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**Abbreviations**: CHZ: Chlorzoxazone ; OH-CHZ: 6-hydroxychlorzoxazone ; CHZ-O-Glc: chlorzoxazone-O-glucuronide ; CHZ-N-Glc: chlorzoxazone-N-glucuronide ; CYP450: cytochrome P450; DMSO: dimethyl sulfoxide ; XMEs: xenobiotic metabolizing enzymes ; UGT: Uridine 5'-diphospho-glucuronosyltransferase ; High Pressure Liquid Chromatography-UV: HPLC-UV ; Liquid-Chromatography-mass spectrometry: LC-MS.

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## ABSTRACT

CYP2E1 activity is measured in vitro and in vivo via hydroxylation of the Chlorzoxazone (CHZ) producing the 6-hydroxychlorzoxazone (OH-CHZ) further metabolized as a glucuronide excreted in urine. Thus, the quantification of the OH-CHZ following enzymatic hydrolysis of CHZ-derived glucuronide appears to be a reliable assay to measure the CYP2E1 activity without direct detection of this glucuronide. However, OH-CHZ hydrolyzed from urinary glucuronide accounts for less than 80% of the CHZ administrated dose in humans leading to postulate the production of other unidentified metabolites. Moreover, the Uridine 5'-diphosphoglucuronosyltransferase (UGT) involved in the hepatic glucuronidation of OH-CHZ has not yet been identified. In this study, we used recombinant HepG2 cells expressing CYP2E1, metabolically competent HepaRG cells, primary hepatocytes and precision-cut human liver slices to identify metabolites of CHZ (300 µM) by High Pressure Liquid Chromatography-UV and Liquid-Chromatography-Mass Spectrometry analyses. Herein, we report the detection of the CHZ-O-glucuronide (CHZ-O-Glc) derived from OH-CHZ in culture media but also in mouse and human urine and we identified a novel CHZ metabolite, the CHZ-N-glucuronide (CHZ-N-Glc), which is resistant to enzymatic hydrolysis and produced independently of CHZ hydroxylation by CYP2E1. Moreover, we demonstrate that UGT1A1, 1A6 and 1A9 proteins catalyze the synthesis of CHZ-O-Glc while CHZ-N-Glc is produced by UGT1A9 specifically. Together, we demonstrated that hydrolysis of CHZ-O-Glc is required to reliably quantify CYP2E1 activity because of the rapid transformation of OH-CHZ into CHZ-O-Glc and identified the CHZ-N-Glc produced independently of the CYP2E1 activity. Our results also raise the questions of the contribution of CHZ-N-Glc in the overall CHZ metabolism and of the quantification of CHZ glucuronides in vitro and in vivo for measuring UGT1A activities.

# 1. Introduction

The microsomal cytochrome P450 enzymes (CYP) constitute a large family of hemoproteins that catalyze phase I monooxygenase reactions resulting in the production of hydroxylated metabolites of potentially toxic endogenous compounds and xenobiotics (Zanger 2013). In subsequent phase II reactions catalyzed by specific transferases, hydroxylated metabolites are conjugated with charged moieties such as glutathione, sulfate or glucuronic acid resulting in more polar metabolites, which facilitates their excretion in bile and urine.

In CYP gene superfamily, the CYP2E1, mainly expressed in the liver, is an ethanol-inducible CYP that oxides ethanol (Koop 1982) and a variety of low molecular weight compounds (Cederbaum 2014) including carcinogenic chemicals (Gonzalez 2007). Some of the CYP2E1 substrates are oxidized into highly reactive metabolites with the production of reactive oxygen species (ROS) that deplete cellular glutathione, form lipid peroxides as well as protein and DNA adducts with deleterious effects in the liver (Gonzalez 2007). The CYP2E1 expression and activity are induced by many of its own substrates including ethanol, acetone and pyrazole through a mechanism of protein stabilization (Song 1989). In addition, several endogenous blood factors including fatty acids and ketone bodies (Zangar 1997, Martin-Murphy 2013), insulin (Woodcroft 2002, Moncion 2002), lipopolysaccharides and inflammatory cytokines (Abdel-Razzak 1993, Rockich 1999, Poloyac 1999) regulate the CYP2E1 expression at the post-transcriptional level by modulating the stability of the CYP2E1 mRNA. The CYP2E1 activity is considered to play an important role in the liver physiopathology for instance during acetaminophen hepatotoxicity, alcohol consumption, non-alcoholic fatty liver disease and diabetes (Chalasani 2003, Aubert 2011, Abdelmegeed 2012). For these reasons, the assessment of the CYP2E1 expression and/or enzymatic activity is of clinical importance to evaluate pharmacotoxicity of xenobiotics (Cederbaum 2014) and to evidence liver dysfunction in humans (Chalasani 2003, Aubert 2011, Abdelmegeed 2012, Gade 2018).

The CYP2E1 activity is measured both *in vitro* and *in vivo* using the Chlorzoxazone [5-chloro-2(3*H*)-benzoxazolone] (CHZ). This synthetic compound is a centrally-acting myorelaxant indicated for the relief of painful musculoskeletal conditions (Olinger 1958, Chou 2004). It is well documented that CYP2E1 is the major CYP involves in the hydroxylation of the CHZ to produce its main phase I metabolite, the 6-hydroxychlorzoxazone (OH-CHZ) (Peter 1990, Kharasch 1993) although other CYP can produce OH-CHZ with a much weaker catalytic

activity (Carriere 1993). The CYP2E1-independent hydroxylation of the CHZ being negligible, CHZ is considered as a selective probe in humans for measuring the CYP2E1 activity (Frye 1998, Lucas 1999, Witt 2016) through the quantification of OH-CHZ. Besides OH-CHZ, two other minor metabolites have been identified, the 5-Chloro-2,4-dihydroxyacetanilide produced from OH-CHZ by ring cleavage and acetylation of the nitrogen, and the 6-hydroxy-benzoxazolone produced by the substitution of the chlorine by hydrogen and hydroxylation of an unidentified neighbour ring-carbon atom (Twele 1982).

The glucuronidation of OH-CHZ and its urinary excretion (Desiraju 1983) were demonstrated by the increase in total glucuronic acid contents in the urine of human subjects who received CHZ (Conney 1960a) and the detection of high concentrations of OH-CHZ in urine following hydrolysis with  $\beta$ -glucuronidase. In contrast, OH-CHZ was barely detectable without hydrolysis of the CHZ glucuronide (Desiraju 1983, Conney 1960b). To the best of our knowledge, the detection of CHZ glucuronide has never been reported. Quantification of OH-CHZ in biological fluids following enzymatic hydrolysis of CHZ glucuronide is thus commonly used to measure CYP2E1 activity. Although in some studies, CYP2E1 activity is measured by direct quantification of OH-CHZ without hydrolyzing CHZ glucuronide. The analytical methods used to quantitate CHZ and OH-CHZ have successively utilized spectrophotometry (Conney 1960b), thin-layer chromatography (Ullah 1970), gas chromatography (Desiraju 1983), high-performance liquid chromatography (Lucas 1993) and mass spectrometry (Witt 2016) with increasing sensitivity levels allowing reproducible and sensitive assessment of hepatic CYP2E1 activity *in vitro* and *in vivo* during various physio-pathological situations (Ernstgård 2004, Witt 2016).

It has been estimated that less than one percent of a dose of CHZ is excreted unchanged in the urine (Conney 1960). Using sensitive analytical technologies to detect OH-CHZ following glucuronide hydrolysis, the urinary excretion of CHZ glucuronide accounts for only 70 to 80% of the CHZ administrated dose in humans and 50% in rats suggesting that hepatocytes could produce other unidentified metabolites (Mehvar 2006). In addition, the Uridine 5'-diphospho-glucuronosyltranferases (UGT) involved in the hepatic glucuronidation of the OH-CHZ have not been identified. The aim of this study was to identify novel CHZ metabolites and to further characterize the metabolic pathway of this compound in human hepatocytes. Herein, we report the detection by HPLC-UV of the CHZ-O-glucuronide deriving from OH-CHZ and identify a novel CHZ metabolite, the CHZ-N-glucuronide, produced independently of CHZ

hydroxylation by CYP2E1, in culture media of HepaRG hepatoma cells and primary human hepatocytes as well as in mouse and human urine. Moreover, we also identified UGT1A family members that catalyze the production of these two CHZ glucuronides.

### 2. Materials and methods

#### 2.1. Cell culture and treatment conditions

Human HepaRG hepatoma cells were cultured as previously described (Cerec 2007) at 37°C and 5% CO<sub>2</sub> atmosphere in William's E medium supplemented with 10% Fetal calf serum, 100 units/mL penicillin, 100 $\mu$ g/mL streptomycin and 2mM L-glutamine. After 2 weeks, cells were cultured in the same medium supplemented with 2% dimethylsulfoxide (DMSO) to obtain differentiated HepaRG hepatocyte-like cells (Cerec 2007). Human liver biopsies and primary hepatocytes were obtained from the processing of biological samples through the Centre de Ressources Biologiques (CRB) Santé of Rennes BB-0033-00,056 under French legal guidelines and fulfilled the requirements of the institutional ethics committee. Primary hepatocytes were isolated by a two-step collagenase perfusion procedure, seeded at a density of  $3x10^5$  cells/cm<sup>2</sup> and cultured in the same medium than HepaRG cells. Precision cut human liver slices were prepared with a Leica VT12008 vibratome and immediately incubated into culture medium containing CHZ for the detection of metabolites. The human HepG2 hepatoma cell line was maintained in Dulbecco's modified Eagle's medium.

# 2.2. Production of stable recombinant cells

The h*CYP2E1wt* cDNA, obtained from Dr. De Waziers (Université Paris Descartes, France), was cloned into the pHAGE-PGK-MCS-IZsGreen lentiviral backbone encoding the Green Fluorescent Protein (GFP) prior to the production of lentiviral particles (2x10<sup>8</sup> Pfu/mL, Vectalys, Toulouse). Lentiviral particles (5.1x10<sup>8</sup> Pfu/mL) produced from the mTTR.hUGT1A1.142T vector encoding the human UGT1A1 under control of the liver-specific transthyretin (mTTR) promoter were kindly provided by Dr. Nguyen (Nantes, France). Lentiviral particles encoding the UGT1A6 and 1A9 (LPP-Lv105-GFP, 10<sup>9</sup> Pfu/mL) were

purchased from Genecoepia. Transductions of HepG2 and HepaRG cells were performed 24h

2.3. *In vitro* production of CHZ-N-Glc and CHZ-O-Glc using Corning® Supersomes<sup>TM</sup> Recombinant Supersomes<sup>TM</sup> for UGT1A1, 1A6, 1A9, 2B4, 2B7, 2B15 were purchased from Corning (New York) and UGT activities were performed following the manufacturer's instructions. CHZ and OH-CHZ were added at the concentration of 50  $\mu$ M and microsomes were added with final protein concentration of 400  $\mu$ g/mL prior incubation at 37°C for 10 to 60 min depending of the UGT isoform. Reactions were stopped using 1/1 volume of acetonitrile prior to vacuum drying and HPLC analysis.

#### 2.4. Mouse and human samples

after cell seeding with a multiplicity of infection at 5.

Ten weeks old Male C57BL/6 mice were maintained under standard conditions (Animal facility, Institute of Biomedical Science, Federal University of Rio de Janeiro, Brazil): controlled temperature  $(21 \pm 2^{\circ}C)$  and humidity  $(50 \pm 10\%)$  subjected to a 12h light/dark cycle and received standard chow and water *ad libitum*. All procedures were performed in accordance with the international guidelines and Brazilian law (the "Arouca" Law). CHZ was administered by gavage at dose of 50 mg/kg and urine was collected during 12 h following treatment.

Human urine samples were collected from children during an explorative pharmacokinetic trial conducted to investigate drug clearance in obese versus non-obese children and approved by the Danish Health and Medicine Authority (EudraCT 2014-004554-34). In the present study, 13 urine samples of non-obese children were used after approval by the Danish authority (Gade 2016) and the French Direction Générale du Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (authorization n° IE-2017-906). Chlorzoxazone (klorzoxazon, Takeda) was given orally (20 mg/kg) with 200 mL of water and urine was collected between 0 and 20 h (34).

#### 2.5. Detection of chlorzoxazone and its metabolites

Chlorzoxazone (CHZ), 6-hydroxychlorzoxazone (OH-CHZ) and pentachlorophenol were purchased from Sigma- Aldrich (Saint Louis, USA). Chlorzoxazone-N-glucuronide lithium salt (CHZ-N-Glc, M02048) was obtained from Bertin Pharma (Montigny le Bretonneux, France) and Chlorzoxazone  $\beta$ -D-Glucuronide (CHZ-O-Glc) from Toronto Research Chemistry (Canada). Hepatoma cells, primary hepatocytes and liver slices were incubated with 300  $\mu$ M CHZ in William's E culture medium without phenol red prior to detection of CHZ metabolites with direct injection of culture media in HPLC column. Enzymatic hydrolysis of CHZ-O-Glc was performed for 2h at 37°C and pH 5 with 200 IU of  $\beta$ -glucuronidase (Sigma Aldrich G0251). Proteins were discarded by adding (v/v) ice-cold acetonitrile and centrifugation (20,000 g for 15 min). Samples were vacuum dried and resuspended in 200 µL of mobile phase prior to injection.

CHZ and its metabolites were analyzed by high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection at  $\lambda = 284$  nm by injecting samples (40µL) on Agilent Series 1100 (Waldbronn, Germany) equipment with a 75 x 3 mm Ace Excel 2 Super C18 column. Elution was performed with a constant flow rate of 0.7 mL/min using a linear gradient elution from 2% to 60% of acetonitrile for 10 min. The eluents were 0.1% acetic acid in water with 0.25% (m/v) of triethylamine hydrochloride. The procedure was slightly modified for urine. Samples (200 µL) were mixed to 1 mL of ice-cold acetone prior to centrifugation at 20,000 g for 15 min to discard protein. Samples were vacuum dried and suspended in 200 µL of mobile phase and filtered (0.5 µm) prior to injection into an accucore Thermo Scientific PFP 150x3 mm column (Waltham, USA). Elution was performed with a constant flow rate (0.650 mL/min) using a segmented gradient at 2, 9.5 and 10 min with 2%, 35% and 90% of acetonitrile. Validation of OH-CHZ, CHZ-O-Glc and CHZ-N-Glc quantification is provided (Supporting information 1). Liquid chromatography-mass spectrometry (LC-MS) analyses were carried out using Orbitrap Q Exactive<sup>TM</sup> mass spectrometer coupled to an Accela 1250 pump (Thermo Scientific, USA). LC separation was performed on a Hypersil GOLD PFP column (150 x 2.1 mm, 5 µm). The mobile phases were composed of ammonium acetate at 10 mM and formic acid 0.1% in water (phase A) and formic acid 0.1% in acetonitrile (phase B). A two-phase LC gradient was performed from 95% to 5% of phase A during 20 min followed by a 7 min plateau with 5%, then a 5 min equilibration step with 95% of phase B. The flow rate was 200 µL/min, the column temperature was maintained at 25°C, the injection volume was 20 µL and the samples were maintained at 15°C in the autosampler. A heated electrospray ionization source (HESI-II) was used for the ionization of the target compounds. Data acquisition, calibration and instrument control were performed using Xcalibur® 2.1 (Thermo Scientific) software. For mass spectrometry, the instrument operated alternately in ESI positive and negative mode in the same run, the range for acquisition was respectively 80-800 m/z in positive mode and 150-800 m/z in negative mode. Ion precursor selection was performed in the data dependent mode of operation where the most intense ion from the previous scan was selected for fragmentation. Full scan (MS1) data were acquired for each ionization mode at a resolution of 70,000 FWHM, with an AGC target of 1e6 and a maximum injection time of 250 ms. Source parameters were as follows: source voltage + 4.0 and - 4.5 kV, sheath gas flow 35 units, auxiliary gas flow 15 units, sweep gas flow 2.5 units, capillary temperature 300 °C, S-Lens RF level 50 units. MS/MS (MS2) data were acquired in profile mode at a resolution of 17,500 FWHM with an AGC target of 1e6, maximum injection time was 250 ms, a TopN of 8 in positive mode and 3 in negative mode, an isolation window of 3 m/z, a normalized collision energy (NCE) of 70 and the dynamic exclusion time set at 10 sec.

#### 2.6. RNA and protein expression studies

RNA purification and reverse transcription were performed using SV total RNA isolation System (Promega, Madison,WI) and High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed with Sybr Green PCR Master Mix (Applied Biosystems) on ABI PRISM 7900HT instrument. The primer sequences are provided (Supporting information 2). Immunoblotting was performed as previously described (Cerec 2007) using the antibodies recognizing CYP2E1 (Oxford Biomedical, USA), HSC70 (sc-7298, Santa Cruz Biotechnology), CYP3A4 (AB1254, Chemicon), UGT1A (sc-25847, Santa Cruz Biotechnology) and UGT2B (sc-271777, Santa Cruz Biotechnology).

### 2.7. Data analysis

Error bars represent the standard error of the mean (SEM). Statistical analyses were performed with the Student's *t*-test using Prismsoftware, version 5.00 (GraphPad Software, La Jolla, CA, USA) and the threshold for statistical significance was set to p < 0.05.

### 3. Results

#### 3.1. CYP2E1 expression and activity in human primary hepatocytes and hepatoma cells.

Three different in vitro hepatic cell models were used to study the production of CHZ metabolites: the HepG2 and HepaRG human hepatoma cells and primary hepatocytes. We first compared the relative expression levels of the CYP2E1 mRNA (Figure 1A) and protein (Figure 1B) in these different *in vitro* models, in human livers and freshly isolated hepatocytes. Despite important differences in CYP2E1 expression between donors, the CYP2E1 mRNA and protein levels were much higher in livers and freshly isolated hepatocytes compared to the amounts detected in primary cultured hepatocytes and differentiated HepaRG hepatocyte-like cells. CYP2E1 mRNA and proteins were undetectable in HepG2 and progenitor HepaRG cells but appeared in differentiated hepatocyte-like HepaRG cells (Figure 1A-B, Supporting information 3) as previously reported (Aninat 2006, Kanebratt 2008, Dumont 2010, Quesnot 2016). The CYP2E1 expression remained, however, statistically weaker in differentiated HepaRG cells compared to that found in primary human hepatocytes. In order to study the production of CHZ metabolites in cells expressing high amounts of CYP2E1, we generated recombinant HepG2 and HepaRG cells using lentiviral particles encoding the human CYP2E1 leading to an enforced expression of CYP2E1 protein in both cell types (Figure 1C). The CYP2E1 expression in HepaRG-CYP2E1 recombinant cells was not dependent upon the status of differentiation since HepaRG progenitor cells expressed CYP2E1 protein from the lentiviral transgene. However, the hepatocyte-like HepaRG-CYP2E1 cells expressed higher amounts of CYP2E1 compared to their wild-type counterparts (Figure 1C-D) by cumulating expression resulting from the transcription of the lentiviral transgene and the endogenous CYP2E1 gene.

# 3.2. Detection of 6-hydroxy-chlorzoxazone, chlorzoxazone-O- and -N-glucuronides by HPLC in culture media of hepatoma cells and human primary hepatocytes.

In culture media, standard CHZ and OH-CHZ compounds were detected by HPLC-UV at retention times of  $8.5\pm0.2$  and  $5.9\pm0.15$  min, respectively (Supporting information 4, Figure 2A). We took advantage of newly available CHZ-O- and CHZ-N-glucuronide synthetic compounds postulating that these molecules could correspond to putative CHZ metabolites

(Supporting information 4). The CHZ-O- and CHZ-N-Glc HPLC standards added to culture medium gave 2 peaks eluting at 3.8 and 5.6 min, respectively (Figure 2A). Importantly, only CHZ-O-Glc was hydrolyzed with  $\beta$ -glucuronidase to produce OH-CHZ demonstrating that CHZ-N-Glc was resistant to hydrolysis (Figure 2A).

After incubation of CHZ with wildtype HepG2 cells that do not express CYP2E1, a single peak corresponding to CHZ was detected in culture medium (Figure 2B) confirming that HepG2 cells did not metabolize CHZ. In contrast, in medium of HepG2-CYP2E1 cells incubated with CHZ, a major peak eluting at  $5.9\pm0.1$  min and two additional minor peaks at  $3.8\pm0.2$  and  $5.6\pm0.2$  min were observed (Figure 2B). The peak eluting at  $5.9\pm0.1$  min was most likely to be OH-CHZ produced by CYP2E1-dependent hydroxylation of CHZ since it eluted with the same retention time than OH-CHZ HPLC standard (Figure 2A) and was not affected by a  $\beta$ -glucuronidase treatment. The peak at  $3.8\pm0.2$  min could be the CHZ-O-Glc since it disappeared after hydrolysis.

In culture media of wild-type and HepaRG-CYP2E1 hepatocyte-like cells incubated with CHZ, two major peaks co-eluting with CHZ-O- and CHZ-N-Glc at 3.8±0.2 and 5.4±0.3 min were found on HPLC chromatograms (Figure 2C). In addition, the peak eluting at 5.4 min was quantitatively similar in media of wild-type and HepaRG-CYP2E1 cells while the peak at 3.8 min was much higher in medium of HepaRG-CYP2E1 cells than in medium of wild-type cells. The peak of OH-CHZ at 5.9 min was not observed in medium of wild-type HepaRG cells and was barely detectable in HepaRG-CYP2E1 cells. Two other minor peaks eluting at 2.3 and 6.5 min were also observed on HPLC profiles of HepaRG cells. In the culture medium of HepaRG-CYP2E1 cells subjected to β-glucuronidase treatment (Figure 2C), the peak initially eluting at 3.8 min was no longer detectable after hydrolysis while a high peak appeared at a retention time of 5.9 min co-eluting with OH-CHZ (Figure 2A). This data confirmed that OH-CHZ was a minor metabolite in HepaRG cells while the metabolite eluting at 3.8 min was most likely the CHZ-O-glucuronide derived from OH-CHZ since it was hydrolyzed by the  $\beta$ -glucuronidase to produce a peak co-eluting with OH-CHZ. The second peak co-eluting with CHZ-N-Glc  $(5.4\pm0.3 \text{ min})$  was not affected by  $\beta$ -glucuronidase hydrolysis. The two major metabolites with retention times of 3.8 and 5.4 min were also detected in culture medium of human hepatocytes while OH-CHZ was barely detectable (Figure 2D). Hydrolysis with  $\beta$ -glucuronidase eliminated the peak at 3.8 min and strongly increased the peak co-eluting with OH-CHZ (data not shown). Together, these data strongly suggested that CHZ-O- and CHZ-N-glucuronides are the two major CHZ metabolites produced by the HepaRG cells and primary hepatocytes in a timedependent manner after CHZ incubation (Supporting information 5). To further confirm this conclusion, culture medium of HepaRG-CYP2E1 cells was analysed by liquid chromatography combined to mass spectrometry (LC-MS). As expected, CHZ and OH-CHZ were found with m/z 167.9857 and 183.9807, respectively, while 2 major metabolites were detected with m/z 344.0173 and 360.133 that fit with the theorical masses of CHZ-N-Glc and CHZ-O-Glc, respectively (Supporting information 6). MS/MS (MS2) data were also acquired and indicated that fragmentation of CHZ-N-Glc with elimination of its Glc moiety gave back CHZ, m/z 167.9857 (Supporting information 6).

We next quantified OH-CHZ, CHZ-O- and CHZ-N-Glc enzymatic activities in hepatoma cells and primary hepatocytes (Supporting information 1, Figure 2E). In absence of  $\beta$ -glucuronidase treatment, OH-CHZ enzymatic activities were much higher for HepG2-CYP2E1 cells (>100 pmoles/min/mg of total cellular proteins) compared to those of HepaRG cells and primary hepatocytes (<10 pmoles/min/mg). Following hydrolysis, OH-CHZ activity was not affected in HepG2-CYP2E1 cells but strongly increased for HepaRG cells and primary hepatocytes. Importantly, OH-CHZ levels in media of HepaRG-CYP2E1 and primary hepatocytes treated with  $\beta$ -glucuronidase were much higher than those of wild-type HepaRG cells. CHZ-O-Glc activities were undetectable in wild-type HepG2 cells (data not shown), very low in HepG2-CYP2E1 cells (<10 pmol/min/mg, Figure 2F) and much higher in wild-type HepaRG cells (>25 pmol/min/mg), HepaRG-CYP2E1 cells and primary hepatocytes (100 to 150 pmol/min/mg). CHZ-N-Glc activities (Figure 2F) were very low in wild-type (data not shown) and HepG2-CYP2E1 cells and much higher but significantly different between HepaRG cells (~200 pmol/min/mg) and primary hepatocytes (~100 pmol/min/mg).

## 3.3. Production of chlorzoxazone N-glucuronide independently of the CYP2E1 hydroxylation

Postulating that CHZ-O-Glc was produced by conjugation of OH-CHZ while CHZ-N-Glc would result from a direct glucuronidation of CHZ, HepaRG cells were incubated for 15, 60 and 90 min with OH-CHZ and the HPLC profiles were determined (Figure 3A). The peak of OH-CHZ disappeared over 90 min from the HPLC chromatograms while the CHZ-O-Glc progressively accumulated in the medium. The quantification indicated that the OH-CHZ was completely metabolized in CHZ-O-Glc within 90 min in parental HepaRG cells (Figure 3B), HepaRG-CYP2E1 cells and primary hepatocytes but not HepG2-CYP2E1 cells (Figure 3C).

The CHZ-N-Glc was not detected in culture media of HepaRG incubated with OH-CHZ (Figure 3A), which confirmed that CHZ-O-Glc is produced by glucuronidation of OH-CHZ while CHZ-N-Glc is a conjugate of CHZ.

We next evaluated the effect of a partially selective inhibitor of UGT, the pentachlorophenol (PCP) (Boström 2000), on the accumulation of CHZ-O- and CHZ-N-Glc in culture media of hepatoma cells and primary hepatocytes (Figure 4). The decreases in CHZ-O- and CHZ-N-Glc activities and the accumulation in OH-CHZ were dose-dependent. At the low dose of 10  $\mu$ M, PCP partially reduced the amounts in CHZ-O-Glc produced by wild type HepaRG and HepaRG-CYP2E1 cells (Figure 4A, D) leading to the appearance of OH-CHZ (Figure 4A-B) while a 6-fold decrease in CHZ-N-Glc was observed (Figure 4C) suggesting that PCP inhibited more efficiently the CHZ-N-Glc catalytic activities than the enzyme(s) producing the CHZ-O-Glc. In HepG2-CYP2E1 cells that produced only OH-CHZ, the PCP did not affect the amounts of this metabolite up to 10  $\mu$ M and slightly reduced them at 50 and 100  $\mu$ M (Figure 4B).

# 3.4. UDP-glucuronosyltransferase 1A family members produce chlorzoxazone-N- and O-glucuronides

In order to identify UGT involved in the CHZ-O- and CHZ-N-Glc catalytic activities, we compared UGT mRNA levels between our 3 hepatic cell models (Supporting information 7A) postulating that differences in UGT expression levels should correlate to CHZ-O- and CHZ-N-Glc catalytic activities (Figures 2-4, Supporting information 7B). We selected 6 UGT candidates (UGT1A1, 1A6, 1A9, 2B4, 2B7 and 2B15) based on their very low expression in HepG2 cells, comparable expression in HepaRG cells and primary hepatocytes (Supporting information 7A). To determine whether these candidates were involved in the glucuronidation of CHZ, we used the recombinant Supersomes<sup>™</sup> expressing these UGT in absence of CYP2E1 and analyzed by HPLC-UV the production of CHZ-N- and CHZ-O-Glc using CHZ or OH-CHZ substrates (Table 1). Variable amounts of UGT proteins were found between the recombinant Supersomes<sup>™</sup> indicating that this assay was only qualitative (Supporting information 8). UGT1A1, 1A6 and 1A9 produced CHZ-O-Glc from OH-CHZ substrate but not from CHZ while the CHZ-N-Glc was found only in the UGT1A9 Supersomes<sup>™</sup> incubated with CHZ. UGT2B did not produce any CHZ-Glc or at very low levels.

To further demonstrate in a cell-based assay that UGT1A1, 1A6 and 1A9 produced CHZ-O-Glc while the CHZ-N-Glc was only generated by UGT1A9, we established recombinant HepG2 cell lines expressing UGT1A1, 1A6 and 1A9 combined or not to CYP2E1 expression (Supporting information 9). The wild-type and the recombinant HepG2 cells were incubated with either OH-CHZ or CHZ and the production of both CHZ-O-Glc and CHZ-N-Glc was analyzed by HPLC (Figure 5). When using OH-CHZ as substrate, only CHZ-O-Glc was detected in media of HepG2 cells expressing UGT1A1, 1A6 and 1A9 but not in wild-type cells (Figure 5A). The expression of CYP2E1 did not significantly affect the amounts of CHZ-O-Glc. Following incubation of HepG2 cells with CHZ, accumulation of OH-CHZ was found only in cells that expressed CYP2E1 with our without UGT, while in cells co-expressing CYP2E1 and UGT1A1, 1A6 and 1A9 high concentrations of CHZ-O-Glc were detected (Figure 5B). Moreover, CHZ-N-Glc was detected predominantly in cells expressing UGT1A9 only or co-expressing CYP2E1 and UGT1A9 (Figure 5B).

These results demonstrated that CHZ-N-Glc synthesis is catalyzed by UGT1A9 activity independently from CYP2E1 while co-expression of CYP2E1 and UGT1A1, 1A6 or 1A9 in human HepG2 cells leads to the production of OH-CHZ phase I metabolite and its downstream CHZ-O-Glc.

#### 3.5. Chlorzoxazone-O- and N-glucuronides are detected in mouse and human urine

We next investigated the production of CHZ-O-Glc and CHZ-N-Glc *in vivo* in mouse and human urine samples collected at a single time-point after CHZ administration (Figure 6). The HPLC-UV procedure was modified to detect CHZ metabolites and to separate the contaminant peaks that remained present on the HPLC profiles following urine extraction. Typical HPLC chromatograms of mouse (Figure 6A) and human (Figure 6B) urine samples without administration of CHZ (-CHZ) and following CHZ administration in absence of  $\beta$ -glucuronidase (+CHZ) or after  $\beta$ -glucuronidase hydrolysis (+CHZ+ $\beta$ -Gluc) were compared to the elution profiles of OH-CHZ, CHZ-O-Glc and CHZ-N-Glc HPLC standard compounds that eluted at 6.9, 8.95 and 9.7 min, respectively, in this modified HPLC procedure. In both mouse and human samples, in absence of  $\beta$ -glucuronidase treatment, a high peak co-eluting with CHZ-

O-Glc HPLC standard at 6.9 min was found. This metabolite disappeared after  $\beta$ -glucuronidase hydrolysis to generate a peak of similar height eluting at the retention time (9.7 min) of OH-CHZ standard compound. In addition, a smaller peak co-eluting with CHZ-N-Glc standard reference at 8.95 min was also found in mouse and human samples (Figure 6A-B). The treatment with  $\beta$ -glucuronidase did not affect its height. The quantification of these two peaks co-eluting with CHZ-O-Glc and CHZ-N-Glc standards, and OH-CHZ (Figure 6B-C) indicated that CHZ-O-Glc was the most abundant CHZ metabolite in mouse (339.02 ± 42.97  $\mu$ M) and human (425.48 ± 53.19  $\mu$ M) urine compared to the lower amounts of CHZ-N-Glc found in mouse (71.15 ± 5.1  $\mu$ M) and human (28.8 ± 7.82  $\mu$ M) samples. These low CHZ-N-Glc amounts detected in urine contrasted with high concentrations of CHZ-N-Glc found in culture media of HepaRG cells and human hepatocytes. To further demonstrate that human liver produced this glucuronide, we analyzed CHZ metabolites in culture media of precision cut human liver slices (Figure 6E). In this organotypic liver model (Supporting Information 10), both CHZ-O-Glc and CHZ-N-Glc were detected at relatively similar levels confirming that CHZ-N-Glc is an abundant metabolite produced by the liver.

## 4. Discussion

This study established that hepatocyte metabolism produces two major CHZ glucuronides. We detected by HPLC-UV and LC-MS analyses of culture media from metabolically competent hepatoma cells and primary hepatocytes incubated with CHZ, the CHZ-O-Glc that derived from the OH-CHZ. More importantly, we identified a novel CHZ glucuronide produced independently of CHZ hydroxylation by CYP2E1, the CHZ-N-Glc, which is resistant to enzymatic hydrolysis. These two glucuronides are also produced *in vivo* since they were found in mouse and human urine. Furthermore, we demonstrate that UGT1A1, 1A6 and 1A9 proteins catalyze the synthesis of CHZ-O-Glc while CHZ-N-Glc is produced by UGT1A9. Together, our results allow revising the hepatic metabolic pathway of the CHZ (Figure 6F).

These data imply several consequences for the measurement of CYP2E1 and UGT1A activities both *in vitro* and *in vivo*. In agreement with other studies, our work confirms that the determination of the CYP2E1 activity in biological samples requires measurement of OH-CHZ with hydrolysis of CHZ-O-Glc reversed into OH-CHZ (Peter 1990, Witt 2016). Indeed in hepatocytes, the overall UGT1A activity producing the CHZ-O-Glc is much higher than the CYP2E1 activity producing OH-CHZ, which results in very low amounts of OH-CHZ in biological fluids. Thus, quantification of OH-CHZ without hydrolysis of CHZ-O-Glc may poorly reflect the CYP2E1 activities in hepatic cells expressing UGT1A.

The second consequence is the identification of a novel CHZ metabolite, the CHZ-N-Glc, found in high amounts in culture media of HepaRG cells, primary hepatocytes and liver slices. The evaluation of CYP2E1 activity *in vivo* is calculated with the metabolic ratio or hydroxylation index corresponding to urinary OH-CHZ (mmole)/plasma CHZ (mmole) CHZ following oral administration of a single dose of CHZ. This ratio does not take into account the fraction of CHZ metabolized into CHZ-N-Glc. In addition, the formation clearance of OH-CHZ accounts for only 50 to 70% of total CHZ clearance (Desiraju 1983, Frye 1998, Ernstgård 2004, Witt 2016), which led authors to postulate that alternative pathways of CHZ metabolism might exist or that there is incomplete intestinal absorption (Frye 1998). Our data demonstrate the existence of this alternative CHZ metabolism and provide a possible explanation for the formation clearance of OH-CHZ significantly lower than the total CHZ clearance. *In vitro*, we found that the CHZ-O-Glc catalytic activities reach ~120 pmoles/min/mg of total proteins in HepaRG CYP2E1 and primary hepatocytes while CHZ-N-Glc catalytic activities were higher in HepaRG

cells (~200 pmoles/min/mg of total proteins) than in human hepatocytes (~100 pmoles/min/mg of total proteins). These data suggest that the amounts of both CHZ-N-Glc and CHZ-O-Glc produced in isolated hepatocytes would be nearly identical. This data was confirmed using the model of precision cut human liver slices. In addition, we found that the HPLC-UV detection was less efficient for CHZ-N-Glc than CHZ-O-Glc with a CHZ-O-Glc/CHZ-N-Glc absorbance signal ratio of 0.762 suggesting that the CHZ-N-Glc catalytic activities might also be underestimated. Thus, the CHZ hydroxylation index used to measure CYP2E1 activity could be partially skewed by the competition with the CHZ-N-Glc and CHZ-O-Glc produced by primary hepatocytes and liver slices are in apparent contradiction with the low amounts of CHZ-N-Glc found in rodent and human urine compared to the high amounts of urinary CHZ-O-Glc. We hypothesize that CHZ-N-Glc could be excreted in the bile. Thus, the evaluation of CHZ-N-Glc clearance might be difficult to implement *in vivo* and additional studies are required to demonstrate the biliary excretion of this novel CHZ-N-Glc metabolite.

Our data also raise the question of the detection of both CHZ glucuronides *in vitro* or *in vivo* for measuring UGT1A activities. A large panel of relatively selective probes has been identified for assessing the CYP450 (Spaggiari 2014) and UGT (Miners 2006, Rowland 2013) activities *in vitro* and *in vivo* but the CHZ has never been proposed as a probe for measuring UGT activities. The demonstration that UGT1A9 exhibits a high metabolic activity towards CHZ to produce CHZ-N-Glc without phase I reaction is of particular interest to measure this UGT activity including in liver microsomes, a cell-free assay used to screen for UGT inhibitors (Walsky 2012).

The human HepaRG hepatoma cells are used for pharmaco-toxicology studies (Aninat 2006, Kanebratt 2008, Dumont 2010, Quesnot 2016), since they have the remarkable ability to differentiate in hepatocyte-like cells (Cerec 2007) that express a broad range of drug metabolizing enzymes (Aninat 2006, Kanebratt 2008). However, controversial data have been reported concerning the levels of CYP2E1 expression and activity in differentiated HepaRG cells (Aninat 2006, Kanebratt 2008). We confirmed that these cells express active CYP2E1 but at a lower level than that found in human primary cells in contrast with other CYP such as CYP3A4, which is strongly expressed in HepaRG cells (Aninat 2006). The HepaRG-CYP2E1 cell line established in this study exhibits CYP2E1 expression levels and catalytic activities similar to those found in primary hepatocytes as demonstrated by the CHZ-O-Glc and OH-CHZ

amounts found in their culture media. In contrast, the OH-CHZ contents measured in absence of CHZ-O-Glc hydrolysis are much weaker in culture medium of HepaRG-CYP2E1 cells compared to those of primary hepatocytes because of higher expression levels of UGT in HepaRG cells compared to that found in primary hepatocytes, which catalyze the production of CHZ-O-Glc. These data further confirmed results reported by others demonstrating high UGT activities in differentiated HepaRG cells (Leite 2012).

Together, our data bring new insights into the hepatic metabolism of CHZ. They emphasize the requirement to convert the CHZ-O-Glc back into OH-CHZ or, alternatively, to quantify the CHZ-O-Glc to reliably measure the CYP2E1 activity. Our results also open new perspectives in the use of CHZ and the detection of these two glucuronides for the evaluation of UGT activities in studies of pharmacology and liver physiopathology.

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### **Conflict of Interest**

The authors declare that there have no conflict of interest.

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### **Figure legends**

# Figure 1: CYP2E1 mRNA and protein levels in human livers, primary hepatocytes and hepatoma cells.

A) The relative amounts of CYP2E1 mRNA were investigated by qPCR in biopsies of human normal livers (n=10), freshly isolated human hepatocytes (FIH, n=6), primary human hepatocytes cultured for 48h (Prim. Hep., n=4), progenitor (Prog. HepaRG, n=5), confluent (14 days post-plating, Conf. HepaRG, n=5) and differentiated Hepatocyte-like (30 days postplating, Diff. HepaRG, n=5) HepaRG cells, HuH7 (n=4) and HepG2 cells (n=4). Statistics: \*\*p < 0.01 mRNA levels higher in primary hepatocytes and differentiated HepaRG cells versus progenitor and confluent HepaRG cells, HuH7 and HepG2, \*\*\*p<0.001 mRNA levels higher in human livers and freshly isolated hepatocytes versus all other conditions. Immunoblotting of CYP2E1 in total extracts prepared from: **B**) human livers (n=4), freshly isolated human hepatocytes (FIH, n=2), primary human hepatocytes cultured for 48h (Prim. Hep., n=2), proliferating at low density (Prolif.) and confluent at high density (Conf.) HepG2 cells, progenitor (Prog.) and differentiated Hepatocyte-like (Diff.) HepaRG cells, C) parental (wild type, WT) and recombinant (HepG2-CYP2E1, HepaRG-CYP2E1) HepG2 and differentiated HepaRG cells, **D**) parental (WT) and recombinant HepaRG-CYP2E1 cells with enforced expression of CYP2E1 during the 30 days culture protocol to obtain the full differentiation of HepaRG cells (Supporting information 3) with 14 days post plating in absence of DMSO (-DMSO) and 2 more weeks in presence of 2% DMSO in culture medium (+DMSO). Loading control for western blot was HSC70.

# Figure 2: HPLC-UV detection of chlorzoxazone metabolites in culture media of hepatoma cells and primary hepatocytes.

Chromatograms of HPLC-UV analysis of: A) Standard compounds CHZ, CHZ-O-Glc (green arrow), CHZ-N-Glc (red arrow) and OH-CHZ added together to culture medium without cell

incubation (Medium W/O cells) and subjected (+  $\beta$ -glucuronidase) or not (-  $\beta$ -glucuronidase) to β-glucuronidase hydrolysis. **B**) Culture medium with CHZ incubated with parental HepG2 cells or recombinant HepG2-CYP2E1 cells without ( $-\beta$ -glucuronidase) or with ( $+\beta$ -glucuronidase) β-glucuronidase hydrolysis prior HPLC-UV analysis. C) Culture medium with CHZ incubated with differentiated parental HepaRG or recombinant HepaRG-CYP2E1 cells without (-βglucuronidase) or with (+ $\beta$ -glucuronidase)  $\beta$ -glucuronidase hydrolysis prior HPLC-UV analysis. **D**) Culture medium with CHZ incubated with primary human hepatocytes. **E**) Quantification of OH-CHZ activities (pmoles/min/mg of total proteins) in primary human hepatocytes (HH), parental and CYP2E1 expressing HepG2 and HepaRG cells following HPLC-UV analysis of culture media, without (- $\beta$ -glucuronidase, left graph) or with (+ $\beta$ glucuronidase, right graph) β-glucuronidase hydrolysis of media. Statistics: 2 or 3 independent experiments with 6 or 9 independent culture wells. Graph without  $\beta$ -glucuronidase hydrolysis (- $\beta$ -glucuronidase), <sup>†</sup>p<0.01: OH-CHZ significantly higher in HepaRG and HepaRG-CYP2E1 versus HepG2,  $^{\dagger\dagger}p$  <0.01: OH-CHZ significantly higher in HH versus HepaRG, HepaRG-CYP2E1 and HepG2, <sup>†††</sup>p<0.01: OH-CHZ significantly higher in HepG2 CYP2E1 than all others cells. Graph with  $\beta$ -glucuronidase hydrolysis (+ $\beta$ -glucuronidase),  $\frac{8}{p} < 0.001$ : OH-CHZ significantly higher in parental HepaRG versus HepG2, <sup>§§§</sup>p<0.001 OH-CHZ significantly higher in HepG2-CYP2E1, HepaRG CYP2E1 and HH versus HepaRG and HepG2 WT. F) Quantification of CHZ-O- and CHZ-N-Glc activities (pmoles/min/mg of total proteins) in HepG2 CYP2E1, HepaRG, HepaRG CYP2E1 cells and primary hepatocytes (HH). Statistics of CHZ-O-Glc graph, \$p < 0.01: CHZ-O-Glc significantly higher in HepaRG versus HepG2 CYP2E1 cells and significantly higher in HepaRG-CYP2E1 and primary hepatocytes (HH) versus HepaRG and HepG2 CYP2E1. Statistics of CHZ-N-Glc graph, <sup>§§§</sup>p<0.001 CHZ-N-Glc significantly higher in primary hepatocytes (HH) versus HepG2 CYP2E1, and <sup>§§</sup>p<0.01 parental HepaRG and HepaRG-CYP2E1 cells versus primary hepatocytes (HH) and HepG2 CYP2E1.

# Figure 3: Conversion of OH-CHZ into CHZ-O-Glc in HepaRG cells and human hepatocytes.

A) HPLC-UV chromatograms of the time-course analysis of OH-CHZ conversion into CHZ-O-Glc by HepaRG cells at 0, 15, 60 and 90 min. B) Quantification in μM of OH-CHZ (plain lane) and CHZ-O-Glc (dashed lane) in culture medium of HepaRG cells during the time-course.
C) Quantification of CHZ-O-Glc activities (pmoles/min/mg of total proteins) after 90 min of

incubation of culture medium containing OH-CHZ by HepG2 CYP2E1, HepaRG, HepaRG CYP2E1 cells and primary hepatocytes (HH). Statistics, \$\$p<0.001: CHZ-O-Glc significantly higher in HepaRG, HepaRG-CYP2E1 cells and primary hepatocytes (HH) versus HepG2 CYP2E1 cells.

### Figure 4: Inhibition of CHZ-O-Glc and CHZ-N-Glc production by pentachlorophenol.

**A)** Chromatograms of HPLC-UV detection of OH-CHZ, CHZ-O- and CHZ-N-Glc in culture media of HepaRG cells after a 90 min incubation with CHZ in absence (0 μM) or presence of pentacholorophenol (PCP) at 10 and 100 μM. The 2 main contaminating peaks are indicated (\*). **B)** Quantification of OH-CHZ activities (pmoles/min/mg of total proteins) in culture of primary hepatocytes (HH), parental and CYP2E1 expressing HepaRG cells and HepG2 CYP2E1 cells in absence and presence of various concentrations of PCP (2.5 to 100 μM). **C)** Quantification of CHZ-N-Glc activities (pmoles/min/mg of total proteins) in culture of primary hepatocytes (HH), parental and CYP2E1 expressing HepaRG cells in absence and presence of various concentrations of PCP (2.5 to 100 μM). **C)** Quantification of CHZ-N-Glc activities (pmoles/min/mg of total proteins) in culture of primary hepatocytes (HH), parental and CYP2E1 expressing HepaRG cells in absence and presence of various concentrations of CHZ-O-Glc activities (pmoles/min/mg of total proteins) in culture of primary hepatocytes (HH), parental and CYP2E1 expressing HepaRG cells in absence and presence of various concentrations of PCP (2.5 to 100 μM). **D**) Quantification of CHZ-O-Glc activities (pmoles/min/mg of total proteins) in culture of primary hepatocytes (HH) and parental HepaRG cells in absence and presence of various concentrations of PCP (2.5 to 100 μM). Statistics of OH-CHZ graph, <sup>\$</sup>p <0.01: OH-CHZ significantly higher in PCP-treated cells versus untreated cells. Statistics of CHZ-N-Glc graph, <sup>†</sup>p<0.01 CHZ-N-Glc significantly lower in PCP-treated cells versus untreated cells. Statistics of CHZ-O-Glc graph, <sup>\*\*</sup>p<0.01 significantly lower in PCP-treated cells versus untreated cells.

### Figure 5: Production of CHZ-O and CHZ-N-Glc in recombinant HepG2-UGT cells.

Quantification (concentration in  $\mu$ M) of CHZ-O-Glc (white bars), CHZ-N-Glc (grey bars) and OH-CHZ (dark bars) in culture medium of recombinant HepG2 cells expressing either UGT1A1, 1A6 or 1A9, and HepG2 cells expression CYP2E1 and UGT1A1, 1A6 or 1A9. Recombinant HepG2 cells were incubated with OH-CHZ (**A**) or CHZ (**B**) and the production of both CHZ-O-Glc and CHZ-N-Glc was analyzed by HPLC and quantified. Statistics, **A**) Chart for cells incubated with OH-CHZ:  $^{\$}p$ <0.05 OH-CHZ significantly lower and  $^{*}p$ <0.01 CHZ-O-Glc significantly higher in cells expressing UGT1A1, 1A6 and 1A9 versus parental/wild type (WT) HepG2 and HepG2-CYP2E1, **B**) chart for cells incubated with CHZ:  $^{*}p$ <0.01 CHZ-O-Glc significantly higher in HepG2 cells expressing CYP2E1 and UGT1A1, 1A6 or 1A9 versus all other conditions,  $^{\$}p$ <0.01 CHZ-N-Glc significantly higher in cells expressing UGT1A9 with

or without expression of CYP2E1 versus all other conditions, p<0.05 OH-CHZ significantly higher in HepG2-CYP2E1 cells versus all other cell conditions and p<0.01 CHZ-O-Glc significantly higher in HepG2 cells expressing CYP2E1 and UGT1A1, 1A6 or 1A9 versus all other cell conditions. Amounts of CHZ were not quantified in samples from chart B.

# Figure 6: Detection of both chlorzoxazone-O- and N-glucuronides in mouse and human urine.

Chromatograms of HPLC-UV detection of OH-CHZ, CHZ-O- and CHZ-N-Glc in **A**) mouse and **B**) human urines. Overlay chromatograms include control urine without administration of CHZ (- CHZ, upper lane), mouse and human urine following CHZ administration without (+ CHZ, blue lane) or with (+ CHZ +  $\beta$ -gluc, red lane)  $\beta$ -glucuronidase hydrolysis of the samples prior HPLC-UV analysis and synthetic OH-CHZ, CHZ-O- and CHZ-N-Glc standard compounds (bottom lane) to indicate the elution times. Inserts are the magnification corresponding to the elution time of CHZ-N-Glc. Charts **C**) and **D**) correspond to the quantifications of OH-CHZ, CHZ-O- and CHZ-N-Glc in mouse (*n*=6) and human (*n*=13) urine samples. **E**) Chromatograms of HPLC-UV detection of OH-CHZ, CHZ-O- and CHZ-N-Glc in culture media of precision cut human liver slices non-incubated control (- CHZ) and incubated with CHZ (+ CHZ). HPLC profiles were overlaid with chromatograms of standard compounds (STD). Contaminating peaks are indicated (\*). **F**) Metabolic pathway of CHZ biotransformation with 2 primary metabolites, the OH-CHZ via the CYP2E1 catalytic activity and the CHZ-N-Glc produced at least by UGT1A9. OH-CHZ is further transformed in CHZ-O-Glc via the catalytic activities of UGT1A1, 1A6 and 1A9 family members.

# Table 1: UGT-dependent CHZ-N-Glc and CHZ-O-Glc catalytic activities using Supersomes<sup>™</sup> incubated with CHZ and OH-CHZ substrates.

CHZ-N-Glc and CHZ-O-Glc catalytic activities were measured using Supersomes<sup>™</sup> and CHZ or OH-CHZ substrates, respectively. Supersomes<sup>™</sup> expressing either UGT1A1, 1A6, 1A9, 2B4, 2B7 or 2B15 were incubated with CHZ or OH-CHZ and HPLC-UV analysis were performed to quantify (pmoles/min/mg) the production of CHZ-N-Glc and CHZ-O-Glc. CHZ-O-Glc was not detected in Supersomes<sup>™</sup> reactions using CHZ as substrate and, vice versa, CHZ-N-Glc was not detected in reactions using OH-CHZ (data not shown). Three independent experiments were performed.



Figure 1



Figure 2



Figure 3



Figure 4

В

С



Figure 5



Figure 6

Activities (pmoles/min/mg prot)	UGT Supersomes					
	1A1	1A6	1A9	2B4	2B7	2B15
CHZ-N-Glc	0	0	62±2	0	0	0
(Substrate : CHZ)						
CHZ-O-Glc	201 : 46	0000 + 2004	2000 - 725			20
(Substrate : OH-CHZ)	$391 \pm 40$	9808 ± 3094	$2898 \pm 633$	0	0	20

Table 1 : UGT activities using Supersomes<sup>™</sup> incubated with CHZ and OH-CHZ substrates.