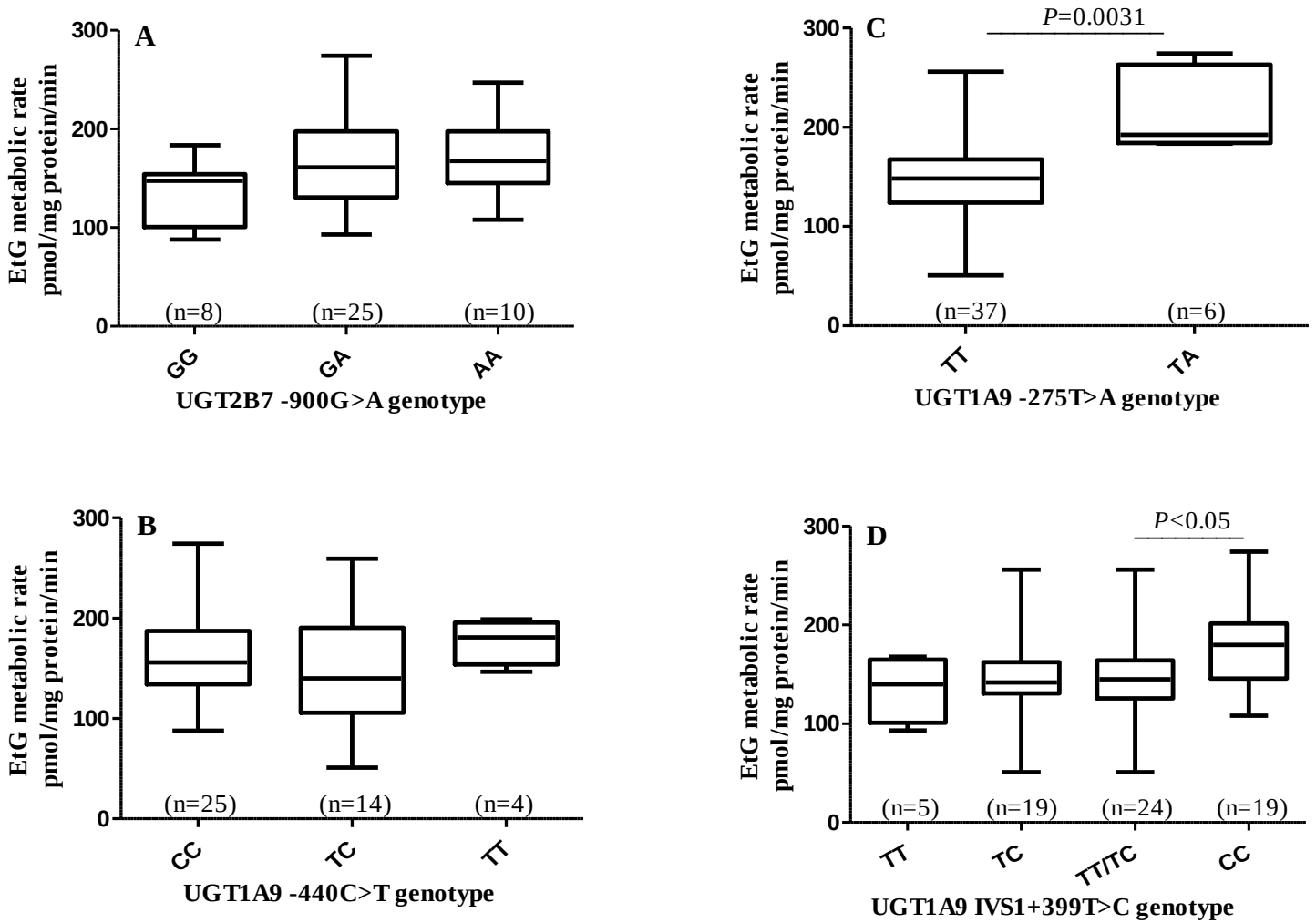

Figure 4. Box and whisker plots of the impact of *UGT2B7* -900G>A (A) and *UGT1A9* -440C>T (B), -275T>A (C), and IVS1+399T>C (D) genotypes on ethylglucuronide metabolic rate by 43 individual HLMs (middle lines represent the median, and the top and bottom extremities of the box represent the 25th and 75th percentiles).

Kinetics of ethanol glucuronidation by pools of genotyped HLMs

1- UGT2B7 -900G>A: Three pools of HLM were constituted for each *UGT2B7* genotype (-900GG, -900GA, and -900AA; n = 6 for each pool) and the kinetics of EtG production was determined using each pool. No difference was found in kinetic parameters (K_m, V_{max}, and Cl_{int}) between carriers and non- carriers of the -900G>A SNP (Table 4; Fig. 5/A).

2- UGT1A9 -275T>A: Similarly, two pools of HLM were constituted for *UGT1A9* homozygous wild-type (-275 TT) and heterozygous (-275TA; n = 6 for both) genotypes, and the kinetics of EtG production was determined using each pool. The pool of heterozygous genotype -275TA showed a 5.1-fold lower maximal velocity (V_{max}) than the homozygous wild-type genotype -275 TT. The apparent K_m was 12.6-fold higher with the pool of homozygous wild-type genotype than with the pool of heterozygous genotype. The resulting intrinsic clearance (Cl_{int}) was 2.5-fold higher in the pools of heterozygous genotype than in that carrying the homozygous wild-type genotype -275TT (Table 4; Fig. 5/B). The linearity of the enzyme kinetics was checked using the Eadie–Hofstee plot.

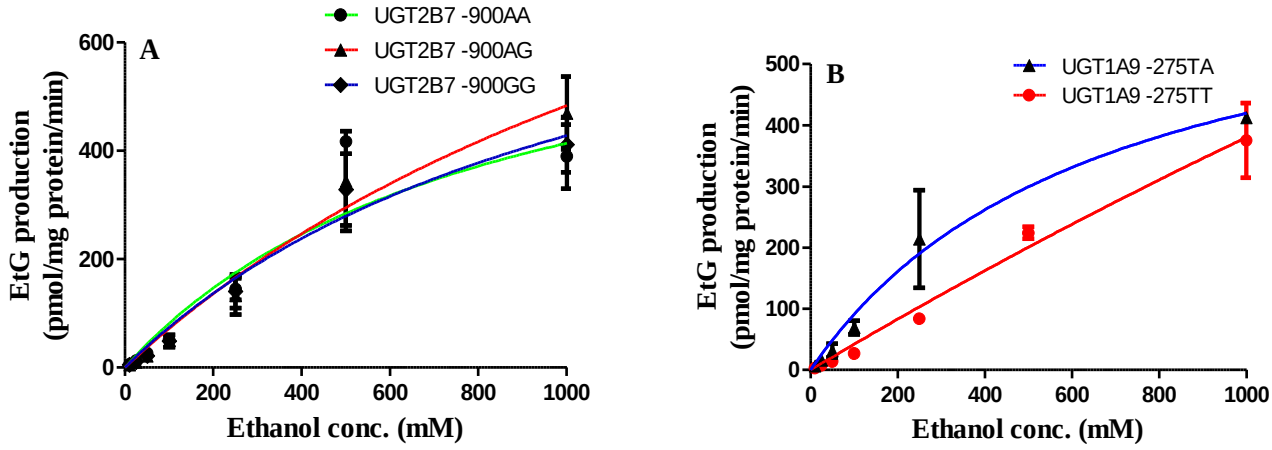

Figure 5. Enzyme kinetic modeling of ethanol glucuronidation in different pools of HLM genotyped for *UGT2B7* -900G>A (A) and for *UGT1A9* -275T>A (B). Glucuronidation activities of microsomes were measured by incubating membrane fractions with increasing concentrations of ethanol at a constant concentration of UDPGA (2 mM). Each point represents the mean ± S.D. of duplicate experiments.

Table 3. Results of *UGT1A9* (-275T>A, -440C>T, and IVS1+399C>T) and *UGT2B7* -900G>A genotyping analysies in 43 human liver samples. All observed frequencies were consistent with the Hardy–Weinberg equilibrium.

SNP genotypes	**UGT1A9 -275T>A**			**UGT1A9 -440C>T**			**UGT1A9 IVS1+399T>C**			**UGT2B7 -900G>A**		
	TT	TA	AA	CC	CT	TT	TT	CT	CC	GG	GA	AA
Human liver samples (n = 43)	37	6	0	25	14	4	5	19	19	8	25	10
Genotype frequency	0.860	0.139	0	0.581	0.325	0.093	0.116	0.441	0.441	0.186	0.581	0.232

Table 4. Kinetic parameters (V_{max}, K_m, and Cl_{int}) of pooled HLM prepared according to their genotypes for *UGT1A9* (TT-275 and T-275A) and *UGT2B7* (-900GG, -900GA, and -900AA).

SNP genotypes	**UGT1A9 -275T>A**		**UGT2B7 -900G>A**		
	Pool of TT-275 (n=6)	**Pool of T-275A** (n=6)	**Pool of GG-900** (n=6)	**Pool of G-900A** (n=6)	**Pool of -900AA** (n=6)
V_{max} (pmol/mg protein/min)	3603	702.0	914.4	1337	760.4
K_m (mM)	8476	672.4	1137	1766	837.6
CL_{int} (nl/min/mg)	0.425	1.044	0.804	0.757	0.907

HLM: human liver microsomes; $Cl_{int}=V_{max}/K_m$.

Discussion & conclusion

To our knowledge, this is the first study that investigates the impact of genetic polymorphisms of both *UGT1A9* and *UGT2B7* on the *in vitro* glucuronidation of ethanol.

We showed previously that ethanol is primarily glucuronidated by the liver, and that kidney and intestine tissues play only a minor role in this metabolic pathway *(Al Saabi et al., 2013)*. For this reason, we limited our study to HLM. After individual incubation of 250 mM ethanol with 43 individual HLM, we found that the distribution of ethanol hepatic glucuronidation followed a normal distribution. However, EtG formation rates were found to be highly variable (5.4-fold difference between extreme values), which is in concordance with the high interindividual variability in EtG levels reported *in vivo (Halter et al., 2008)*. Several factors, including both genetic and non-genetic factors, could be responsible for this variability in ethanol hepatic glucuronidation.

HLMs were prepared from liver samples collected in a pathological department from surgical specimens. Sample collection was organized by Biopredic International as part of a collaboration contract. Each specimen was examined by a pathologist and any macroscopical signs of liver abnormality (such as steatosis) led to the exclusion of the specimen. We found no difference in EtG production by HLM derived from males and females. We also found that ethanol glucuronidation was not affected by HLM donors' age. However, many other characteristics of the donors, not recorded here, might influence liver enzyme activity and specifically the metabolism of ethanol (medication prior to the surgery, ethanol consumption, underlying diseases…). This has to be considered as a limitation of this study.

Two different approaches were chosen for this study:

We investigated the impact of known UGT genetic polymorphisms on the glucuronidation rate of ethanol in the 43 HLMs. We restricted this study to 4 SNPs (UGT1A9 -275T>A, -440C>T, and IVS1+399T>C; and UGT2B7 -900G>A), which are known to affect either expression or activity of their respective encoded-enzyme *(Bernard and Guillemette, 2004;*

Bernard et al., 2006; Court et al., 2003; Djebli et al., 2007; Duguay et al., 2004; Girard et al., 2006, 2004). Also, these polymorphisms has a relatively high allele frequency sufficient for a study using a rather limited number of HLMs (n = 43).

Our results indicate that the *UGT1A9* -275T>A SNP seems to affect the *in vitro* formation rate of EtG; the presence of the -275A variant allele (TA and AA genotypes) was associated with higher EtG metabolic rate in HLM compared with homozygous wild-type TT genotype. Furthermore, we found that the Cl_{int} obtained after incubation with homozygous wild-type microsomes (-275 TT) are about 4-fold lower than that obtained with heterozygous microsomes (-275 TA). Accordingly, it was previously reported that this SNP is associated with increased UGT1A9 protein content in human liver microsomes *(Girard et al., 2004)*. It is noteworthy that the -275T>A (rs6714486) and -2152C>T (rs17868320) SNPs are in complete linkage disequilibrium and that their allelic frequency in the Caucasian population in relatively low (6%) *(Girard et al., 2004).*

EtG production by HLM was not influenced by the presence of the *UGT1A9* -440C>T polymorphism (rs2741045). This SNP is in complete linkage disequilibrium with the -331C>T (rs2741046) polymorphism. Although none of them significantly affected UGT1A9 protein levels in human liver, both were reported to significantly increase mycophenolic acid phenyl-glucuronide (MPAG) production by HLMs *(Girard et al., 2004).*

Concerning the intronic *UGT1A9* IVS1+399T>C polymorphism, our experiment using HLM showed that EtG metabolic rates in CC carriers were significantly higher than those in wild-type TT and heterozygous TC subjects. No kinetic evaluation had been performed for this allelic variant. In the literature, this intronic SNP has been particularly studied. It was found to be associated *in vitro* with increased protein expression and 7-ethyl-10-hydroxycamptothecin (i.e., SN-38; irinotecan metabolite) hepatic glucuronidation *(Girard et al., 2006).* Other polymorphisms that were not considered in the present study might also influence ethanol glucuronidation and should be investigated in further studies.

The last SNP studied here was the *UGT2B7* -900G>A (rs7438135). Similarly to the *UGT1A9* -440C>T SNP, we showed that there was no significant association between the *UGT2B7* -900G>A polymorphism and the interindividual variability in EtG metabolic rates by HLM. This result is in concordance with the result reported by Agteren *et al.,* (2008), that showed no influence of the -900G>A SNP on the glucuronidation of mycophenolic acid (an immunosuppressive drug) *(van Agteren et al., 2008).* However, in another study, Darbari *et al.,* (2008) showed that the presence of the -900G allele (GG and GA genotypes) was associated with lower morphine metabolites/morphine AUC ratio compared with the AA genotype (*P*=0.03), and that the presence of *UGT2B7* -900G allele was associated with significantly reduced glucuronidation of morphine *(Darbari et al., 2008).* Djebli *el al*., (2007) also showed that mycophenolate acyl-glucuronide (AcMPAG) production velocity rate was significantly increased in microsomes carrying an adenosine at position -900 as compared to those carrying a guanine *(Djebli et al., 2007).* It is worth to note that this SNP is in complete linkage disequilibrium with the 802C>T exonic SNP (rs7439366; *UGT2B7*2*) *(Sawyer et al., 2003).UGT2B7* -900G>A SNP is also strongly linked to other promoter polymorphisms, including the -1248A>G, -268 A>G, and -102 T>C SNPs. Duguay *et al*., had observed a two-fold increase in the transcriptional activity of hepatic and colon cell lines carrying the variant haplotype of the promoter region compared to the wild-type promoter *(Duguay et al., 2004).*

In a second approach, and in order to find further explanations of the large interindividual variability in EtG hepatic production, we performed a screening for additional variants in coding and regulatory regions of the *UGT1A9* and *UGT2B7* genes by direct sequencing of 10 genomic DNAs from unrelated individuals, which were selected according to their rate of EtG production. For an initial screen, we selected 2 individual HLM preparations with low EtG production rate (< 100 pmol/mg protein/min), 5 with medium EtG production rate (160-200 pmol/mg protein/min), and 3 with high EtG production rate (> 200 pmol/mg protein/min). This strategy increases the probability of detecting functional *UGT1A9* and *UGT2B7* polymorphisms. In the 5'-regulatory region of *UGT1A9*, 9 SNPs were found. One SNP in

exon 1 of *UGT1A9* (c.98 T>C; rs72551330) was identified in one sample only, which belongs to an individual with a low rate of EtG production (Table 2).

In the literature, at least eleven SNPs have been reported in the proximal promoter region of *UGT1A9 (Girard et al., 2004)*. Only two of them, -275T>A (rs6714486) and -2152C>T (rs17868320), were associated with increased UGT1A9 protein content in human liver microsomes *(Girard et al., 2004)*. The *UGT1A9* c.98T>C SNP (exon 1) is a non-synonymous polymorphism ($Met^{33}Thr$). It was reported that this SNP decreases the glucuronidation of both SN-38 and mycophenolic acid, presumably through decreased enzyme affinity *(Bernard and Guillemette, 2004; Villeneuve et al., 2003)*.

A total of 13 SNPs, three in the 5'-regulatory region and ten within the exonic sequences of *UGT2B7* gene, were found. No polymorphism was found in exons 3, 5 and 6. Similarly to *UGT1A9*, several SNPs have been described previously in *UGT2B7*. Among them, the non-synonymous c.802C>T SNP (rs7439366), in exon 2, results in a histidine to tyrosine substitution at codon 268 ($His^{268}Tyr$). However, the functional impact of this SNP remains controversial and might be linked to the -900G>A promoter SNP described above. No sufficient data were reported about other identified SNPs. Their frequencies are relatively low in Caucasians. Because of the low frequency of a number of genetic polymorphisms in the *UGT1A9* and *UGT2B7* genes, larger studies are needed to investigate their potential contribution to the variability of EtG levels. As shown by the data presented in Table 2, *UGT1A9* and *2B7* genotypes were randomly and quasi-evenly distributed between individuals with low and high EtG formation rates which make the sequencing interpretation somewhat difficult. We could not find any SNP that was characteristic of the low or high rate of *in vitro* EtG production. The population and number of HLM studied needs to be increased to draw a more definitive conclusion. Further studies, investigating the *in vivo* relevance of our results, and taking other potential factors into account, are required to explain the interindividual variability of ethanol glucuronidation.

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Impact of cannabis and drug consumption on ethylglucuronide (EtG) concentrations in *post-mortem* blood (article 4, in preparation)

Alaa Al Saabi, Gilles Tournel, Nicolas Picard, and Delphine Allorge

Introduction / Context

Ethylglucuronide (EtG) has been proposed in several *post-mortem* situations, as a marker of *ante-mortem* ingestion of alcohol *(Appenzeller et al., 2008; Høiseth et al., 2007b)*. However, there are some challenges associated with the use of this marker, especially the fact that many factors could alter EtG concentration (bacterial degradation and production of EtG post-collection, potential interactions with other glucuronidated substances…). Furthermore, high interindividual variability in the pharmacokinetics of its production has already been reported *(Halter et al., 2008)*.

Wurst *et al.* (2004) showed that factors including race, smoking status, body mass index, liver cirrhosis, age at which regular drinking began, packs of cigarettes smoked in the past month, and total body water were not associated with changes in urine EtG concentration *(Wurst et al., 2004)*. However, they showed that some other factors were identified to significantly influence EtG concentrations. These factors include age, male gender, tetrahydrocannabinol (THC) use, kidney disease, and total grams of ethanol consumed in the last month *(Wurst et al., 2004)*. Male gender and kidney disease were associated with decreases in urine EtG concentration, whereas THC use was associated with an increase. The authors suggested that these factors need to be taken into account when interpreting EtG concentrations. In another study, Paul *et al.*, (2008) compared the mean concentration of EtG in hair samples between a group of narcotic and drug 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 *versus* 0.011 ng/mg of hair; $P < 0.05$) *(Paul et al., 2008)*. The origin of this difference could not be explained.

Several drugs are inhibitors of UGT-mediated glucuronidation *(Kiang et al., 2005)*, but drug interactions with ethanol glucuronidation have not been studied so far. It is possible that competition (inhibition or induction) with drugs conjugated by UGT enzymes might decrease or increase EtG formation.

We showed previously that morphine, codeine, nicotine, and cotinine did not alter EtG formation rate *in vitro*, and that lorazepam and oxazepam produced a minor, but not significant, increase of EtG formation by human liver microsomes (HLMs) *(Al Saabi et al., 2013)*. In contrast, cannabidiol exhibited a strong noncompetitive inhibition of ethanol glucuronidation, whereas cannabinol increased ethanol glucuronidation in a concentration-dependent manner.

The aim of the present work was 1) to investigate the impact of *ante-mortem* consumption of cannabis and other illegal and/or medicinal drugs on EtG levels in *post-mortem* blood samples, and 2) to evaluate the correlation between EtG and ethanol concentrations in *post-mortem* blood samples.

Materials and Methods

Chemicals and reagents

EtG (reference EGL-332-10) and its deuterated analog EtG-D5 (reference EGL-780-10), which was used as an internal standard, were obtained from Lipomed (Souffelweyersheim, France). Methanol (MeOH, ref: 20837.320) was obtained from VWR Prolabo (Fontenay-sous-Bois, France). The derivatization agent, pentafluoropropionic anhydride (PFPA 99%, ref: 206-604-2), was obtained from SigmaAldrich (Saint-Quentin Fallavier, France). Milli-Q water and all other solvents were of high-performance liquid chromatography grade.

Experimental set-up

The laboratory of forensic toxicology (Centre Hospitalier Régional Universitaire de Lille) in collaboration with the institute of legal medicine performs several toxicological analysis and interpretation on human material derived from forensic autopsy cases in Lille. EtG levels were determined in selected peripheral blood samples (n = 117) received for toxicological analysis between 2010 and 2013. The cases were divided into five groups according to the presence of ethanol, cannabis, and other illegal and medicinal drugs (antidepressants, antipsychotics, sedatives, hypnotics, analgesics, etc.). Clinical information and details about the corpse and scene of death were recorded. The collected samples were stored at -20 °C until analysis. The use of preservatives was not required since the specimens were frozen shortly after collection. All data was handled confidentially.

Group 1: no ethanol, cannabis, illegal and/or medicinal drugs most likely ingested and no *post-mortem* ethanol production: 14 subjects with no ethanol detected in blood and in other body fluids. Here, we termed this group: N.

Group 2: ethanol, but neither cannabis nor illegal and/or medicinal drugs (antidepressants, antipsychotics, sedatives, hypnotics, analgesics, etc.), most likely ingested: 35 subjects with blood alcohol concentration (BAC) ranging from 0.17 to 3.6 g/L, an equal or higher ethanol concentration in vitreous humor and fulfillment of all the following other inclusion criteria: no report of putrefaction of the corpse, no *n*-propanol detected, and no trauma leading to perforation. We termed this group: E.

Group 3: ethanol and other illegal and/or medicinal drugs, but no cannabis, most likely ingested: 35 subjects with BAC ranging from 0.14 to 4.7 g/L. Similar other inclusion criteria as in group 2. We termed this group: E/D.

Group 4: ethanol and cannabis, but no illegal and/or medicinal drugs, most likely ingested: 20 subjects with BAC ranging from 0.24 to 2.94 g/L. Similar other inclusion criteria as in groups 2 and 3. We termed this group: E/C.

Group 5: ethanol, cannabis, illegal and/or medicinal drugs most likely ingested: 13 subjects with BAC ranging from 0.18 to 3.1 g/L. Similar other inclusion criteria as in groups 2, 3 and 4. We termed this group: E/C/D.

Analytical methods

1. Ethanol (EtOH)

EtOH concentrations in whole blood were measured using HS-GC-FID (Headspace gas chromatography equipped with a flame ionization detector) using tert-butanol as an internal standard. Ethanol determination was performed using linear calibration with 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5 g/L aqueous ethanol solutions for HS-GC/FID. The limit of quantitation (LOQ) corresponded to the lowest calibrator concentration (0.05 g/L).

2. Ethylglucuronide

2.1. Samples preparation

An aliquot of 100 µL of whole blood was mixed with 25 µL internal standard solution (10 mg/L) and 1 mL cold methanol. The samples were immediately agitated for 1 min and thereafter centrifuged at 4 500 rpm for 10 min. Methanol layer was then transferred and evaporated to dryness under a stream of nitrogen using a heated metal block at 60°C. The residue was derivatized with 50 µL of pentafluoropropionic anhydride (PFPA) which had been previously shown to be the best agent for EtG derivatization *(Jurado et al., 2004)*. The tubes were tightly closed, mixed by vortexing (10 s), heated for 30 min at 60°C, then dried under N_2 and finally, the residue was reconstituted in 50 µL of isooctane and transferred to auto-sampler vials. One µL of extracted samples was injected in the GC-MS/MS system.

2.2. Analytical validation

EtG concentrations were determined using our previously developed method *(Al Saabi et al., 2011)*. The method was revalidated according to the guidelines of the French Society of Analytical Toxicology *(Groupe de travail « Accréditation », 2005)* and of the French Accreditation Committee COFRAC *(LAB SH-GTA 04 report, 2011)*. 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).

Data analysis

Differences between EtG concentrations from the different groups studied (with and without potential interacting drugs) were considered statistically significant when the *P* value was less than 0.05. All statistical analyses were performed using nonparametric tests (Kruskall-Wallis or Mann-Whitney U tests) using GraphPad Software (Prism version 5.01; GraphPad Software Inc., La Jolla, CA).

Results

Analytical validation

Quantitative results were obtained by peak-height calculations. EtG was detected at a retention time of 6.47 min, following three specific multiple reaction monitoring transitions (Fig. 1). The LOD and LOQ were 0.05 and 0.1 mg/L, respectively. The values were calculated as a mean of background noise + 3 standard deviations (S.D.) and + 10 S.D., respectively. EtG calibration curve was linear in the concentration range from the LOQ up to 20 mg/L ($r > 0.999$). Intra- and inter-day precisions and mean relative errors showed relative standard deviations that were lower than 10% over the calibration range. The analytical specificity for EtG was tested by analysing autopsy blood samples containing no ethanol, but various illegal and medicinal drugs (antidepressants, antipsychotics, sedatives, hypnotics, analgesics, etc.). No interfering substances were detected.

Detection of EtG in the studied groups

The detections of EtG in groups 1, 2, 3, 4, and 5 are summarized in Table 1. Group 1 (N) consisted of subjects with most likely no *ante-mortem* ingestion of ethanol. EtG was detected in a single case (out of 14 blood samples) at a concentration of 0.22 mg/L. In group 2 (E), which consisted of subjects with most likely *ante-mortem* ingestion of ethanol, EtG was detected in 34 cases (out of 35 blood samples), with concentrations between 0.57 and 16.91 mg/L and a median of 3.50 mg/L. In group 3 (E/D), where BAC was between 0.14 and 4.7 g/L, EtG was detected in all cases, with concentrations between 0.36 and 28.70 mg/L, median 3.61 mg/L. In group 4 (E/C), which consisted of subjects with most likely *ante-mortem* consumption of ethanol and cannabis, EtG was also detected in all cases, with concentrations between 0.79 and 15.39, median 2.21 mg/L. Finally, in group 5 (E/C/D), EtG was detected in all cases with concentrations between 0.42 and 12.78 mg/L, median 3.24 mg/L.

Thus, the sensitivity and specificity of EtG in *post-mortem* blood samples as a marker of *ante-mortem* ingestion of ethanol were in these groups about 93 and 99%, respectively, according to our inclusion criteria.

Impact of cannabis and drug consumption

No statistically significant difference in EtG concentrations was observed among tested groups (2, 3, 4, and 5; Kruskall-Wallis test, P=0.3130). However, although the concentrations of ethanol in the second group (E) were significantly higher than those in the third group (E/D) (Mann-Whitney U test, P=0.045; Fig. 2/A), the concentrations of EtG were slightly (but not significantly) higher in group 3 compared to group 2 (Table 1 and Fig. 2/B). When presenting our results as a ratio between EtG and ethanol concentrations, this EtG/EtOH ratio was significantly variable among the groups (Kruskall-Wallis test, $P < 0.05$). More precisely, this ratio was significantly higher in group 3 (E/D) compared to group 2, and compared to group 4 (Mann-Whitney U test, P=0.0036 and 0.0107, respectively; Fig. 2/C); but not to group 5 (P=0.1571). No significant effect of cannabis consumption on EtG levels was found ($P > 0.05$; Fig. 2/B and 2/C), according to our inclusion criteria.

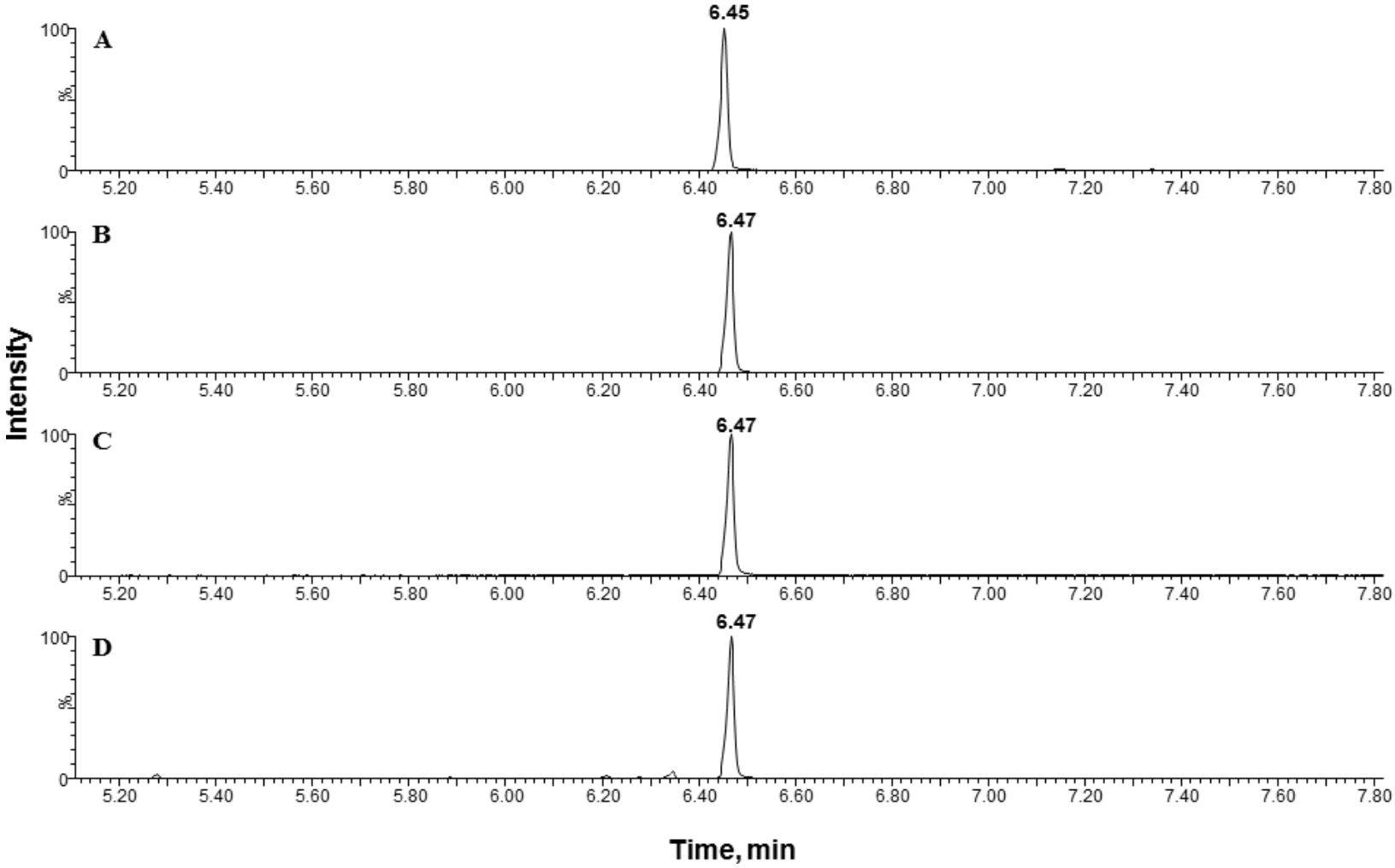

Figure 1. Multiple reaction monitoring chromatograms of ethylglucuronide (EtG) of a blank blood sample spiked by EtG at a concentration of 0.1 mg/L. Transition of m/z 500.9→163.0 was used for internal standard (A), 496.0→163.0 and 496.0→119.0 transitions were used for EtG identification (B and C), and transition 347.0→163.0 was used for EtG quantification (D).

Table 1. Detection of blood EtG in groups 1, 2, 3, 4, and 5 (see text)

Group	Description	No.	No. of cases with EtG detected	Blood ethanol concentration (g/L)			Blood EtG concentration (mg/L)		
				range	median	average	range	median	average
1	Neither ethanol nor other illicit substances were ingested	14	1	<LOQ	<LOQ	<LOQ	/	/	/
2	ethanol, but not other illicit and/or medicinal substances, was ingested	35	34	0.17 - 3.6	1.82	1.91	0.57 - 16.91	3.50	4.50
3	ethanol and other illegal and/or medicinal drugs, but not cannabis, were ingested	35	35	0.14 - 4.7	1.02	1.52	0.36 - 28.70	3.61	6.53
4	ethanol and cannabis, but not other illegal and/or medicinal drugs, were ingested	20	20	0.24 - 2.94	1.60	1.44	0.79 - 15.39	2.45	3.77
5	ethanol, cannabis, and other illegal and/or medicinal drugs were ingested	13	13	0.18 - 3.1	1.40	1.33	0.42 - 12.78	3.24	4.10

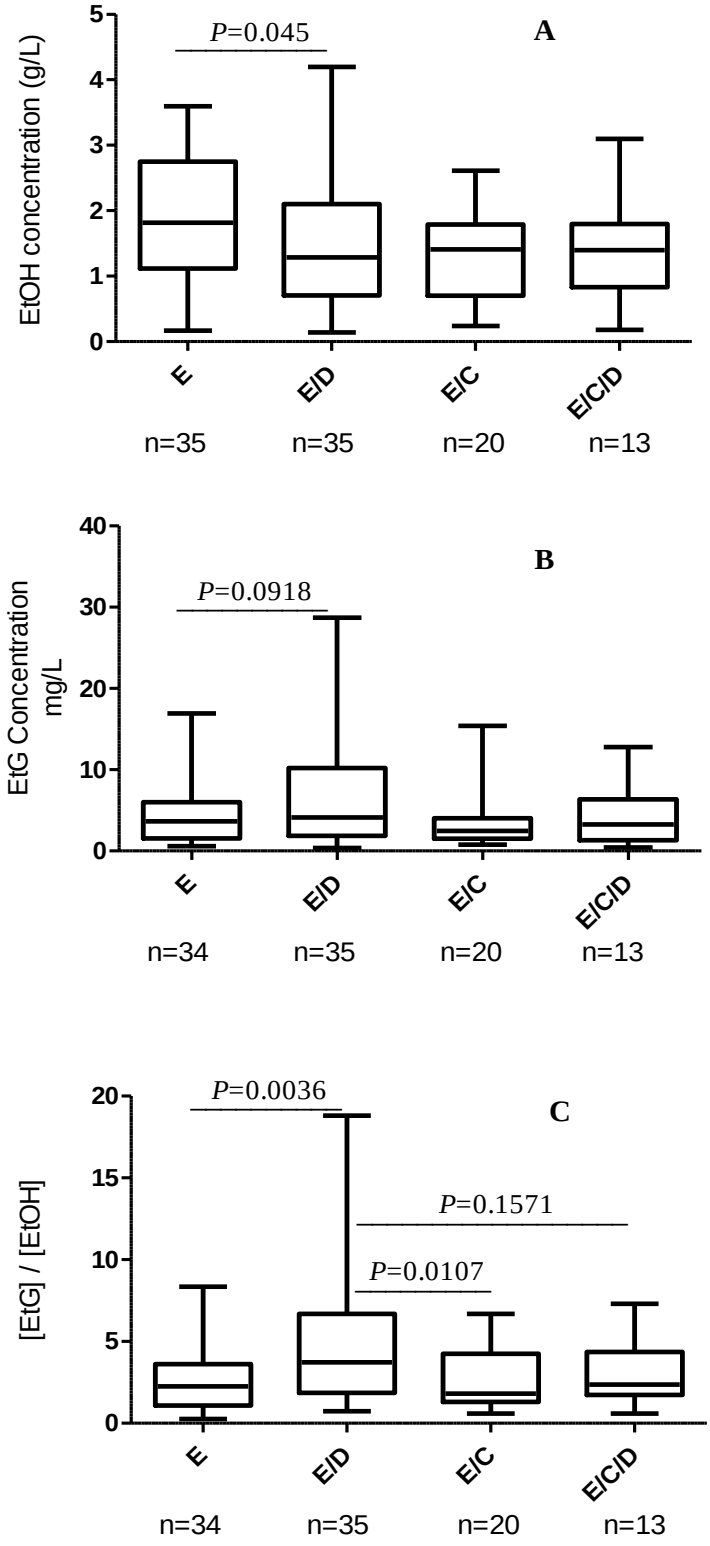

Figure 2. Comparison of ethanol concentrations (A), EtG concentrations (B), and [EtG]/[ethanol] ratios (C) among tested groups. E: ethanol, but not other illicit and/or medicinal substances was ingested; E/D: ethanol and other illegal and/or medicinal drugs, but not cannabis were ingested; E/C: ethanol and cannabis, but not other illegal and/or medicinal drugs, were ingested; E/C/D: ethanol, cannabis, and other illegal and/or medicinal drugs were ingested.

EtG and ethanol correlation

In all tested groups, significant correlations were found between ethanol and EtG, when comparing their blood concentrations (Spearman R test, $P < 0.05$). This result is presented in Fig. 3 (A, B, C, and D for group 2, 3, 4, and 5, respectively). This correlation was higher in group 3 (where both ethanol and drugs were ingested).

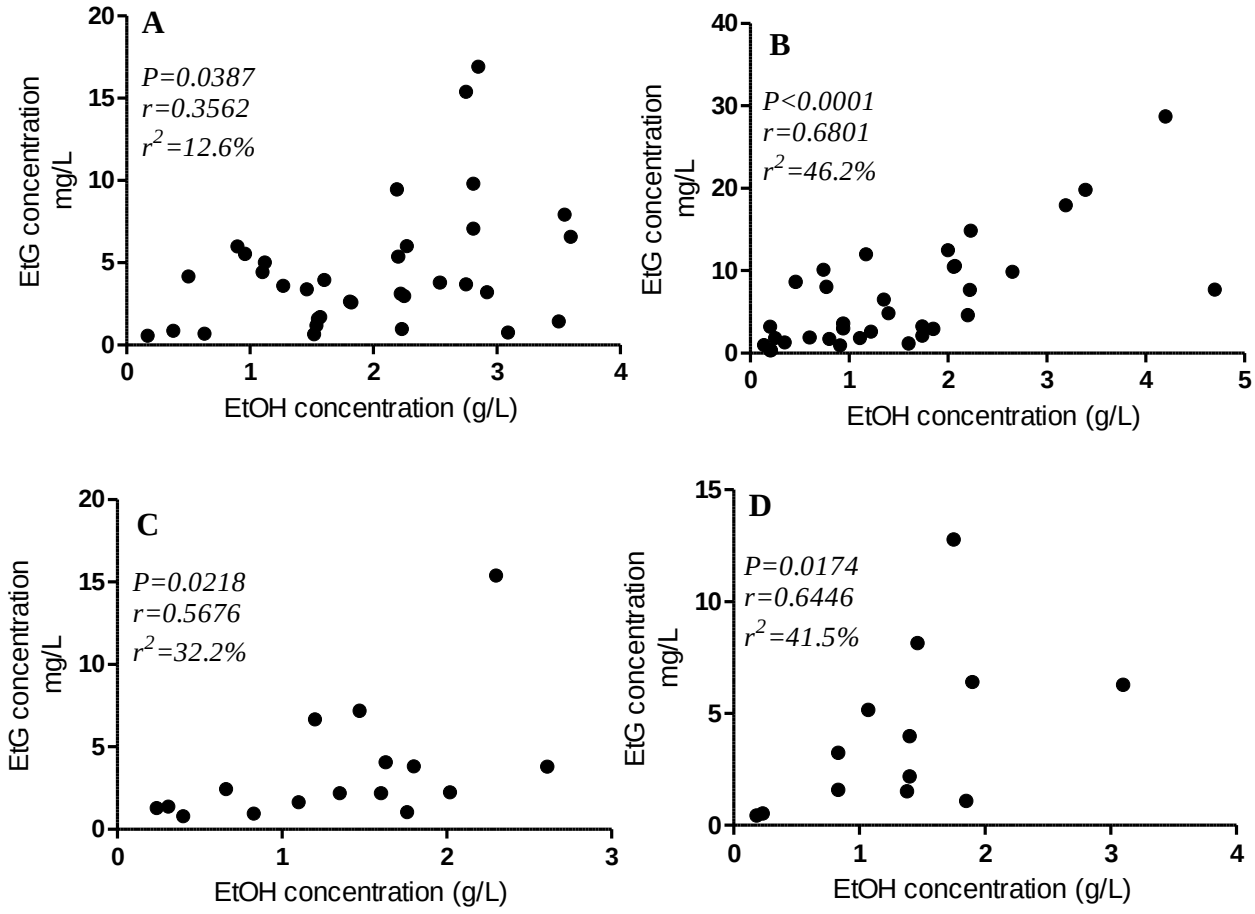

Figure 3. Correlation between ethanol and EtG blood concentrations (Spearman R test). A, B, C, and D represent group 2 (E), 3 (E/D), 4 (E/C), and 5 (E/D/C), (see text).

Discussion & Conclusion

In the present study, we investigated, for the first time, the impact of *ante-mortem* consumption of cannabis and other illegal and/or medicinal drugs on EtG levels in *post-mortem* blood, and evaluate the correlation between EtG and ethanol concentrations.

Our results confirmed that the presence of EtG in blood is a reliable marker of *ante-mortem* ingestion of alcohol in forensic autopsy cases. The results from all tested groups indicated that EtG has a high sensitivity and specificity for alcohol ingestion (about 93 and 99%, respectively), even when ethanol concentrations, and thus expected EtG concentrations, are low. Furthermore, a significant correlation between ethanol and EtG concentrations was found in each tested group, (Spearman R test, $P < 0.05$).

We found that ethanol concentrations in group 2 (mean = 1.91, median = 1.82) were higher than those observed in group 3 (mean = 1.52, median = 1.02) (Mann-Whitney U test, P=0.045). However, mean and median of EtG concentrations in group 3 (drug-positive group) were higher than those observed in the group 2 (Table 1). This difference between groups 2 and 3 did not reach statistical significance ($P > 0.05$). No difference was found in EtG concentrations between groups 2 and 4, or between groups 2 and 5. Thus, cannabis consumption does not seem to affect EtG levels according to our inclusion criteria.

The mean ratio of EtG/EtOH concentration in the drug-positive group (group 3) was 5.1 compared to 2.6 in the group 2. By comparing these ratios between tested groups, we found that difference between groups 2 (E) and 3 (E/D) reached statistical significance (Mann-Whitney U test, P=0.0036). However, the difference between groups 2 and 4, or between groups 2 and 5, did not reach significance ($P > 0.05$), which strongly suggests that drug consumption increase the formation levels of EtG, and that cannabis consumption does not have a significant effect on EtG levels according to our inclusion criteria.

Presenting results as a ratio between EtG and ethanol concentrations has already been used *(Høiseth et al., 2007b)*. When comparing between two groups with low and high BACs, they found that this EtG/ethanol ratio was significantly higher in the group with low BAC.

In our study, the 'drug-positive' group (group 3; n = 35), included samples that were tested positive for one or more drugs. The drugs tested for were different combinations of: opiates, cocaine, benzodiazepines, antidepressants, analgesics, etc. We showed in a previous work that benzodiazepines (lorazepam and oxazepam) produced a minor, but not significant, increase of EtG formation by human liver microsomes *(Al Saabi et al., 2013)*. It is noteworthy here that benzodiazepines were present in about 75% of tested blood samples (26 out 35), which suggests that benzodiazepines could be responsible for this effect. However, the population and number of cases studied need to be increased to draw a more definitive conclusion.

This result is in discordance with data published by Paul *et al.*, (2008), who found that the mean concentration of EtG was significantly higher in the drug-negative group than that found in the drug-positive group *(Paul et al., 2008)*. However, the context and the studied populations in our study are different.

There are many other variables outside our control, which may have affected the results. These variables include liver and/or kidney diseases, total grams of ethanol consumed before death, *post-mortem* delay, the interval between last drinking and the death, etc. The delayed elimination of EtG from blood compared to ethanol may lead to false-positive results. If a person drinks alcohol, and dies a few hours later, ethanol could be totally eliminated from blood. However, the time difference between total elimination of ethanol and EtG in blood is quite short *(Schmitt et al., 1997)*. This source of error could be more problematic if EtG is analysed in urine, since EtG might be found much longer in this matrix *(Dahl et al., 2002)*.

The short time lag after ethanol ingestion before EtG is detected in blood, may lead to false-negative results. This time lag, being up to only 45 min *(Schmitt et al., 1997)***,** will probably not represent an important practical problem.

In cases with positive ethanol and EtG, it is always possible that some of the detected ethanol is formed *post-mortem* and it can therefore not be concluded that the detected level of ethanol is the true *ante-mortem* BAC. A very low EtG/ethanol ratio may lead to the suspicion that the *ante-mortem* BAC was lower than the detected one, and that some of the detected ethanol was produced *post-mortem*. However, another possibility could be that the death occurred soon after ethanol intake. In our study, we had no information about the time of ethanol ingestion, and, therefore, the concentration of EtG compared to that of ethanol gives little information.

This study has some obvious weaknesses. The high sensitivity and specificity of EtG depends on the correct assignment of the cases into groups with and without alcohol ingestion. It is impossible to be absolutely sure whether ethanol was ingested or not, but we believe that our strict inclusion criteria minimized this source of error.

In conclusion, this study indicates that ethanol and EtG concentrations in *post-mortem* blood were significantly correlated; thus EtG in blood could be used as a marker of *ante-mortem* alcohol ingestion. Drugs consumption (especially benzodiazepines) seems to be associated with higher levels of EtG in *post-mortem* blood, whereas cannabis consumption does not seem to have any significant effect on these levels. Additional *in vivo* studies are required to assess the potential contribution of drug consumption to the interindividual variability of EtG production.

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GENERAL DISCUSSION

Approximately one third of people aged 18 or more have an ethanol consumption considered at risk *(Rehm et al., 2009)*. Death, disease and injury caused by alcohol consumption have high socioeconomic impacts *(Kintz et al., 2009; WHO report, 2011)*. Thus, alcoholism is a major public health problem, often underestimated and whose diagnosis is based on clinical and biological lines of evidence.

Biological state markers remain suboptimal with regard to sensitivity and specificity for monitoring recent alcohol consumption in various settings. Furthermore, these biomarkers can be influenced by many factors (e.g. age, gender, a variety of substances, and non-alcohol-associated diseases) and do not cover fully the time axis for alcohol intake *(Conigrave et al., 2002; Fleming et al., 2004)*.

Ethylglucuronide (EtG), as a direct metabolite of ethanol, seems to meet the need for a sensitive and specific marker to elucidate alcohol use not detected by standard testing. It can be detected in various body fluids, tissues, and hair covering a unique and important time spectrum for acute and/or chronic alcohol intake *(Halter et al., 2008; Helander et al., 2009a; Kharbouche et al., 2012; Morini et al., 2008)*.

The present work aimed 1) to identify, through *in vitro* approaches the UGT isoforms involved in the glucuronidation of ethanol and their relative contribution in the major biotransformation organs (liver, kidneys, and intestinal tract), and 2) to investigate through *in vitro* and *in vivo* (*post-mortem*) approaches, factors that could potentially affect the production of EtG in humans and, therefore, that could change the interpretation of its concentrations.

First of all, to perform our work, we needed analytical methods to dose EtG in different contexts. We developed and validated a gas chromatography negative chemical ionization-

tandem mass spectrometry (GC-NCI-MS/MS) method to measure EtG levels in human urine, serum, and whole blood with both high sensitivity and specificity. We also developed a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure EtG in the supernatant of microsomal incubations for *in vitro* experiments. Both methods seem sensitive and reliable with a LOQ sufficient to measure low concentrations of EtG, and they could be routinely used in various clinical and forensic contexts. The use of these sensitive and specific analytical techniques was an asset in this work.

Using human microsomes, as well as recombinant UGT enzymes, one of the main new finding of our work was the demonstration that UGT1A9 and 2B7 isoforms are predominantly involved in ethanol glucuronidation (50% of the overall EtG formation), and that ethanol is primarily glucuronidated by the liver, whereas kidney and intestine tissues play only minor roles in this metabolic pathway. In the literature, only one study showed that multiple UGT isoforms (especially UGT1A1 and 2B7) are competent for EtG hepatic formation, without investigating their specific contributions to hepatic ethanol glucuronidation *(Foti and Fisher, 2005)*. The major limitation of recombinant enzymes or transfected cells in drug metabolism studies is that extrapolation of the results to humans requires taking into account specific factors, such as differences in membrane compositions between expression models and hepatocytes, the absence of competing enzymes, and, above all, the relative expression level of enzyme isoforms in the liver. To overcome this limitation, we used the relative activity factor (RAF) approach, which is proposed for scaling enzymatic activities obtained using cDNA-expressed enzymes to human microsomes *(Crespi and Miller, 1999; Rouguieg et al., 2010; Toide et al., 2004)*. This approach allows the extrapolation of recombinant enzyme formation rates to native human liver enzyme activity. We calculated RAFs for UGT1A3, 1A4, 1A9, and 2B7, to scale velocities obtained using UGT BD-Supersomes to HLM. Using this approach makes our results more relevant than those

published by Foti and Fisher, (2005). The RAF approach predicted a limited role of UGT1A3 and 1A4 (~ 3%) in EtG hepatic production, and no activity was found with UGT1A1, 1A6, and 1A10 (EtG formation rate < LOQ). However, RAFs could not be calculated for other UGT isoforms because no adequate probe has been identified to date. It is noteworthy that several other hepatic and extra-hepatic UGTs (including UGT1A5, 2B10, 2B11, 2B28, 2A1, 2A2, and 2A3) were not studied here, because they are not commercially available as recombinant enzymes. However, as their expression in human liver, kidney, and intestine, is low, their contribution to ethanol metabolism is likely to be minor *(Court et al., 2012; Ohno and Nakajin, 2009; Sneitz and others, 2009).*

These preliminary experiments were necessary to then consider the study of factors potentially involved in the variability of EtG production, such as UGT genetic polymorphisms, or other non-genetic factors, such as age, gender, and the impact of co-consumed drugs on the production of EtG.

In our work, HLMs were prepared from liver samples collected in a pathological department from surgical specimens. During their recruitment, each specimen was examined macroscopically and any signs of liver abnormality (such as steatosis) led to their exclusion. However, it is worth to note that many other variables, which could influence liver enzyme activity and, consequently, ethanol metabolism, were outside of our control. Having not enough characteristic information on the donors, such as body mass index, smoking, liver cirrhosis, kidney disease, medication prior to surgery, and ethanol consumption, is considered as a limitation of this study.

As a part of this work, we also evaluated the distribution of ethanol hepatic glucuronidation *in vitro* using 43 HLMs, which were individually incubated with 250 mM of ethanol. Our results showed a normal distribution, but with a high interindividual difference between extreme values. This variability is in concordance with that reported *in vivo* by Halter *et al.,* (2008). Several genetic and/or non-genetic factors could be responsible for this variability. We

showed that neither age, nor gender seems to affect EtG production by HLM. These data confirm that EtG concentration interpretation does not require any gender or age adjustments. These findings approve the trends previously reported *(Kharbouche et al., 2012; Morini et al., 2009)*. In the first study, Kharbouche *et al*., (2012) assessed the relevance of hair EtG, as a marker of alcohol consumption in a well-characterized population, which consisted of one hundred and twenty-five subjects who were classified according to their self-reported alcohol consumption. By using a univariate regression analysis of age and gender, they found that none of these variables affected the diagnostic performance of EtG in identifying chronic and excessive alcohol consumption *(Kharbouche et al., 2012)*. In the second study, Morini *et al*., (2009) analysed hair samples from 98 volunteers among teetotallers, social drinkers, and heavy drinkers, whose ethanol daily intake was estimated by means of a written questionnaire. They showed that neither age nor gender was found to significantly influence marker performance *(Morini et al., 2009)*.

The catalytic activity of UGT enzymes, particularly of UGT1A9 and 2B7, is highly variable in the general population, and these enzymes are well known to be polymorphic enzymes *(Argikar et al., 2008; Guillemette, 2003)*. Functional polymorphisms in *UGT1A9* and/or *UGT2B7* genes may therefore be important determinants of EtG production. However, up to now, no study has evaluated the potential impact of UGT genetic polymorphisms on the glucuronidation of ethanol. Our work is the first report assessing the influence of genetic polymorphisms on ethanol hepatic glucuronidation. Altogether, our metabolic experiments allow us to narrow down the investigation of candidate genetic polymorphisms with potential consequences on ethanol glucuronidation. In our research, two different approaches were used: in the first one, the impact of 4 known SNPs on the *in vitro* production of EtG was investigated using 43 genotyped HLM. These polymorphisms were previously shown to affect either expression or activity of the encoded enzyme *(Bernard and Guillemette, 2004; Bernard et al., 2006; Court et al., 2003; Djebli et al., 2007; Duguay et al., 2004; Girard et*

al., 2006, 2004). Their allelic frequencies in our population were comparable to those previously reported in Caucasians, and were sufficiently high to be studied considering the limited number of HLM. We found that 2 *UGT1A9* SNPs (-275T>A and IVS1+399T>C) affect the formation rate of EtG. The presence of the -275A allele was associated with higher EtG metabolic rate, whereas the presence of the +399T allele was associated with lower EtG metabolic rate in HLM. Neither *UGT1A9* -440C>T, nor *UGT2B7* -900G>A SNP, seem to influence the *in vitro* glucuronidation of ethanol. The clinical relevance of these genetic variants of UGT2B7 and UGT1A9 remain to be assessed by further *in vivo* studies.

In the second approach, in order to increases the probability of detecting functional *UGT1A9* and *UGT2B7* polymorphisms; we investigated other potential sequence variations by using a PCR-sequencing strategy. In total, 10 different SNPs were identified in the *UGT1A9* gene, comprising 9 in the 5'-regulatory region and 1 in exon 1. A total of 13 SNPs were identified in the *UGT2B7* gene, 4 of them (in exon 4) corresponding to novel polymorphisms. Three SNPs were identified in the 5'-regulatory region and ten within the exonic sequences (exon 1, 2, and 4). No polymorphism was found in exons 3, 5 and 6. However, as the frequency of most of these SNPs is relatively low in Caucasians, larger studies are needed to investigate their potential contribution to the variability of EtG levels. Consequently, our sequencing results did not identify any characteristic SNP for samples with low or high rate of EtG production. The restricted number of HLM studied (n = 43) is an obvious weakness of our study.

Several illegal and/or medicinal drugs are eliminated by glucuronidation *(Chen et al., 2010; Chung et al., 2008; Coffman et al., 1997; Court et al., 2002; Mazur et al., 2009),* and may alter the production rate of EtG by enzyme inhibition or induction. These potential interactions have not received enough attention. As a part of our work, we evaluated, for the first time, the impact of the co-incubation of eight compounds (morphine, codeine, lorazepam, oxazepam, cannabidiol (CBD), cannabinol (CBN), nicotine, and cotinine) on HLM-catalyzed

ethanol glucuronidation. No modification in EtG formation rate was observed when ethanol was incubated with morphine, codeine, nicotine, and cotinine. Lorazepam and oxazepam produced a minor, but not significant, increase of EtG formation by HLM. Interestingly, CBD and CBN significantly affected ethanol glucuronidation. CBN significantly increased the glucuronidation of ethanol in a concentration-dependent manner, whereas CBD significantly inhibited EtG production. We completed these results by an inhibition kinetics, which demonstrated a noncompetitive inhibition mechanism.

The observed increase in EtG formation by HLM after incubation with CBN appears to result from the modulation of UGT2B7 activity. It has been previously demonstrated that enzyme activation might result from heterotropic activation, which has been observed with UGT2B7 *(Uchaipichat et al., 2008)*. For cytochrome P450, such activation has become widely accepted; it may be due to the binding of multiple molecules to the enzyme, either within the active site *(Domanski et al., 2000; Korzekwa et al., 1998; Shou et al., 1994)*, or at separate, distant locations on the enzyme *(Schwab et al., 1988; Ueng et al., 1997)*. However, the precise mechanism of interaction between UGT2B7-catalyzed CBN and ethanol glucuronidation remains to be further examined. The main limitation inherent to this study is that tested substance concentrations were very likely exceeding expected *in vivo* concentrations. For this reason, the impact of benzodiazepine consumption, as well as the contradictory effects of CBN and CBD, need to be further investigated, as the simultaneous consumption of these substances with ethanol is very common *(Patton et al., 1995)*.

In the literature, only two studies have dealt with this question. In the first one, EtG concentrations in hair of non-users of drugs were shown to be significantly greater than those found in drug users *(Paul et al., 2008)*. The authors mentioned that their results add considerable support to the anecdotal belief that the alcohol consumption of non-drug users is higher than the alcohol consumption by drug users. However, in a second study, tetrahydrocannabinol (THC) use was associated with an increase of urinary EtG levels. The authors suggested that the association between THC use and increased EtG concentrations

simply reflects that THC users also tend to drink more ethanol than non-users *(Wurst et al., 2004)*.

In order to further elucidate and clarify these interacting factors, the last part of our work was aimed to investigate, for the first time, the impact of *ante-mortem* consumption of cannabis and other illegal and/or medicinal drugs on EtG levels in *post-mortem* blood samples. EtG concentrations were determined in peripheral blood samples (n = 117) received for toxicological analysis in the period 2010–2013. Samples were classified into five groups according to the presence of alcohol, cannabis, and other illegal and medicinal drugs (antidepressants, antipsychotics, sedatives, hypnotics, analgesics, etc.). Ethanol concentrations, EtG concentrations, and the ratio of EtG/EtOH concentrations were compared among all tested groups. Although the concentrations of ethanol in the second group (E) were significantly higher than those in the third group (E/D) (Mann-Whitney U test, P=0.045), the concentrations of EtG were slightly, but not significantly, higher in group 3 compared to group 2. EtG concentrations did not seem to be affected by cannabis consumption. Furthermore, we compared the [EtG]/[EtOH] ratios among tested groups using the Kruskall-Wallis test and found significant differences ($P < 0.05$). Specifically, these ratios in the drug-positive group were significantly higher than those found in the alcohol consumer group (P=0.0036), as well as in the alcohol and cannabis consumer group (P=0.0107), but not to those found in the alcohol, cannabis, and drugs consumers (P=0.1571). No significant difference in [EtG]/[EtOH] ratios was found neither between alcohol consumers and alcohol and cannabis consumers, nor between alcohol consumers and alcohol, cannabis and drugs consumers. These preliminary results strongly suggests that drugs consumption increase the formation levels of EtG, and that cannabis consumption does not seem to have any significant effect on EtG levels according to our inclusion criteria. Hoiseth *et al.*, (2007) have already used the ratios between EtG and ethanol concentrations in order to evaluate the usefulness of the measurement of EtG in *post-mortem* blood samples, as a marker of *ante-mortem* alcohol

ingestion *(Høiseth et al., 2007b)*. When comparing between two groups with low and high BAC, they found that this EtG/ethanol ratio was significantly higher in the group with low BAC. Thus, we do not have a clear explanation for our discrepant findings. The association between drug use and increased EtG concentrations may simply be due to a higher ethanol intake in this group before death. However, interestingly, in the drug-positive group, benzodiazepines were present in 75% of tested samples (26 out 35). We showed previously that benzodiazepines produced a minor, but not significant, increase of EtG formation using human liver microsomes. Thus, this observation in *post-mortem* samples deserve to be further investigated in order to verify if benzodiazepines are responsible of this effect. Our results are in discordance with data published by Paul *et al.,* (2008), who found that the mean concentration of EtG was significantly higher in a drug-negative group than that found in a drug-positive group *(Paul et al., 2008)*. However, the context and the studied populations in our study are different.

We are aware that this study has many limitations. Although putrefaction of corpse was an exclusion criteria, endogenous formation of ethanol could not be totally excluded. Moreover, we did not have any information about the quantity of ingested ethanol and the time interval between last drinking and death. However, this latter limitation could be managed by the fact that the time difference between total elimination of ethanol and EtG in blood is quite short *(Schmitt et al., 1997)*. The low number of cases in each studied group was also another weakness. Consequently, the relevance of this interaction on EtG formation remains uncertain and needs to be further investigated.

PERSPECTIVES & CONCLUSION

From our data, there are several open questions concerning factors potentially affecting EtG production *in vivo*. The first issue is the contribution of UGT1A9 and 2B7 genetic polymorphisms, as well as of cannabis and/or drugs consumption, to the interindividual variability of EtG production and, consequently, to the relevance of its proposed cut-offs in clinical and forensic practices. It might be of great interest to know which factors have to be taken into consideration for EtG interpretation, but our work did not provide a definitive conclusion.

Throughout this work, the use of *in vitro* models (HLM and recombinant enzymes) has allowed us to answer a number of questions, to develop or support new hypotheses that need to be subsequently tested clinically. Concerning genetic polymorphisms, *UGT1A9* -275T>A and IVS1+399T>C SNPs could potentially be of interest when interpretating EtG concentrations. Cannabis and other drug consumption could also alter the EtG formation rate. However, the precise mechanism behind these effects is not definitely clarified in our work. There are other potential factors of variability which were not considered in this work and need to be further assessed. Multiple-factors and controlled studies, involving both genetic and non-genetic factors, may help to make further progress towards a better interpretation of EtG concentrations.

At the beginning of this work, we also raised the question of the variability factors affecting EtS production. It was originally proposed to carry out the same experiments on EtG and EtS together. However, because of methodological and technical reasons, our work was limited to the study of EtG. In all biological matrices, EtS provides an excretion profile relatively comparable to that of EtG. It is worth to note that the simultaneous urinary analysis of EtG and EtS increases the sensitivity of the investigation. It is reported that EtG, but not EtS, can

be produced after sampling if samples are infected with bacteria (e.g. *E. coli, E. cloacae, K. pneumonia*, or *Clostridium sordelli*) *(Alt et al., 1997; Helander and Dahl, 2005)* and contain ethanol formed by fermentation. Thus, testing for EtG and EtS in parallel, which is not more time-consuming than EtG alone, could minimize false identification of alcohol consumption and possibly identify a possible alteration of metabolism of ethanol. Therefore, it is recommended for clinical and forensic practice. Nevertheless, the identification of the human SULT isoforms involved in ethanol sulfoconjugation reaction, the impact of genetic polymorphisms affecting implicated SULT enzymes, and the potential interactions with other drugs on this metabolic pathway still need to be further studied. Furthermore, the determination of reliable and consensual cut-off values for EtS is still premature and need more investigations.

In conclusion, EtG detection in different biological matrices is a reliable marker of alcohol consumption. It can improve the knowledge of drinking patterns by differentiating moderate/social drinkers from problematic/harmful drinkers. It also helps to support differential diagnosis (e.g. elevated transaminases, elevated GGT and/or CDT, *post-mortem* formation of ethanol), to evaluate treatment programs and drug trials, and to elucidate the role of neuropsychological impairment after alcoholization (i.e. hangover state).

Although further work on factors of EtG interindividual variability is needed, our preliminary data suggest that interpretation of EtG concentrations should be made cautiously, especially in a forensic context.

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Intérêt de l'éthylglucuronide comme marqueur d'alcoolisation : développement de méthodes de dosage et étude des sources de variabilité de sa production

Définitions & épidémiologie de l'éthylisme

L'alcoolisme (au sens d'éthylisme) est la conséquence de la consommation régulière et excessive de boissons alcooliques ou alcoolisées. L'Organisation Mondiale de la Santé (OMS) en donne la définition suivante : « Les alcooliques sont des buveurs excessifs dont la dépendance à l'égard de l'alcool est telle qu'ils présentent soit un trouble mental décelable, soit des manifestations affectant leur santé physique ou mentale, leur relation avec autrui et leur bon comportement social et économique, soit des prodromes des troubles de ce genre. Ils doivent être soumis à un traitement ». En réalité, il n'existe pas de définition « scientifique et médicale » précise et consensuelle de l'alcoolisme, mais il est habituel de considérer que la consommation de boissons alcoolisées devient excessive lorsqu'elle dépasse 3 verres (soit 30 grammes d'éthanol) par jour. D'un point de vue médical, il est usuel de considérer qu'un patient souffre d'alcoolisme chronique (« alcoolo-dépendance ») lorsqu'il consomme plus de 50 grammes d'éthanol par jour. Pour certains auteurs, une consommation excessive et chronique d'alcool est avérée lorsque celle-ci atteint ou dépasse 21 verres « standards » par semaine chez l'homme et 14 chez la femme.

L'abus d'alcool est un problème majeur de santé publique bien connu pour les nombreuses pathologies et les problèmes sociaux qu'il cause à travers le monde. En 2004, l'OMS a estimé que plus de 2 milliards de personnes dans le monde consommaient des boissons alcoolisées, dont plus de 76 millions souffraient de troubles dûs à l'abus d'alcool *(WHO report, 2004)***.**

L'impact de la consommation excessive d'alcool sur la santé en France demeure élevé, en termes de mortalité, de morbidité et de dommages sociaux. Au total, les problèmes liés à l'alcoolisme touchent environ 5 millions de personnes, dont environ 2 millions sont

dépendantes. L'Institut de cancérologie Gustave Roussy (IGR) estimait à 49000 le nombre de décès attribuables à l'alcool en 2009, ce qui en faisait la deuxième cause de mortalité évitable après le tabac, avec 15000 décès dus à un cancer, 12000 à une maladie cardiovasculaire, 8000 à une pathologie digestive, 8000 à une cause externe (accident, chute, suicide, homicide), 3000 liés à des troubles mentaux et comportementaux, et enfin 3000 dûs à des causes diverses *(Guérin et al., 2013)*. Les décès attribuables à l'alcool étaient de 22 % et 18 % de la population âgée de 15 à 34 ans et de 35 à 64 ans, respectivement, comparativement à 7 % chez les personnes âgée de 65 ans ou plus *(Guérin et al., 2013)*. Par ailleurs, le syndrome d'alcoolisation fœtale concerne chaque année entre 5 et 7 000 nouveau-nés *(Kintz et al., 2009)*.

Au vu de ces données épidémiologiques et des conséquences sanitaires et sociales de l'éthylisme, il est donc important de disposer de marqueurs, sensibles et spécifiques, permettant une meilleure évaluation de la consommation d'éthanol. En tant que métabolite direct de l'éthanol, l'éthylglucuronide (EtG) présente une grande spécificité. Il est détectable dans l'organisme uniquement à la suite d'une consommation d'éthanol. Son apparition rapide, sa détection pendant une longue période après l'élimination complète de l'éthanol, et sa particularité de se fixer dans les cheveux, font de l'EtG un marqueur pertinent et prometteur en toxicologie clinique et médico-légale.

Marqueurs biologiques de l'alcoolisme

Ces dernières années, la recherche a porté sur l'utilisation de marqueurs permettant simultanément l'identification du profil de consommation d'un sujet et un diagnostic spécifique et sensible. Différents types de marqueurs sont communément utilisés pour identifier une consommation d'alcool. On distingue ainsi des marqueurs indirects et des marqueurs directs de la consommation d'éthanol :

- Les marqueurs indirects sont issus de modifications métaboliques et/ou biochimiques liées à la consommation régulière et excessive d'éthanol (souvent en relation avec une souffrance hépatocytaire) : volume globulaire moyen (VGM), activité plasmatique de la gamma glutamyl transférase (GGT) et teneur en transferrine déficiente en carbohydrate (CDT) dans le plasma.

- Les marqueurs directs sont principalement constitués de l'éthanol lui-même et de métabolites mineurs de l'éthanol, tels que l'EtG que l'on peut rechercher notamment dans le sérum, les urines et les cheveux.

Métabolisme de l'éthanol

Après ingestion, 90 à 95 % de l'alcool éthylique absorbé est métabolisé par réactions d'oxydation, principalement au niveau hépatique, via l'alcool- et l'aldéhyde-déshydrogénase (ADH et ALDH respectivement), le cytochrome P450 CYP2E1 et la catalase *(Fraser, 1997; Pawan, 1972)* (Fig. 5). L'ADH est une enzyme cytosolique qui catalyse la formation de l'acétaldéhyde ou aldéhyde acétique. L'ALDH est une enzyme à la fois cytosolique et mitochondriale, responsable de 95 % de la transformation de l'acétaldéhyde en acétate. Une proportion mineure de l'éthanol ingéré est également excrétée, sous forme inchangée, par voie rénale, pulmonaire et cutanée.

Les autres voies métaboliques de l'éthanol sont accessoires et représentent généralement moins de 5 % du métabolisme total de l'éthanol. Elles aboutissent cependant à la formation de marqueurs « directs » potentiellement intéressants dans le dépistage de l'éthylisme. La biotransformation de l'éthanol en EtG par conjugaison avec l'acide glucuronique activé ne représente que 0,02 à 1,5 % de son élimination totale *(Goll et al., 2002)*. Cette réaction de conjugaison est catalysée par des UDP-Glucuronyl Transférases (UGT), enzymes pour lesquelles un polymorphisme génétique à l'origine de variations fonctionnelles a été décrit *(Miners et al., 2002)*. La sulfoconjugaison de l'éthanol, *via* des sulfotransférases, est à

l'origine d'un autre métabolite « de phase II » de l'éthanol : l'éthylsulfate (EtS) *(Schneider and Glatt, 2004)*. Enfin, l'action des enzymes FAEE-synthétase et acylCoA-Ethanol-O-Acyltransférase est à l'origine d'une autre voie mineure du métabolisme de l'éthanol qui aboutit à la formation d'éthyl-esters d'acides gras (ou FAEE pour *Fatty Acid Ethyl Esters*). Les FAEE sont également considérés par certains auteurs comme intéressants dans le suivi et le diagnostic de l'éthylisme *(Kinnunen and Lange, 1984;Kaphalia and Ansari, 2001; Laposata and Lange, 1986)*.

Marqueurs indirects

Marqueurs classiques et conventionnels: VGM, GGT, ASAT, ALAT ...

Le VGM, la GGT et les aminotransférases (l'alanine aminotransférase (ALAT) et l'aspartate aminotransférase (ASAT)) sont des paramètres anciens, largement utilisés comme marqueurs de consommation et de surveillance de la désintoxication alcoolique. Deux informations conditionnent leur pertinence : spécificité et délai de normalisation après abstinence. L'augmentation du volume globulaire moyen (VGM) au-dessus de 98 fl (normales entre 82 et 98 fl) constitue un argument en faveur d'une consommation chronique d'éthanol. Le VGM peut augmenter (macrocytose) chez certains patients qui ont une carence en vitamine B12 et/ou folates ou des troubles de la lignée érythroblastique. La spécificité est de l'ordre de 40 à 90 % et la normalisation après abstinence se fait en 10 à 12 semaines.

La gamma glutamyl transférase (GGT) est une enzyme inductible par l'éthanol, mais aussi par certains médicaments (barbituriques, phénytoïne, imipraminiques, antihypertenseurs, contraceptifs oraux...). La GGT est élevée dans 35 à 90 % des cas d'alcoolisme, mais aussi dans toutes les pathologies hépatobiliaires. L'abstinence permet un retour à la normale sous une quinzaine de jours. Les aminotransférases, l'ASAT et l'ALAT, sont des marqueurs d'une souffrance hépatique. Localisées dans les hépatocytes périportaux, leur élévation sérique

traduit une altération de la membrane cellulaire. Pour la détection de l'éthylisme chronique, leur détermination est d'un intérêt limité.

L'ASAT est un mélange de 2 iso-enzymes, chacune étant codée par un gène distinct. L'iso-enzyme la plus importante est l'isoforme mitochondriale (ASATm) dont l'activité représente environ 80 % de l'activité aspartate aminotransférase totale (ASATt) au niveau hépatique ; les 20 % restants sont constitués par l'autre iso-enzyme, d'origine cytosolique (ASATc). Par contre, au niveau sérique, l'ASATm ne représente qu'une faible partie (moins de 10 %) de l'activité ASATt. Chez les alcooliques, la fraction sérique ASATm est augmentée (du fait de l'action toxique directe de l'éthanol sur les mitochondries) et il a été proposé que le rapport ASATm/ASATt puisse être un critère de discrimination entre une population de buveurs, qu'ils aient ou non une hépatopathie, d'une population sobre ayant un foie sain ou non. Si ce test a fait ses preuves sur des patients hospitalisés, les conclusions semblent plus réservées sur la population générale. Le rapport ASATm/ASATt se normalise en 15 jours au cours de l'abstinence. Enfin, la mesure de l'alpha gluthation S-transférase, augmentée lors de l'intoxication alcoolique aiguë, ne semble pas pertinente pour mettre en évidence une atteinte hépatique liée à l'alcool. La mesure de l'acétate sanguin, métabolite final de l'oxydation hépatique de l'alcool, permet en théorie de distinguer un alcoolisme chronique (augmentation de la concentration par induction enzymatique) d'une alcoolisation aiguë (qui n'affecte que très peu la concentration).

Transferrine déficiente en carbohydrates

La transferrine est une glycoprotéine synthétisée par le foie et impliquée dans le transport du fer. Elle possède 2 chaînes polysaccharidiques plus ou moins ramifiées (de 0 à 6) avec des résidus d'acide sialique. La forme tétrasialo-transferrine (n = 4) est majoritaire, et seules les formes asialo- (n = 0), monosialo- (n = 1) et disialo-transferrine (n = 2) sont augmentées en

cas d'exposition prolongée à l'éthanol, ce qui conduit davantage à la formation d'isoformes de la transferrine déficiente en carbohydrates (CDT ou formes avec 0, 1 ou 2 résidus). Chez un sujet sobre, le taux de CDT est de l'ordre de 2 %, chiffre qui va rapidement augmenter lorsque la consommation d'éthanol pur est supérieure à 50 g par jour pendant au moins 8 jours. La demi-vie de la CDT est de l'ordre de 17 jours, ce qui en fait un marqueur intéressant dans les cas d'alcoolisme intermittent. C'est un marqueur très sensible pour repérer une rechute chez les personnes alcoolo-dépendantes. Après 3 semaines d'imprégnation éthylique, le dosage de la CDT est normalisé. La sensibilité de ce test est estimée en moyenne à 70 %.

La spécificité de la CDT est supérieure à celle observée pour les marqueurs classiques, mais quelques facteurs sont susceptibles de conduire à une augmentation de la CDT, sans exposition à l'éthanol, comme les carences martiales et la grossesse.

Marqueurs directs

Éthanol

La présence de la molécule elle-même, l'éthanol, est un marqueur spécifique d'une consommation récente. Du fait de sa demi-vie relativement courte dans l'organisme, son utilisation est limitée en pratique au diagnostic de l'alcoolisation aiguë et non de l'abus chronique d'alcool. La concentration sanguine d'éthanol maximale est obtenue 30 minutes à une heure après l'ingestion d'éthanol, puis l'élimination à partir du sang s'effectue en moyenne à raison de 0,10 à 0,15 g/L/heure chez un buveur occasionnel et 0,20 à 0,40 g/L/heure chez un buveur chronique. L'éthanol lui-même constitue donc, bien évidemment, un marqueur direct d'une consommation récente de boissons alcoolisées. Toutefois, il ne permet pas de se prononcer sur l'éventualité, ou non, de consommations importantes et régulières d'éthanol au cours des jours ou des semaines précédant le prélèvement sanguin.

Les Ethyl-Esters d'Acides Gras

Les esters éthyliques d'acides gras (FAEE) sont des métabolites mineurs de l'éthanol formés par conjugaison de l'éthanol aux acides gras libres, triglycérides, lipoprotéines ou phospholipides. Cette réaction est principalement catalysée par l'enzyme FAEE-synthétase présente au niveau hépatique, mais également au niveau du pancréas, du cœur, du tissu adipeux, du cerveau et des globules blancs (*Laposata and Lange, 1986*). Ces métabolites sont suspectés d'être responsables, en partie du moins, des atteintes organiques liées à l'alcool.

Dans le sang, les FAEE apparaissent rapidement après ingestion de boissons alcoolisées et leur dosage permet la détection d'une prise aiguë, immédiate ou récente d'éthanol jusqu'à environ 24 heures après élimination complète de l'éthanol par l'organisme (*Doyle et al., 1996)*. Plus de 15 FAEE ont été identifiés et il apparaît que les concentrations de 4 d'entre eux sont intéressantes pour le diagnostic et/ou le suivi d'un alcoolisme chronique : éthyl-myristate, éthyl-palmitate, éthyl-oléate et éthyl-stéarate. Une valeur « seuil » de 400 pg/mg de cheveux pour la somme de ces 4 FAEE a été proposée pour distinguer une consommation excessive et chronique d'éthanol d'une consommation modérée, avec une sensibilité de 100 % et une spécificité proche de 95 %. Des travaux récents ont cependant démontré que l'utilisation quotidienne de certains traitements capillaires, en particulier shampooings, laques et surtout lotions capillaires alcoolisées, pouvait affecter les résultats observés et conduire à des « faux-positifs ». La chromatographie en phase gazeuse couplée à la spectrométrie de masse est communément utilisée pour doser ces FAEE. Il n'existe pas de test immuno-chimique pour les FAEE.

Phosphatidyléthanol

Le phosphatidyléthanol est formé par action de la phospholipase D en présence d'éthanol. Il s'agit donc d'un marqueur spécifique, dont la normalisation est de l'ordre de 15 jours après le début de l'abstinence.

L'Ethylsulfate

L'éthylsulfate (EtS) est un métabolite mineur de l'éthanol, formé par transfert d'un groupement sulfate provenant de la 3'phosphoadenosine-5'phosphosulfate sur l'éthanol, réaction catalysée par les sulfotransférases hépatiques (SULT) 1A1 et 1A2 *(Wurst et al., 2006)*. L'EtS présente un profil d'excrétion urinaire relativement comparable à celui de l'EtG avec une apparition dans les urines environ 1 heure après ingestion de boissons alcoolisées, un pic 4 à 5 heures après celui de l'éthanol et une fenêtre de détection de l'ordre de 30 heures *(Wurst et al., 2006)*. Il n'existe pas de test immuno-chimique pour l'EtS.

Éthylglucuronide

En tant que métabolite direct de l'éthanol, l'éthylglucuronide (EtG) présente une grande spécificité. Il n'est détectable dans l'organisme que suite à une consommation d'alcool. Son apparition rapide, sa présence dans l'organisme bien après l'élimination de l'éthanol, et sa particularité de se fixer dans les cheveux, font de l'EtG un marqueur prometteur en toxicologie médico-légale et clinique.

L'EtG résulte de la conjugaison de l'éthanol avec l'acide UDP-glucuronique (UDP-GA). Cette réaction est catalysée par une famille d'isoenzymes, les UDP-glucuronyl transférases (UGT). Elle se déroule principalement dans le réticulum endoplasmique des cellules hépatiques, mais également, dans une moindre mesure, dans les cellules des muqueuses intestinale et pulmonaire.

L'EtG est majoritairement retrouvé dans l'urine, le foie, la bile et le sang. Il est également retrouvé en moindre quantité dans le liquide céphalorachidien, la moelle osseuse, le tissu musculaire, le tissu adipeux, le cerveau et les cheveux *(Høiseth et al., 2007b; Schloegl et al., 2006b; Wurst et al., 1999b)*.

L'EtG apparaît dans le sang moins de 45 min après une consommation d'éthanol. Sa concentration maximale est atteinte après environ 3,5 heures. L'EtG y est détecté jusqu'à 17 heures après une consommation d'éthanol *(Halter et al., 2008; Høiseth et al., 2007a; Schmitt et al., 1997)*. L'EtG apparaît dans l'urine moins d'une heure après une consommation d'éthanol. Sa concentration maximale dans l'urine est atteinte après 5,5 heures *(Dahl et al., 2002; Halter et al., 2008; Høiseth et al., 2007a)*. L'élimination de l'EtG est essentiellement rénale. La quantité d'EtG excrétée dans les urines représente moins de 0,1 % de la dose d'éthanol ingérée. L'EtG y est détectable jusqu'à 21 heures après une faible consommation d'éthanol et plus de 5 jours après une consommation élevée d'éthanol. La concentration maximale et la fenêtre de détection de l'EtG sont dépendantes de la dose d'éthanol ingérée et du métabolisme de chaque individu.

Intérêt de l'éthylglucuronide

L'EtG apparaît rapidement dans le sang et l'urine où il est détectable même suite à une faible consommation d'alcool (10 g d'alcool ou un verre standard). L'EtG est éliminé beaucoup plus lentement que l'éthanol. Il présente donc l'intérêt majeur d'augmenter la fenêtre de détection observée avec l'éthanol. Après l'élimination complète de l'éthanol hors de l'organisme, l'EtG est encore détectable jusqu'à environ 8 h dans le sang et 80 h dans les urines. La présence de l'EtG dans le sang ou dans l'urine permet donc d'objectiver une consommation récente d'alcool même lorsque que l'éthanol n'est plus présent dans l'organisme.

L'utilisation de l'EtG comme marqueur d'une consommation chronique d'éthanol a été rendue possible dès lors que son accumulation dans les cheveux a été démontrée *(Sachs, 1997)*. L'utilisation des cheveux en tant qu'échantillon biologique permet d'augmenter de façon considérable la fenêtre de détection. L'EtG est ainsi détectable plusieurs semaines, voire plusieurs mois, en fonction de la longueur des cheveux. La détermination de la

concentration d'EtG dans les cheveux permet ainsi le suivi de la consommation d'alcool sur une longue période.

Jusqu'à aujourd'hui, seule l'analyse d'échantillons de cheveux provenant d'individus ayant consommé de très grosses quantités d'alcool permettait d'obtenir un résultat positif pour l'EtG. Avec l'amélioration de la sensibilité des méthodes analytiques, notamment la chromatographie liquide ou gazeuse couplée à la spectrométrie de masse en tandem, la détection de l'EtG a depuis été rendue possible dans les cheveux de consommateurs même « modérés ». A ce jour, l'EtG n'a jamais été détecté dans les cheveux d'abstinents. Sa présence dans les cheveux reflète immanquablement une consommation d'alcool. Pour éviter toute ambiguïté, un seuil de 7 pg/mg a été proposé par la « Society of Hair Testing » *(SoHT report, 2012)* en dessous duquel une consommation d'alcool peut être écartée.

Le pouvoir discriminant de l'EtG repose sur sa particularité de pouvoir différencier une consommation dite « sociale » ou à faible risque d'une consommation excessive et chronique d'alcool. Dans ce but, un seuil de positivité de 30 pg/mg, basé sur l'élimination des faux positifs, a aussi été proposé par la « Society of Hair Testing » *(SoHT report, 2012)* afin de distinguer ces deux types de consommateurs. Il a été établi que des concentrations d'EtG supérieures à ce seuil traduisaient une consommation excessive, alors que des concentrations d'EtG inférieures à ce seuil indiquaient une consommation à faible risque. En tenant compte de ce seuil, on diminue le risque que ces derniers soient considérés, à tort, comme consommateurs excessifs et chroniques; ce qui augmente ainsi la spécificité.

Travaux personnels (Résultats)

Ces travaux de recherches s'inscrivent donc dans un cadre à la fois et clinique et médicolégal, et visent ainsi à valider l'utilisation de l'EtG comme marqueur d'alcoolisation, marqueur qui tend à supplanter la quasi-totalité des autres marqueurs conventionnels utilisés jusqu'ici pour l'évaluation qualitative et quantitative de la consommation d'alcool.

Les objectifs de nos travaux ont ainsi consisté à (1) développer et valider une méthode de dosage de l'EtG dans différentes matrices biologiques par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem, (2) identifier les UGT humaines impliquées dans la glucuronoconjugaison de l'éthanol et étudier leur contribution relative au niveau hépatique, (3) évaluer l'impact de la consommation de cannabis et d'autres drogues sur la production d'EtG *in vitro* et à l'aide de prélèvements *post-mortem*, et enfin (4) étudier l'impact de polymorphismes génétiques fonctionnels des UGT sur la production hépatique d'EtG.

La première partie de nos travaux, consacrée aux aspects expérimentaux, concerne la mise au point d'une méthode de dosage de l'EtG dans les matrices urinaire et sérique par chromatographie gazeuse couplée à la spectrométrie de masse en tandem (GC-MS/MS). Cette partie a fait l'objet d'une publication parue dans les Annales de Toxicologie Analytique en 2011. Ces travaux constituent la première utilisation d'un tel équipement (GC-MS/MS) dans ce cadre précis, permettant ainsi une amélioration significative des limites de détection et de quantification par rapport aux méthodes de dosage déjà proposées.

La deuxième partie des travaux expérimentaux, qui a également fait l'objet d'une publication, a consisté en l'identification précise des isoformes d'UDP-glucuronyltransférases (UGT) humaines impliquées dans la glucuronoconjugaison de l'éthanol, ainsi que sur l'étude de la contribution relative de chaque isoforme active au niveau hépatique.

Ce travail a été réalisé à l'aide de préparations microsomales d'origine humaine (hépatiques (MFH), rénales (MRH) et intestinales (MIH)) et d'UGT humaines recombinantes (Supersomes® ; Gentest). Les résultats des études de cinétique enzymatique, réalisées à l'aide de pools de microsomes, ont montré que les trois organes étudiés produisent de l'EtG avec une très faible affinité, ce qui est cohérent avec la contribution mineure de la glucuronoconjugaison dans le métabolisme global de l'éthanol chez l'homme. La clairance intrinsèque (Cl_{int}) des MFH est 4 fois et 12,5 fois plus élevée que celles observées avec les MRH et les MIH, respectivement. Ce résultat suggère ainsi que l'éthanol est, quantitativement, principalement glucuronoconjugué par le foie, puis par les reins et que l'intestin joue un rôle mineur dans cette voie métabolique.

L'incubation avec les principales UGT recombinantes humaines (UGT1A1, 1A3, 1A4, 1A6, UGT1A7, 1A8, 1A9, 1A10, 2B7, 2B4, 2B15 et 2B17) a montré que toutes les isoformes testées, excepté les UGT1A1, 1A6 et 1A10, sont capables de produire de l'EtG en quantités détectables. De plus, ces travaux, publiés dans la revue Drug Metabolism and Disposition en 2013, ont mis en évidence que l'essentiel de la production d'EtG à partir d'éthanol est réalisé au niveau hépatique (en comparaison des intestins et reins) et ont montré au travers de tests *in vitro*, que les UGT1A9 et UGT2B7 sont les deux enzymes majoritairement impliquées dans la glucuronoconjugaison de l'éthanol, quel que soit l'organe considéré. Au niveau hépatique, l'UGT1A9 et l'UGT2B7 contribuent respectivement à 17,2 % et 33 % de la production d'EtG. La contribution des UGT1A3 et 1A4 s'élève à environ 3 %. Ces travaux suggèrent ainsi la possibilité que le polymorphisme génétique des UGT, notamment celui des UGT1A9 et 2B7, puisse influencer la formation d'EtG.

Cette étape du travail s'avérait indispensable pour réaliser **la troisième partie de nos travaux** qui correspond à l'étude de l'influence de la consommation de cannabis, d'autres substances illicites et de certains médicaments psychoactifs, sur la glucuronoconjugaison de l'éthanol *in vitro* et à l'aide de prélèvements *post-mortem*.

- Le premier volet de cette troisième partie correspond à une étude de l'impact de la co-administration de huit drogues ou médicaments fréquemment utilisés par les consommateurs d'alcool (opiacés: morphine, codéine; benzodiazépines: oxazépam, lorazépam ; tabac: nicotine, cotinine ; et cannabis: cannabinol, cannabidiol) sur la production d'EtG *in vitro* en utilisant un pool de MFH. Les huit molécules sélectionnées pour ces études d'interaction sont toutes des substrats des UGT. En effet, la consommation de médicaments ou de substances illicites également métabolisés par glucuronoconjugaison est susceptible de modifier la production d'EtG, par des mécanismes d'inhibition ou d'induction enzymatique (interactions d'ordre pharmacocinétique). Divers médicaments sont connus pour être des inhibiteurs de la glucuronoconjugaison *(Kiang et al., 2005)*. Cependant, à notre connaissance, les interactions avec la glucuronoconjugaison de l'éthanol n'ont pas été étudiées jusqu'ici. Paul *et al*. (2008) ont comparé les concentrations d'EtG dans des échantillons de cheveux entre un groupe de consommateurs de stupéfiants ou de psychotropes (opiacés, cocaïne, amphétamines, méthamphétamine, benzodiazépines et cannabis) et un groupe de non-consommateurs. Ces auteurs ont montré, mais sans y apporter d'explication, que les concentrations moyennes d'EtG étaient significativement plus élevées dans le groupe des non-consommateurs *(Paul et al., 2008)*.

Ce travail a été réalisé à l'aide d'un pool de 44 préparations microsomales hépatiques d'origine humaine. Les incubations ont été réalisées à des concentrations d'éthanol de 25, 100 et 250 mM (correspondant à des concentrations de 1,15 ; 4,6 et 11,5 g/L), soit des concentrations plus ou moins proches des concentrations sanguines attendues dans le cadre

d'une consommation aiguë abusive de boissons alcoolisées. Les inhibiteurs potentiels ont été testés à des concentrations allant de 1 à 30 fois les concentrations sanguines habituellement mesurées dans un contexte clinique ou toxicologique.

L'incubation avec les drogues/médicaments testés a révélé que la morphine, la codéine, la nicotine et la cotinine n'entraînent aucune modification des taux de production d'EtG. Le lorazépam et l'oxazépam ont produit une légère augmentation, mais non significative, du taux de formation d'EtG par les MFH ($p = 0,2$ et 0,065, respectivement). Seuls le cannabinol et le cannabidiol affectent significativement la production de l'EtG *in vitro*. Le cannabidiol a ainsi montré une forte inhibition de la glucuronoconjugaison de l'éthanol par un mécanisme non-compétitif (CI_{50} = 1,17 mg/L; K_i = 3,1 mg/L), alors que le cannabinol augmentait cette glucuroconjugaison de manière concentration-dépendante ($p < 0,05$). Le mécanisme de cette induction par le cannabinol reste à étudier. Les résultats concernant l'influence du cannabinol et du cannabidiol, bien que plus difficiles à interpréter et à extrapoler à un organisme complet, montrent également l'influence potentielle de certaines substances fréquemment rencontrées sur le plan médico-légal, dans la formation de l'EtG et attirent l'attention sur les réserves à apporter dans l'interprétation des résultats d'analyse.

- Le deuxième volet de cette troisième partie, basé sur l'analyse d'échantillons sanguins collectés en *post-mortem*, est une étude des relations entre EtG et éthanol sanguin, et plus particulièrement de l'influence potentielle de la consommation *ante-mortem* de « substances illicites », dont notamment le cannabis, et de médicaments, sur la production d'EtG. Cette étude n'a pas réellement permis de mettre en évidence un effet notable de la co-consommation des différentes substances sur la production d'EtG. Néanmoins, le rapport des concentrations EtG/éthanol apparaît significativement plus élevé chez des co-consommateurs de drogues que chez des consommateurs d'alcool seul, ou des co-consommateurs d'alcool et de cannabis. La limitation principale de cette étude réside dans la non-connaissance

(information non disponible) du délai entre la consommation *ante-mortem* d'éthanol et l'heure du prélèvement, ce qui induit fort probablement un biais dans l'interprétation du rapport EtG/éthanol. Des travaux complémentaires sont nécessaires afin de mieux interpréter ces résultats.

La quatrième partie correspond à une étude de la variabilité interindividuelle d'origine génétique de la glucuronoconjugaison de l'éthanol et, plus particulièrement, de l'influence de certains polymorphismes génétiques des UGT1A9 et 2B7 sur la glucuronoconjugaison de l'éthanol. L'intérêt principal de cette étude tient au fait qu'elle ait été réalisée à partir de prélèvements hépatiques humain (n = 43). Cette étude, focalisée sur les UGT1A9 et 2B7, représente un prolongement logique de l'étude préalablement publiée dans Drug Metabolism & Disposition qui avait mis en évidence l'importance de ces deux UGT dans la glucuronoconjugaison de l'éthanol.

La variabilité interindividuelle de la glucuronoconjugaison de l'éthanol a été évaluée par l'incubation de chacune des 43 préparations microsomales hépatiques avec 250 mM d'éthanol. Les résultats ont montré une distribution a priori unimodale, mais ont confirmé néanmoins la large variabilité interindividuelle de la production d'EtG. Afin d'examiner l'hypothèse d'une origine génétique de cette variabilité interindividuelle, le génotypage des *UGT1A9* et *2B7* a été entrepris, avec notamment l'identification de quatre polymorphismes fonctionnels (UGT2B7 -900G>A ; UGT1A9 : IVS1+399T>C, -275T>A et -440C>T) relativement fréquents chez les caucasiens. Afin d'évaluer l'impact de chacun de ces polymorphismes sur le taux de production de l'EtG, nous avons comparé les concentrations moyennes d'EtG en fonction des différents génotypes pour chaque SNP étudié (homozygote sauvage, hétérozygote et homozygote muté) par un test de comparaison de moyennes (Mann-Whitney).

Ce travail a montré que les génotypes pour l'UGT1A9 -440C>T et l'UGT2B7 -900G>A n'entraînent aucune modification significatives des taux de production d'EtG. Les SNP c.-275T>A et IVS1+399T>C affectant l'*UGT1A9* modifient, par contre, significativement le taux de formation d'EtG *in vitro*. Ces résultats préliminaires ont été complétés par la réalisation d'un séquençage complet des *UGT1A9* et *2B7* appliqué en premier lieu aux échantillons qui présentent les valeurs d'activité enzymatique les plus basses et les plus hautes. Cependant, l'interprétation de ce séquençage n'a pas permis d'identifier de SNP ou d'allèles caractéristiques des productions basses et/ou hautes d'EtG. Le nombre restreint de microsomes étudiés est une faiblesse évidente de notre étude et doit être augmenté afin de conclure de manière plus définitive sur l'influence du polymorphisme génétique des UGT1A9 et 2B7 sur la production d'EtG.

Par ailleurs, l'absence d'effet du sexe et de l'âge sur la production d'EtG a été montrée. Ces résultats sont également des éléments particulièrement intéressants qui viennent conforter les résultats publiés antérieurement par d'autres équipes.

Cette étude, mettant en évidence la grande variabilité dans la production d'EtG à partir d'éthanol par les cellules hépatiques provenant de différents individus, apporte des informations concrètes particulièrement pertinentes vis-à-vis de l'utilisation de l'EtG comme biomarqueur de consommation d'alcool.

L'ensemble de ces travaux démontre l'existence de plusieurs facteurs pouvant potentiellement influencer la production d'EtG, et qui devraient donc être pris en considération lors de l'interprétation de sa concentration *in vivo*. Ces travaux confirment cependant le rôle intéressant de l'EtG comme marqueur *post-mortem* d'une consommation *ante-mortem* d'éthanol, et viennent une fois encore renforcer l'intérêt de ce marqueur dans un contexte médicolégal.