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Optimization of Experimental Conditions of Automated Glucuronidation Assays in Human Liver Microsomes using a Cocktail Approach and Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Running title: Automated glucuronidation assay optimization

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Introduction: 750

Discussion/Conclusion: 1463

Abbreviations:

BSA: bovine serum albumin, CYP: cytochrome P450, HLM: human liver microsomes, Km: Michaelis-Menten constant, $MgCl_2$: magnesium chloride, MS: mass spectrometry, UDPGA: uridine 5'-diphosphoglucuronic acid, UGT: UDP-glucuronosyltransferases, UHPLC: ultra-high performance liquid chromatography

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Abstract

UDP-glucuronosyltransferase (UGT)-mediated metabolism is possibly the most important conjugation reaction for marketed drugs. However, there are no generally accepted standard incubation conditions for UGT microsomal assays and substantial differences in experimental design and methodology between laboratories hinders cross-study comparison of *in vitro* activities. This study aimed to define optimal experimental conditions to determine glucuronidation activity of multiple UGT isoforms simultaneously using human liver microsomes. Hepatic glucuronidation activities of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B15 and UGT2B17 were determined using cocktail incubations of 10 UGT probe substrates. Buffer components and co-substrates were assessed over a range of concentrations including magnesium chloride ($MgCl_2$, 0-10 mM) and uridine 5'-diphosphoglucuronic acid (UDPGA, 1-25 mM) with either Tris-HCl or potassium phosphate buffer (100 mM, pH 7.4). Greater microsomal glucuronidation activity by different hepatic UGT isoforms was obtained using: 10 mM $MgCl_2$ and 5 mM UDPGA with 100 mM Tris-HCl buffer. The influence of bovine serum albumin (BSA, 0.1-2% w/v) on glucuronidation activity was also assessed. Enzyme- and substrate-dependent effects of BSA were observed, resulting in decreased total activity of UGT1A1, UGT1A3, UGT2B17 and increased total UGT1A9 and UGT2B7 activity. Inclusion of BSA did not significantly reduce the between-subject variability of UGT activity. Future *in vitro* UGT profiling studies under the proposed optimized experimental conditions would allow high quality positive control data to be generated across laboratories, effective control of high degree of between-donor variability for UGT activity and for chemical optimization towards lower clearance drug molecules in a pharmaceutical drug discovery setting.

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Introduction

In recent years, the interest in glucuronidation reactions mediated via UDP-glucuronosyltransferase (UGT) enzymes has significantly increased as a clearance mechanism of new chemical entities (Di and Obach, 2015). In humans, UGTs comprise a large family of membrane-bound enzymes. They play a major role in the detoxification and elimination of endogenous substrates (i.e. bilirubin, bile acid, steroids) and xenobiotics (i.e. drugs, toxins, carcinogens) by catalyzing the transfer of a glucuronic acid moiety from their co-substrate, uridine 5'-diphosphoglucuronic acid (UDPGA) (Tukey and Strassburg, 2000; Rowland *et al.*, 2013).

In vitro glucuronidation studies are routinely conducted to determine the enzymes involved in conjugation as well as the fraction metabolized via this route using human liver microsomes (HLM). Because of their reproducible nature, capacity for long-term storage, and rapid availability, HLM are used as as the first-line screening model for high throughput assays for determination for determination of UGT metabolism. They may be used to assess expression and activities within a population of individual donors (Miyagi and Collier, 2011; Gill *et al.*, 2012; Walsky *et al.*, 2012; Slovak *et al.*, 2017). Methodological advances have in recent years improved the accuracy and reproducibility of microsomal UGT activity determination using selective or partially selective UGT probe substrates. This includes the optimization of incubation conditions for 5 important UGT isoforms (UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7) (Walsky *et al.*, 2012). Additionally, cocktail incubation approaches coupled with liquid chromatography–tandem mass spectrometry quantification, typically under low HLM concentration suited to inhibition assays, have been developed (Gagez *et al.*, 2012; Joo *et al.*, 2014; Seo *et al.*, 2014; Gradinaru *et al.*, 2015). The *in vitro* cocktail incubation of multiple substrates approach has been well described for determining the activity of CYPs in HLM (Patten, 2006; Dixit *et al.*, 2007; Pillai *et al.*, 2013; Spaggiari *et*

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al., 2014). In contrast, the determination of glucuronidation activity of individual UGT isoforms can be challenging due to the lack of selective and validated UGT probe substrates (Donato *et al.*, 2010). To date, a method for the simultaneous determination of glucuronidation activities of major hepatic UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B15 and UGT2B17) has not been developed for use with microsomal preparations (Badée *et al.*, 2018). This results in substantial inter-laboratory technical and methodological differences that have hindered the cross-study comparison of glucuronidation activity. Therefore, the understanding of the differential contribution of analytical, technical and between-subject variability in glucuronidation activity remains challenging. The variations observed in experimental conditions can be listed as follows: (i) microsomal protein concentrations, (ii) use of (validated and selective) UGT probe substrates, (iii) incubation buffer to adjust and stabilize the desired pH of the reaction, (iv) pre-incubation of microsomes with pore-forming agents (i.e. alamethicin) or detergents, (v) a wide concentration range of UDPGA (1-30 mM) and differing buffer components (magnesium chloride ($MgCl_2$), 0-10 mM) and (vi) supplementation or not with bovine serum albumin (BSA) (0.1-4%).

Walsky and colleagues addressed the substrate characterization challenge and, thereby proposed the best guide so far for *in vitro* glucuronidation assays to measure the glucuronidation activity of UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 in HLM (Walsky *et al.*, 2012). Glucuronidation activities were enhanced by conducting incubations of alamethicin-treated HLM with Tris-HCl buffer (100 mM, pH 7.4) and 10 mM $MgCl_2$. However, controversy exists on the use of BSA. The use of 2% BSA was shown to enhance the glucuronidation activity of UGT1A9 and UGT2B7, which was explained by the sequestration of inhibitory long-chain fatty acids released during microsomal preparation and incubation. However, reduced or unchanged glucuronidation activity was observed for

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UGT1A1, UGT1A4 and UGT1A6 (Walsky *et al.*, 2012). Therefore, the utility of BSA supplementation in the profiling of new drug candidate molecules needs to be addressed due to the competing effects of enzyme-selective enhancement versus compound-dependent reduction of free incubation concentration and reaction rates.

The objective of this study was to optimize the incubation conditions of *in vitro* UGT glucuronidation assays using hepatic microsomal preparations under conditions suited to UGT phenotyping for low to moderate clearance compounds. For this purpose, automated glucuronidation experiments were conducted to determine simultaneously the metabolic activity of major hepatic UGT isoforms by incubation of 10 UGT probe substrates in cocktails. First, we investigated the effect of a wide concentration range of co-substrates and buffer components. Then, we explored whether increasing BSA concentrations enhanced glucuronidation activity. Protein binding assays were carried out to correct activities for the unbound concentration of probe substrates. Finally, we evaluated whether the incubation of individual HLM with BSA reduced between-subject variability.

Material and Methods

Chemicals and Reagents

β-estradiol, chenodeoxycholic acid, 5-hydroxytryptophol, propofol, gemfibrozil, zidovudine, amitriptyline, oxazepam (racemic), testosterone, dimethyl sulfoxide (DMSO), alamethicin, uridine 5'diphosphateglucuronic acid (UDPGA), and fatty acid free bovine serum albumin (BSA) (≥98%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Trifluoperazine was purchased from LKT laboratories (St Paul, MN, USA). β-estradiol-3-glucuronide, chenodeoxycholic acid-glucuronide, trifluoperazine-glucuronide, propofol-glucuronide, gemfibrozil-glucuronide, zidovudine-glucuronide, and amitriptyline-glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada). 5-hydroxytryptophol-

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glucuronide was purchased from Synlnnova (Edmonton, Canada). Magnesium chloride ($MgCl_2$) was purchased from Merck (Darmstadt, Germany). Tris buffer was purchased from AppliChem GmbH (Darmstadt, Germany). Hexa-deuterated midazolam was obtained from Hoffmann-La Roche (Basel, Switzerland). High purity acetonitrile for ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) were obtained from BioSolve (Lexington, MA, USA).

Human Liver Microsomes

Mixed-gender pooled HLM derived from 150-donors (lot 38290, 75 male and 75 female) were purchased from Corning Life Sciences (Tewksbury, MA, USA). Individual human liver microsomes (HLM) derived from single adult donors were purchased from Sekisui XenoTech (Kansas City, KS, USA). The individual HLM donor panel comprised of 15 donors with 14 Caucasian, 1 African American, 6 males, 9 females and the average age was 46.8 years (range, 21-74 years). Microsomes were stored at -80°C.

Automated Glucuronidation Assay Program

The highly flexible automated glucuronidation assay program used to perform these studies was developed at Hoffmann-La Roche Ltd and implemented on a Tecan Fluent system (Tecan Group Ltd, Männedorf, Switzerland) which was controlled by Fluent Control software, version 1.5. The Tecan Fluent system was equipped with an eight-channel air displacement liquid handling arm using disposable tips for pipetting liquids, a 96 / 384 multichannel arm using disposable tips and a robotic manipulator arm for transporting plates. Metabolism time course experiments were performed using the Tecan Fluent system allowing the incubation of microsomal preparations with 11 probe substrates combined with up to 12

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different enzyme / buffer / enzyme-specific cofactor conditions within one 96-well plate and up to 8 incubation time points from 0.5 to 30 min.

Cocktail Incubations of UGT Probe Substrates

Glucuronide formation of β-estradiol, chenodeoxycholic acid, trifluoperazine, 5-hydroxytryptophol, propofol, gemfibrozil, zidovudine, amitriptyline, oxazepam and testosterone was used to determine the hepatic metabolic activity of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4/2B7, UGT2B7, UGT2B10, UGT2B15 and UGT2B17, respectively (Supplemental Fig. 1). Oxazepam was incubated as a racemic preparation of *S*- and *R*-stereoisomers. β-estradiol-3-glucuronide was used to assess the microsomal glucuronidation activity of UGT1A1.

Glucuronidation activities were simultaneously evaluated by incubating the UGT probe substrates in several cocktails, in a similar manner to other cocktail studies previously described (Gagez *et al.*, 2012; Joo *et al.*, 2014; Seo *et al.*, 2014; Gradinaru *et al.*, 2015). β-estradiol was incubated alone due to inhibition and mass spectrometry interference effects which made it unsuitable for use in the cocktails. The glucuronidation rate of each substrate in the individual incubation was compared to that in the cocktail incubation to identify potential metabolic and analytical interferences between selected UGT probe substrates. The final substrate concentration was close to or below their corresponding Michaelis-Menten constant (K_m) reported in HLM (Supplemental Table 1, references herein). The final concentration of the UGT substrates used was 5 µM with the exception of zidovudine, for which 100 µM was used. In order to minimize the effect of organic solvent on UGT activity in the incubation, the cocktails of substrates were dissolved in DMSO and serially diluted to the required concentrations. The final concentration of DMSO was 0.5% (v/v).

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Optimization of Incubation Buffer and $MgCl_2$ Concentration

Experimental settings for optimizing the incubation conditions of glucuronidation assays using 10 UGT probe substrates are summarized in Table 1. Pooled HLM (at fixed final microsomal protein concentration of 1 mg/mL) were pre-treated with alamethicin (10 µg/mL) for 30 min on ice at 4°C. Quench solutions containing ice-cold acetonitrile with hexa-deuterated midazolam as the internal standard (final concentration of 500 ng/mL) were placed in the individual reservoirs on the Tecan deck. 160 µL of quench were transferred in 384-deep well plates. 2 µL of each test compound solution was added in the incubation mixtures and placed in a 96-well plate on the Tecan deck. Incubation mixtures containing alamethicin treated-HLM, in either potassium phosphate or Tris-HCl buffer (100 mM, pH 7.4) and $MgCl_2$ at different concentrations (0, 1, 2, 5, 8 and 10 mM) were placed in a 96-well plate on the Tecan desk. 360 µL of the incubation solutions along with the 96-well plate containing UDPGA (50 mM) were pre-incubated for 5 min with shaking (800 rpm) at 37°C.

After 5 min of pre-incubation, reactions were initiated by the addition of 40 µL of UDPGA (final concentration of 5 mM) to obtain a final incubation volume of 0.4 mL. Reactions were terminated by transferring 80 µL of incubation mixture at each time point (0.5, 3.5, 6.5, 10, 15, 20, 25 and 30 min) into the 384-deep well plates preloaded with quench solutions. Plates were then sealed with adhesive aluminum sealing foils using an automated plate sealer (Agilent Technologies, Santa Clara, CA, USA). Samples were centrifuged (Heraeus Multifuge X3R centrifuge, ThermoFisher Scientific, Waltham, MA, USA) at 4°C and 6000 g for 10 min. 50 µL of supernatant was transferred into 384-well plates. All reaction rate calculations were performed using multiple timepoint data, and following linear initial rate conditions. Glucuronidation reactions were carried out in triplicate, unless mentioned.

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Optimal Concentration of UDPGA

UGT probe substrates were co-incubated with UDPGA at different concentrations (1, 2.5, 3.75, 5, 8, 12, 16 and 25 mM) as well as with optimal incubation buffer (100 mM Tris-HCl) and 10 mM $MgCl_2$ concentration in pooled HLM (at fixed final microsomal protein concentration of 1 mg/mL). All reactions were terminated after 3.5 to 15 min of incubation to ensure linearity and measurability using this microsomal protein concentration. Glucuronidation reactions were carried out in triplicate, unless otherwise mentioned.

Protein Binding to HLM and BSA

Protein binding for all selected UGT probe substrates was measured at a low drug concentration and under optimized incubation conditions of the *in vitro* glucuronidation assays (10 m$MgCl_2$, 5 mM UDPGA and 100 mM Tris-HCl buffer). The binding of aforementioned compounds to BSA at different concentrations (0, 0.1, 0.2, 0.5, 1, 1.5 and 2% w/v) in pooled HLM (at fixed final microsomal protein concentration of 1 mg/mL) was determined via high-throughput equilibrium dialysis, a commonly used technique as previously described by (Kariv *et al.*, 2001; Vanstraelen *et al.*, 2014). After equilibrating for 5 hours, solutions from both chambers were removed, quenched and stored at −20˚C until further analysis. The unbound fraction of UGT substrates is assumed to be comparable in the incubation mixture and under the aforementioned conditions in pooled and individual HLM.

Effect of Bovine Serum Albumin on Hepatic UGT Activity.

UGT probe substrates were co-incubated with BSA at different concentrations (0, 0.1, 0.2, 0.5, 1, 1.5 and 2% w/v) as well as with optimal incubation buffer and concentrations of $MgCl_2$, UDPGA and UGT probe substrates in pooled HLM (at fixed final microsomal protein concentration of 1 mg/mL). Reactions were terminated after 0.5-30 min of incubation with

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measurements at eight time points (0.5, 3.5, 6.5, 10, 15, 20, 25 and 30 min). At each BSA concentration, the unbound glucuronidation rate values were obtained by dividing measured total glucuronidation rates by unbound fraction values. In addition, effect of BSA on the between-subject variability in activity of selected UGT isoforms, for which BSA significantly influences glucuronidation activity, was assessed. Incubations were conducted in the presence of BSA at different concentrations (0, 0.1 and 0.5% w/v) and using 15 individual HLM derived from single adult donors.

UHPLC-MS/MS Analysis of Glucuronide Metabolites

The UHPLC–MS/MS systems used in this study were a LC-30-A-series UHPLC system (Shimadzu, Kyoto, Japan) combined with an API 6500 mass spectrometer (AB Sciex LLC, Framingham, MA,USA) and an Nexera X2 autosampler (Shimadzu, Kyoto, Japan). Samples (2 µL) were injected onto an Ascentis Express C18 HPLC column (2.7 µm, 2.1 x 20 mm, Sigma-Aldrich, St Louis, MO, USA) and eluted with aqueous solution contains 0.1% formic acid (v/v) (A) and acetonitrile solution contains 0.1% formic acid (v/v) (B). The flow rate was set at 0.9 mL/min over a period of 1 minute. Compounds were eluted using the following gradient conditions: initial flow rate begins at 95% of solvent A and changes to 2% at 0.50 min, and 95% at 0.66 min to stop at 1.00 min. Mass detection, ionization mode and retention time of investigated compounds are summarized in Table 2. Data acquisition, data handling and instrument control were performed using Analyst® 1.6 software package (AB Sciex LLC, Framingham, MA, USA).

To determine the optimal incubation buffer and $MgCl_2$ concentration, the relative UGT activities using specific probe substrates were expressed as relative rate percentage and assessed by dividing the glucuronide metabolite to internal standard peak area ratio. The peak area ratio obtained in the presence of Tris-HCl buffer and 10 mM $MgCl_2$ was defined as

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100%. To assess the impact of BSA on the microsomal UGT activities, the relative UGT activities using specific probe substrates are expressed as relative rate percentage and assessed by dividing the glucuronide metabolite to internal standard peak area ratio, with the highest peak area ratio obtained defined as 100%. The results of triplicate incubations are presented as the mean ± standard deviation (S.D.). Parent drug concentration (or relative concentration) was assessed for all incubations to ensure correct spiking of test compounds and no detectable depletion of drug substrate.

Kinetic Data Analysis

Kinetic constants for substrate glucuronidation using human liver microsomes were generated by fitting non-linear models to experimental data: Michaelis-Menten equation for a single-enzyme model, Hill and substrate inhibition equations, as described previously (Hutzler and Tracy, 2002). GraphPad Prism version 7.3 for Windows (GraphPad Software, La Jolla CA, USA) was used. Relative activity corresponding to glucuronide over internal standard peak area ratio was plotted against UDPGA concentration varying from 1 to 25 mM. Visual inspection of fitted functions was used to select the best-fit enzyme kinetic model. The results of triplicate incubations are presented as the mean ± S.D.

Statistical Analysis

Comparison of the mean glucuronidation rate of UGT isoforms between different experimental conditions and the between-subject variability in the absence and presence of BSA (0.1% and 0.5% w/v) was performed using a two-tailed t-test. P-value (P) < 0.05 was considered as statistically significant. Statistical comparisons were performed using GraphPad Prism version 7.3 for Windows (GraphPad Software, La Jolla CA, USA).

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Results

Cocktail Incubations of UGT Probe Substrates using UHPLC-MS/MS

We aimed to determine different combinations of UGT probe substrates suitable to measure simultaneously the glucuronidation activity of multiple UGTs in human hepatic microsomal preparation. For the first time, a set of cocktail assays were developed using the Tecan Fluent system to measure hepatic UGT activity simultaneously for a panel of 10 UGT probe substrates using UHPLC-MS/MS. Four cocktail incubations and one individual incubation (β-estradiol) of UGT probe substrates were performed simultaneously within the same run applying similar experimental conditions (Table 3). The simultaneous incubation of two substrates (i.e., cocktail 1: trifluoperazine and gemfibrozil, cocktail 2: amitriptyline and oxazepam and cocktail 3: propofol and zidovudine) or three substrates (i.e., cocktail 4: chenodeoxycholic acid, 5-hydroxytryptophol and testosterone) revealed no changes in glucuronidation activity (< 1.2-fold) in comparison to individual incubations. In addition, no metabolic or analytical interactions among selected UGT probe substrates were detected for any metabolites during cocktail incubations in pooled HLM. The relative standard deviations ranged from 4.4 to 24.0% for the results of the cocktail incubation procedure, which were comparable to those of the individual incubations (3.3-19.6%).

Optimal Incubation Buffer and $MgCl_2$ Concentration

A panel of 10 selective UGT probe substrates was incubated to determine the glucuronidation activity of hepatic UGT isoforms in alamethicin-pretreated pooled HLM. The effect of incubation buffer (i.e. Tris-HCl and potassium phosphate buffer 100 mM, pH 7.4) as well as increasing $MgCl_2$ concentrations (0, 1, 2, 5, 8 and 10 mM) was assessed on the glucuronidation activity of major hepatic UGT isoforms. Incubation of UGT probe substrates showed that the rate of glucuronide formation in pooled HLM (1 mg/mL) was linear up to 3.5

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min. A fixed microsomal protein concentration of 1 mg/mL was selected to represent high enzyme activity conditions under which most (relatively stable) candidate drugs are likely to be assessed and where monitoring the UGT enzymes is subject to the highest challenge in terms of probe substrate metabolism linearity. For amitriptyline (UGT2B10) and oxazepam (UGT2B15), incubation time data up to 15 minutes was used. These conditions were applied in subsequent experiments. All glucuronidation activity values were expressed as relative rate percentage i.e. peak area ratio of glucuronide over internal standard and, normalized with the ratio obtained using Tris-HCl buffer in the presence of 10 mM $MgCl_2$, which was defined as 100%.

Incubations of pooled HLM formed the glucuronides of all selected UGT substrates in the absence and presence of $MgCl_2$, and with both incubation buffers (Fig. 1). The glucuronidation rates increased with increasing $MgCl_2$ concentrations with either Tris-HCl or potassium phosphate incubation buffers. Greatest glucuronidation rates were observed in the presence of 10 mM $MgCl_2$, with the exception of oxazepam (UGT2B15) and testosterone (UGT2B17), for which the maximum glucuronidation activity was obtained at 5 and 8 mM $MgCl_2$, respectively. However, the 10 mM condition was less than 10% lower than the maximum activity observed for these substrates. In Tris-HCl incubations and in the absence of $MgCl_2$, the activity of UGT1A9 (propofol) and UGT1A6 (5-hydroxytryptophol) represented 13% and 74% of the activity obtained in the presence of 10 mM $MgCl_2$, respectively.

In the presence of 10 mM $MgCl_2$, glucuronidation activities were significantly greater with Tris-HCl buffer compared to potassium phosphate buffer for chenodeoxycholic acid (UGT1A3), trifluoperazine (UGT1A4), propofol (UGT1A9), gemfibrozil (UGT2B4/2B7) and testosterone (UGT2B17) (Fig. 1). In contrast, no significant differences in glucuronidation rates were detected for β-estradiol (UGT1A1), 5-hydroxytryptophol (UGT1A6), zidovudine

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(UGT2B7), amitriptyline (UGT2B10) and oxazepam (UGT2B15). Interestingly, a smaller coefficient of variability was associated with the glucuronidation activities measured with Tris-HCl buffer compared to potassium phosphate buffer although the reason for this observation is not currently known.

Optimal Concentration of UDPGA

Incubations of UGT probe substrates were conducted using pooled HLM with Tris-HCl buffer, 10 mM of $MgCl_2$ and UDGPA concentration ranging from 1 to 25 mM. Kinetic profiling revealed that the formation of β-estradiol-3-glucuronide (UGT1A1), propofol-glucuronide (UGT1A9), and zidovudine-glucuronide (UGT2B7) followed hyperbolic Michaelis-Menten kinetics, as illustrated in Fig. 2. However, the formation of glucuronides of chenodeoxycholic acid (UGT1A3), trifluoperazine (UGT1A4), amitriptyline (UGT2B10), oxazepam (UGT2B15), and testosterone (UGT2B17) exhibited sigmoidal kinetics and were modelled by the Hill equation. When co-incubated with 5 mM UDPGA, the glucuronidation activity of those UGT isoforms represented typically more than 65% of the maximal activity, with the exception of amitriptyline (52%). However, for some compounds, the maximal velocity was not fully reached at 25 mM concentration of UDPGA, the formation rate of trifluoperazine-glucuronide did not reach a plateau at high UDPGA concentrations; UGT1A4 activity represented only 47% of maximal activity at 5 mM UDPGA. Chenodeoxycholic acid, trifluoperazine and testosterone glucuronidation, via UGT1A3, UGT1A4 and UGT2B17 respectively, exhibited positive cooperativity in cofactor binding. The detailed kinetic parameters are summarized in Table 4.

The formation of 5-hydroxytryptophol-glucuronide (UGT1A6) and gemfibrozil-glucuronide (UGT2B4/2B7) displayed substrate inhibition kinetics when co-incubated with increasing UDPGA concentrations. In contrast to other UGT probe substrates, the

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glucuronidation rates started to decrease above concentrations of 3.75-5 mM UDPGA. At 8 and 25 mM UDPGA, a 21% and 46% reduction in UGT1A6 activity was observed in comparison to that at 5 mM UDPGA, respectively (Fig. 2 panel K). Similarly, a 6% to 12.5% reduction in gemfibrozil-glucuronide formation was observed above concentration of 5 mM UDPGA. As a result, 5 mM was defined as an optimal concentration of UDPGA to measure simultaneously the microsomal glucuronidation activity of hepatic UGT isoforms in an automated glucuronidation assay whilst also ensuring that reagent costs are not prohibitive.

Effect of Bovine Serum Albumin on Hepatic Activity of UGT Isoforms.

The present study clearly demonstrated that the effect of BSA supplement on hepatic glucuronidation rates was enzyme- and substrate-dependent (Fig. 3). The total glucuronidation rate of UGT1A9 and UGT2B7, using propofol and zidovudine as probe substrates, increased by 1.6- and 3.4-fold respectively with 2% BSA supplementation. However, compared with the absence of BSA, the total metabolic activity of UGT1A4 (trifluoperazine glucuronidation) in HLM decreased by 12.5% in the presence of 2% BSA (Fig. 3 Part I). For the remaining compounds, the metabolic activity of UGT1A1, UGT1A3, UGT2B15, and UGT2B17 decreased by 75%, 91%, 87%, and 60% with the 2% BSA supplementation, respectively. The total glucuronidation activity of UGT1A6 (5-hydroxytryptophol glucuronidation) and UGT2B10 (amitriptyline glucuronidation) remained unchanged with increasing BSA concentrations.

In the absence of BSA, the unbound fraction of UGT probe substrates ranged from less than 2% for trifluoperazine to 100% for zidovudine and 5-hydroxytryptophol (Fig. 3 Part I). The effect of BSA on the unbound fraction varied between substrates. No changes in unbound fraction was observed for trifluoperazine, propofol and amitriptyline with increasing BSA concentrations up to 2%. Unbound fraction for gemfibrozil reached values near the

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lower limit of quantification with increasing BSA concentrations. In contrast, for the remaining probe substrates, the fraction unbound measured in the incubation mixture decreased in the presence of BSA, between 1.5-fold and 6.3-fold for 5-hydroxytryptophol and chenodeoxycholic acid respectively. However, unbound fractions could not be determined for β-estradiol with increasing BSA concentrations.

Glucuronide formation rates of UGT substrates were corrected for experimentally measured unbound fraction (Fig. 3 Part II). Our results indicated that the optimal BSA concentration at which the greatest unbound glucuronidation activity was obtained differed across UGT isoforms being both enzyme- and substrate-dependent. With the exception of UGT1A6, the greatest unbound glucuronidation rates were not systematically obtained in the presence of 2% BSA. The greatest unbound glucuronidation activities were obtained in the presence of 0.2% BSA (testosterone via UGT2B17), 0.5% BSA (chenodeoxycholic acid via UGT1A3) and 1% BSA (propofol via UGT1A9, zidovudine via UGT2B7 and oxazepam via UGT2B15), in comparison to those without BSA supplement. In addition, BSA inhibitory effects on unbound glucuronidation activities were observed for several UGT isoforms from 0.1% BSA for UGT1A4, 0.5% BSA for UGT2B17 or 1% BSA for UGTs 1A3, 1A9, 2B7 and 2B15.

Effect of Bovine Serum Albumin on Between-Subject Activity of UGT Isoforms.

The effect of BSA on the between-subject variability in the formation rate of chenodeoxycholic acid-glucuronide (UGT1A3), trifluoperazine-glucuronide (UGT1A4), propofol-glucuronide (UGT1A9) and zidovudine-glucuronide (UGT2B7) was assessed using 15 individual HLM characterized with low, moderate and high corresponding glucuronidation activity (in-house data). As illustrated in Fig. 4, the between-subject variability in glucuronidation activity was not significantly reduced in the presence of either

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0.1% or 0.5% BSA compared to that to 0% BSA ($P > 0.05$). Between-subject variability of zidovudine-glucuronide formation (UGT2B7) decreased up to 12% in the presence of 0.5% BSA. In contrast, the variability in UGT1A9 activity (propofol-glucuronide) across individuals slightly increased by 5%.

Discussion

In our study, we aimed to develop and optimize an automated UHPLC-MS/MS-based UGT profiling assay to assess simultaneously the glucuronidation activity of 10 hepatic UGT isoforms using a cocktail incubation approach in human liver microsomes. We identified and implemented methodological and technical improvements to reduce the discrepancies in glucuronidation activity observed across studies, which are in part due to lack of standardization (Badée *et al.*, 2018).

We developed an automated assay using a Tecan Fluent system at Hoffman-La Roche Ltd. Due to its flexible and configurable architecture, a large number of experimental condition combinations could be achieved and this facilitated the systematic testing which is required in order to improve the measurement of *in vitro* glucuronidation activity. Our proposed UGT profiling assay is intended for UGT clearance optimization and phenotyping and prediction of human drug clearance by *in vitro* to *in vivo* scaling data has not been the objective.

Cocktails and individual incubations of selected UGT probe substrates using HLM were compared to ensure that interactions among substrates were avoided. The proposed substrate cassetting approach allows efficient monitoring of 10 glucuronidation activities with minimal effect on enzyme activity or analytical interactions among substrates even under high metabolism conditions (Table 3). However, in comparison to the situation for cytochromes P450 where selective substrates and inhibitors exist (Zientek and Youdim,

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2015), UGT isoform-selective substrates/inhibitors are either limited or not available. Our study highlighted the critical challenge of obtaining selective UGT probe substrates. Whilst some of the UGT probe substrate reactions used in this study show good enzyme selectivity, such as for β-estradiol, trifluoperazine, 5-hydroxytryptophol, propofol and zidovudine, other probe substrates such as gemfibrozil, oxazepam and chenodeoxycholic acid are less selective. Gemfibrozil is metabolized by both UGT2B4 and UGT2B7 isoforms (Mano *et al.*, 2007). In these studies, no selective UGT2B7 inhibitor was co-incubated with gemfibrozil but this could be done in the future to enhance the selectivity of gemfibrozil for UGT2B4. In addition, oxazepam was formulated as a racemic preparation of *S*- and *R*-enantiomers, as *S*-oxazepam was not available. *S*-oxazepam is principally metabolized by UGT2B15, while *R*-oxazepam is also metabolized by additional UGTs, mainly UGT1A9 and UGT2B7 (Court, 2002). However, we demonstrated in stereoselective analyses that *S*-oxazepam glucuronide was mostly generated by pooled HLM (80.7 ± 2.9%) in comparison to *R*-oxazepam glucuronide under the incubation conditions used in this study (in-house data), which allowed the use of the racemic oxazepam to assess the microsomal glucuronidation activity of UGT2B15. Chenodeoxycholic acid is mainly metabolized by UGT1A3, with a minor relative contribution of UGT2B7 and UGT1A1 (Gradinaru *et al.*, 2015). The relative activity of recombinantly expressed UGT1A1 and UGT2B7 isoforms were less than 1.5% of UGT1A3 activity in chenodeoxycholic acid glucuronidation (in-house data), indicating a good selectivity of chenodeoxycholic acid for UGT1A3. However, chenodeoxycholic acid would not be a useful UGT1A3 probe substrate in a more complex system such as hepatocytes due to very high taurine and glycine conjugation rates (Thakare *et al.*, 2018). Raloxifene, considered as a partial substrate of UGT1A8 and UGT1A10 (Kemp *et al.*, 2002), was not included in the present report, as neither UGT isoforms were detected in HLM (Fallon *et al.*, 2013; Margaillan *et al.*, 2015). However, we found that raloxifene could be combined with

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trifluoperazine and gemfibrozil in cassette (at 5 µM) (in-house data) and may be a useful additional probe for investigations of extra-hepatic raloxifene metabolism using human intestinal microsomes, where UGT1A8 and UGT1A10 are highly expressed (Tukey and Strassburg, 2000).

The lack of standardization of incubation conditions of *in vitro* glucuronidation assays is one of the main sources of inter-study variability. Therefore, we examined whether standardized incubation conditions would enhance the determination of glucuronide formation rates of multiple hepatic UGT isoforms in HLM. Our results indicated that greater microsomal glucuronidation activity by different hepatic UGT isoforms was obtained using: 10 mM $MgCl_2$ and 5 mM UDPGA with 100 mM Tris-HCl buffer. This is in agreement with a previous study by Walsky and colleagues (reference?). They demonstrated greater activity of five hepatic UGT isoforms (UGTs UGT1A1, 1A4, 1A6, 1A9 and 2B7) using 10 mM $MgCl_2$ with 100 mM Tris-HCl buffer in comparison with those obtained with potassium phosphate buffer and 0 to 5 mM $MgCl_2$. Our results further revealed that glucuronidation activities of previously less characterized UGT isoforms (UGTs UGT1A3, 2B10, 2B15 and 2B17) were enhanced under the aforementioned optimized conditions (Fig. 1).

A 5 mM UDPGA concentration is generally used in UGT phenotyping assays in HLM although the impact of varying UDPGA concentration has not been systematically examined. A recent publication from Lu and colleagues demonstrated that the maximal metabolic activity of UGT1A4 and UGT2B10 was reached when 8 mM UDPGA was used, which was the highest UDPGA concentration tested (Lu *et al.*, 2017). In this study, we demonstrated that the microsomal activity of hepatic UGT isoforms increased as UDPGA concentration increased. The selected substrates panel, with the exception of 5-hydroxytryptophol and gemfibrozil, followed either hyperbolic Michaelis-Menten kinetics or sigmoidal kinetics reaching 52-96% of maximal activity at 5 mM UDPGA (Fig. 2). Thus, we

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demonstrated that the concentration of the main co-substrate had a significant impact on the microsomal UGT metabolic capacity. The reasons that the activity of UGT1A6 and UGT2B4/2B7 decreases with UDPGA concentrations above 5 mM remain to be determined but a recent paper by Walia and colleagues support our findings (Walia *et al.*, 2018). They found that the combination of 5 mM UDPGA and 10 mM $MgCl_2$ led to maximal activities in both HLMs and recombinantly expressed UGT1A1 and UGT1A4 isoforms, but not in UGT1A6 and UGT1A9, for which the specific mechanism appeared to be susceptibility to inhibition by UDP (a product of the co-substrate). Of note, saccharolactone was not included in this optimization. Walsky and colleagues demonstrated that saccharolactone supplementation did not significantly affect the microsomal activity of UGT1A1, UGT1A6 and UGT2B7, whilst decreased activity of UGT1A4 and UGT1A9 was observed (Walsky *et al.*, 2012). Nevertheless, inclusion of saccharolactone is favoured by some laboratories and its addition may be helpful where significant microsomal glucuronidase activity towards individual drug compounds exists.

The inclusion of BSA is thought to sequester inhibitory fatty acids produced during microsomal incubation, and enhanced the turnover of some UGT substrates whilst inhibiting that of others (Rowland *et al.*, 2007, 2008; Walsky *et al.*, 2012). Our results are consistent with published data. Total and unbound UGT1A9 and UGT2B7 glucuronidation activity, using propofol and zidovudine as probe substrates, increased respectively by up to 74% and 343% in the presence of 2% BSA in comparison to that in absence of BSA (Fig. 3). Although the addition of BSA enhanced the unbound glucuronidation activity of a number of UGT isoforms, the recommended BSA concentration of 2% did not systematically produce the greatest unbound activity. We demonstrated that the BSA inhibitory effects on either total or unbound glucuronidation activities UGTs were BSA concentration-, isoform- and substrate-dependent. We noted that the variability in activity was not significantly reduced between

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different HLM donors, as might have been expected if each donor sample contained an individual and varied concentration of fatty acids (Fig. 4). We hypothesized that this is due to the lack of donor-dependent fatty acid concentrations present in the individual donor microsomal incubations, although the concentrations and identified fatty acids released during incubation were not investigated. According to our results, we consider that the inclusion of BSA is not advantageous when considering the simultaneous characterization of microsomal glucuronidation activities of hepatic UGT isoforms. This is especially true for new drug molecules of moderate to high lipophilicity as the greatly enhanced protein binding significantly out-weighed the 2- to 3-fold activity enhancement of a small number of UGT enzyme activities and especially if intrinsic clearance of parent drug is the main measurement. Therefore, high total glucuronidation activity tools may be a more suitable for the characterization of metabolically stable new drug candidates especially when the effect of BSA binding and the identity of the UGT enzymes involved in metabolism had not yet been determined, and where BSA-related binding may substantially diminish metabolism measurability.

Conclusion

To date, few *in vitro* UGT profiling studies have been reported using conditions optimized for such a wide variety of UGT isoform activities. In addition, only small numbers of positive control compounds are routinely deployed as markers of UGT activity in phenotyping experiments. In a pharmaceutical industry setting, use of the proposed substrates panel will allow control of multiple glucuronidation reactions in the automated screens used for compound optimization. The substrate cassetting approach enables more data to be generated using the same amount of (sometimes scarce) biological material and analytical resource. Automated glucuronidation assays making use of these control cassettes and

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optimized experimental settings also enable more effective performance of multiple-donor activity studies e.g. for correlation analyses, ontogeny investigations or assessment of UGT polymorphism effects on drug clearance.

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Authorship contributions

Participated in research design: Badée, Qiu, Parrott, Collier, Schmidt and Fowler.

Conducted experiments: Badée and Qiu.

Performed data analysis: Badée, Qiu and Fowler.

Wrote or contributed to the writing of the manuscript: Badée, Qiu, Parrott, Collier, Schmidt and Fowler.

Conflicts of Interest

Justine Badée, Nahong Qiu, Neil Parrott, Abby C. Collier, Stephan Schmidt and Stephen Fowler declare that they have no conflicts of interest that might be relevant to the content of the manuscript.

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Legends of Figures

Figure 1. Effect of incubation buffer and increasing $MgCl_2$ concentrations on hepatic UGT activities in pooled human liver microsomes. Bars represent the relative glucuronidation rates of UGT isoforms determined using 10 UGT probe substrates in the absence and presence of $MgCl_2$ at different concentrations (1, 2, 5, 8 and 10 mM), and with either potassium phosphate buffer (black) or Tris-HCl buffer (grey) (100 mM, pH 7.4). For each substrate, the relative UGT activities were expressed as relative rate percentage and assessed by dividing the glucuronide metabolite to internal standard peak area ratio, with the peak area ratio obtained in the presence of Tris-HCl buffer and 10 mM $MgCl_2$ defined as 100%. Paired t-test between phosphate and Tris-HCl incubation buffers, *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Each bar represents mean ± standard deviation (S.D.) (n=3).

Figure 2. Kinetics of 10 UGT probe substrates over a wide UDPGA concentration range (1, 2.5, 3.75, 5, 8, 12, 16 and 25 mM) in pooled human liver microsomes. Symbols represent the mean peak area ratio of glucuronide metabolite to internal standard ± S.D. (n=3). Solid line represents the fit of the reaction velocity plotted against increasing UDPGA concentrations using nonlinear least-squares regression analysis. Experimental data were fitted by using a Michaelis-Menten kinetic model (A-C), an allometric sigmoidal kinetic model (D-H) and a substrate inhibition kinetic model (I-J). Relative glucuronidation rate of UGT isoforms using selective UGT probe substrates over a wide UDPGA concentration range (1, 2.5, 3.75, 5, 8, 12, 16 and 25 mM) in pooled human liver microsomes (K). Relative UGT activities were expressed as relative rate percentage, and assessed by dividing the glucuronide metabolite to internal standard peak area ratio, with the peak area ratio obtained in the presence of 5 mM UDPGA defined as 100%. Dotted and dashed lines represent the relative glucuronidation formation of 5-hydroxytryptophol (UGT1A6) and gemfibrozil (UGT2B4/2B7).

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Figure 3. Effect of increasing BSA concentrations on the total (Part I) and unbound (Part II) glucuronidation rates of hepatic UGT isoforms in pooled human liver microsomes. Black bars represent total relative rate percentage, which were determined by dividing the glucuronide metabolite to internal standard peak area ratio. Circles and dashed line represent the unbound fraction of UGT probe substrates measured in HLM in the absence and presence of increasing BSA concentrations (0.1, 0.2, 0.5, 1, 1.5 and 2% w/v). Protein binding were determined via high-throughput equilibrium dialysis. Protein binding in HLM was not measurable for β-estradiol. Grey bars represent the unbound glucuronidation relative rates corrected by the experimentally measured unbound fraction. For each substrate, the glucuronide metabolite to internal standard peak area ratio obtained without BSA supplement was defined as 100%. Paired t-test compared to 0% BSA, *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Each bar represents mean ± S.D. (n=3).

Figure 4. Between-subject variability of UGT1A3 (A), UGT1A6 (B), UGT1A9 (C) and UGT2B7 (D) activity in the absence and presence of BSA (0.1% and 0.5% w/v) and using a panel of 15 individual human liver microsomes. Hepatic glucuronidation activity data were reported as relative rate percentage with the average activity rate defined as 100% (dashed line). Each point represents mean ± S.D. (n=3).

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Tables

Table 1. Experimental settings for optimizing incubation conditions of automated UGT phenotyping assay using a panel of 10 UGT probe substrates in human liver microsomes.

Experimental conditions	
Material	Pooled human liver microsomes (150-donors) 15 human liver microsomes from single donors
Enzyme concentration (mg/mL)	1
Incubation volume (mL)	0.4
Alamethicin treatment (µg/mL)	10, for 30 min at 4°C
Pre-incubation step (min)	5, shaking at 37°C
Incubation time (min)	0.5-30, shaking at 37°C
Incubation buffer	Potassium phosphate (100 mM at pH 7.4) Tris-HCl (100 mM at pH 7.4)
$MgCl_2$ (mM)	0, 1, 2, 5, 8 and 10
UDPGA (mM)	1, 2.5, 3.75, 5, 8, 12, 16 and 25
BSA (w/v %)	0, 0.1, 0.2, 0.5, 1.0 1.5 and 2

$MgCl_2$: Magnesium chloride, UDPGA: uridine 5'-diphosphoglucuronic acid, BSA: Bovine serum albumin

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Table 2. UHPLC-MS/MS conditions used to determine the glucuronidation activity of hepatic UGT isoforms based on the appearance of metabolite glucuronides of 10 probe substrates in human liver microsomes.

Analyte	Q1 mass (m/z)	Q3 mass (m/z)	MRM	Declustering Potential (V)	Collision Energy (V)	Retention Time (min)
β-Estradiol-3-glucuronide	447	113	Negative	-55	-30	0.52
Chenodeoxycholic acid-glucuronide	568	382	Negative	-80	-50	0.46
Trifluoperazine-glucuronide	584	408	Positive	75	45	0.42
5-Hydroxytryptophol-glucuronide	352	176	Negative	-30	-35	0.18
Propofol-glucuronide	353	117	Negative	-40	-30	0.43
Gemfibrozil-glucuronide	425	249	Negative	-70	-30	0.49
Zidovudine-glucuronide	442	125	Negative	-80	-26	0.25
Amitriptyline-glucuronide	454	278	Positive	80	30	0.44
Oxazepam-glucuronide *	464	288	Positive	80	30	0.44
Testosterone-glucuronide	465	289	Positive	80	30	0.40
Hexa-deuterated midazolam **	332	297	Positive	110	25	0.37

MRM: Multiple reaction monitoring, *: Incubation of racemic oxazepam, **: Used as internal standard

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Table 3. Cocktail incubation approach using pooled human liver microsomes and Tecan Fluent System. 10 UGT probe substrates were incubated either individually or in cocktails.

Cocktail group	UGT probe substrates	UGT isoforms	Incubation concentration (μM)	Relative rate % (CV%)		Fold difference
				Individual incubation	Cocktail incubation	
1	Trifluoperazine	UGT1A4	5	100 ± 3.3	111.5 ± 7.3	1.1
	Gemfibrozil	UGT2B4/2B7	5	100 ± 12.9	92.1 ± 13.6	0.9
2	Amitriptyline	UGT2B10	5	100 ± 10.7	94.2 ± 20.0	0.9
	Oxazepam *	UGT2B15	5	100 ± 2.0	104.7 ± 21.2	1.0
3	Propofol	UGT1A9	5	100 ± 13.3	105.1 ± 11.8	1.1
	Zidovudine	UGT2B7	100	100 ± 4.4	97.5 ± 4.4	1.0
4	Chenodeoxycholic acid	UGT1A3	5	100 ± 10.9	98.0 ± 7.6	1.0
	5-Hydroxytryptophol	UGT1A6	5	100 ± 12.0	101.0 ± 6.1	1.0
	Testosterone	UGT2B17	5	100 ± 3.9	87.1 ± 7.2	0.9
5	β-Estradiol	UGT1A1	5	N.A.	N.A.	N.A.

N.A.: not applicable, *: Incubation of racemic oxazepam. Data were reported as relative rate % (Coefficient of variation %) (n=3). Relative rate measured in individual incubation was defined as 100% for each substrate.

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Table 4. Hepatic glucuronidation activities using 10 UGT probe substrates in pooled human liver microsomes and in the presence of increasing UDPGA concentrations (1, 2.5, 3.75, 5, 8, 12, 16 and 25 mM).

UGT substrate concentration (5 µM)	K_m (mM)	Maximal reaction rate	S_{50} (mM)	n	K_i (mM)	% of maximal activity at 5 mM UDPGA	Model
β-Estradiol	1.77 ± 0.14	0.64 ± 0.01	N.A.	N.A.	N.A.	73	MM
Chenodeoxycholic acid	4.79 ± 0.80	0.27 ± 0.01	2.29 ± 0.12	1.89 ± 0.18	N.A.	80	SMM
Trifluoperazine	9.13 ± 0.99	0.34 ± 0.02	5.59 ± 0.56	1.29 ± 0.11	N.A.	47	SMM
5-Hydroxytryptophol	2.79 ± 1.74	1.02 ± 0.40	N.A.	N.A.	3.69 ± 2.02	84 +	SI
Propofol	0.80 ± 0.17	0.09 ± 0.00	N.A.	N.A.	N.A.	76	MM
Gemfibrozil	0.04 ± 0.10	0.40 ± 0.02	N.A.	N.A.	113.7 ± 61.8	89 +	SI
Zidovudine *	2.47 ± 0.33	0.29 ± 0.01	N.A.	N.A.	N.A.	65	MM
Amitriptyline	3.07 ± 0.19	1.19 ± 0.07	4.47 ± 0.83	0.75 ± 0.07	N.A.	52	SMM
Oxazepam **	1.02 ± 0.12	0.07 ± 0.00	1.04 ± 0.18	0.68 ± 0.14	N.A.	75	SMM
Testosterone	1.63 ± 0.20	1.59 ± 0.03	1.28 ± 0.07	1.94 ± 0.21	N.A.	96	SMM

MM: Hyperbolic Michaelis-Menten model, SMM: Sigmoid Michaelis-Menten model, SI: Substrate inhibition model, N.A.: Not available, K_m: UDPGA affinity constant, S_{50}: UDPGA concentration at which half of maximal activity is reached, n: Hill coefficient, K_i: Substrate inhibition constant, *: Incubation of 100 µM Zidovudine, **: Incubation of racemic oxazepam, $^+$: Maximal activity reached at 2.5 mM UDPGA. Data are reported as mean ± S.D. (n=3).

Figures

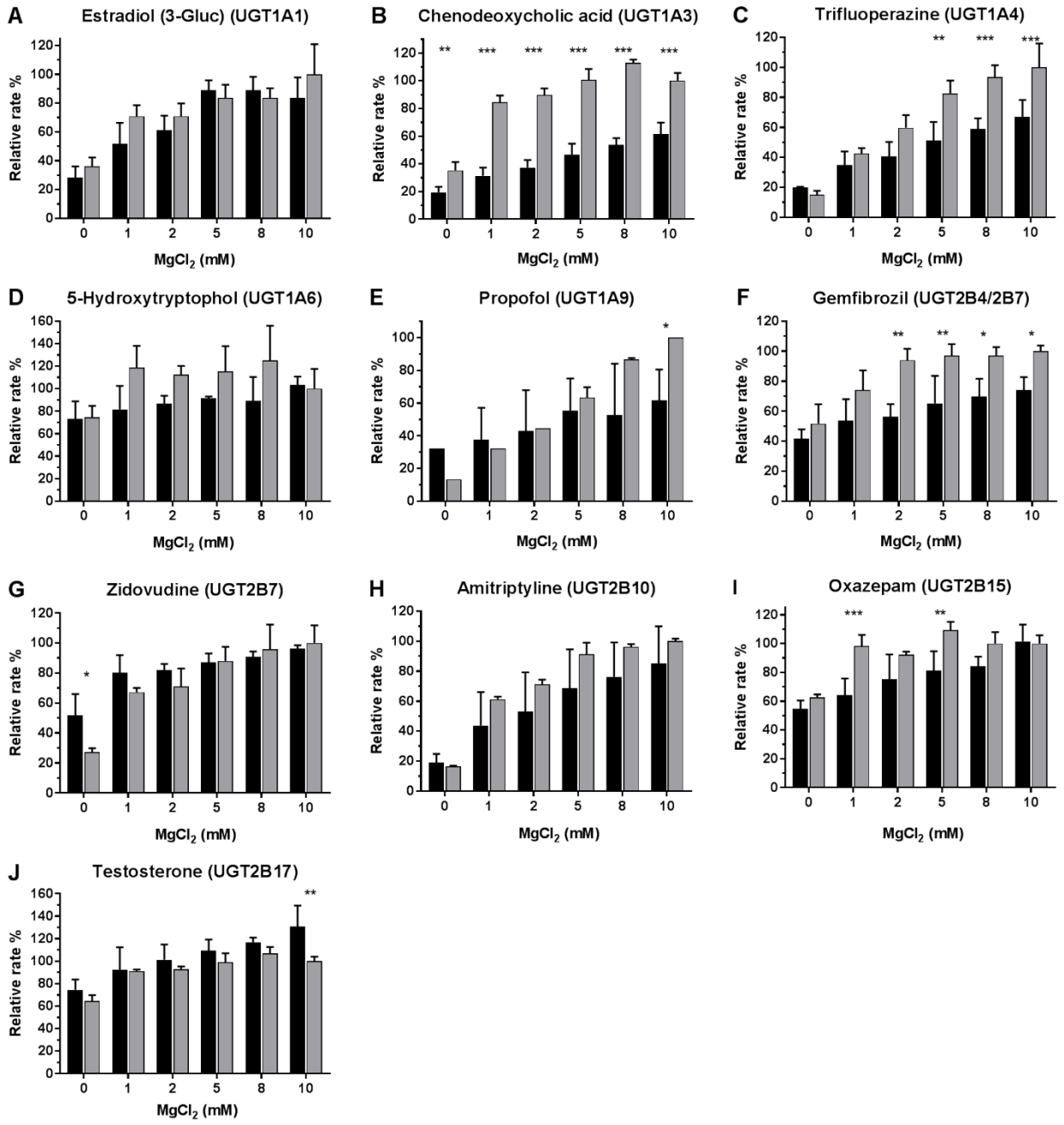

Figure 1.

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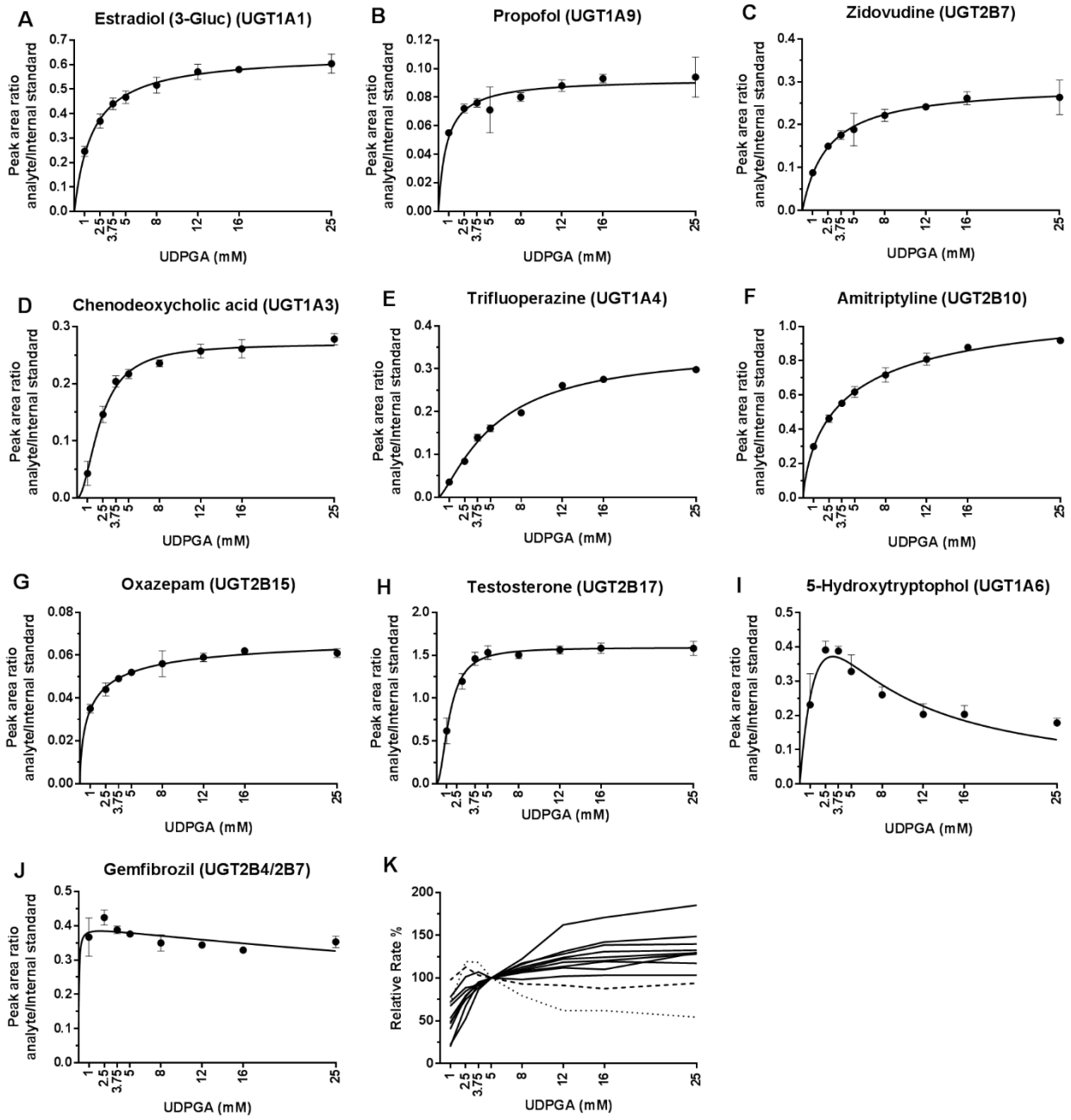

Figure 2.

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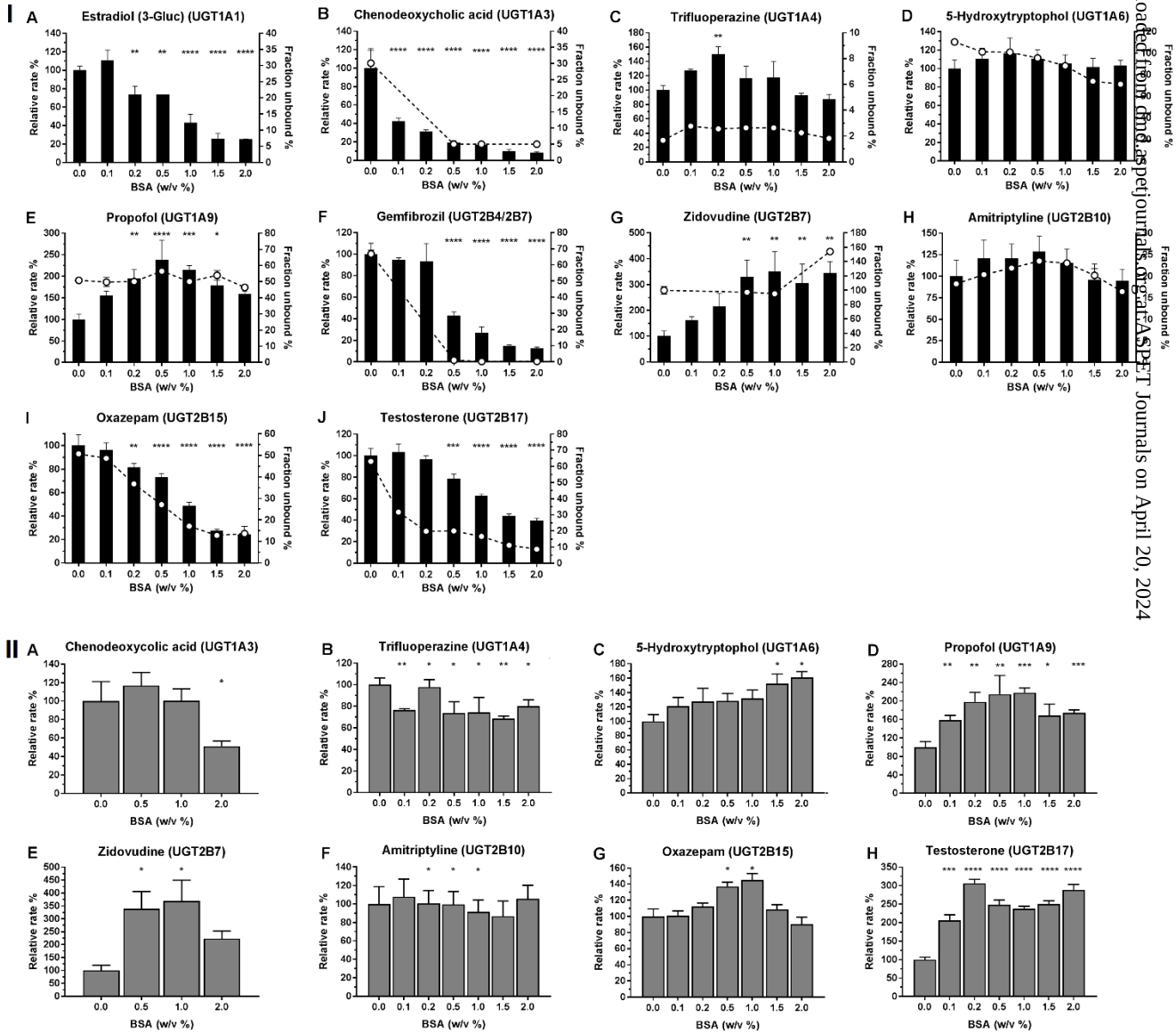

Figure 3.

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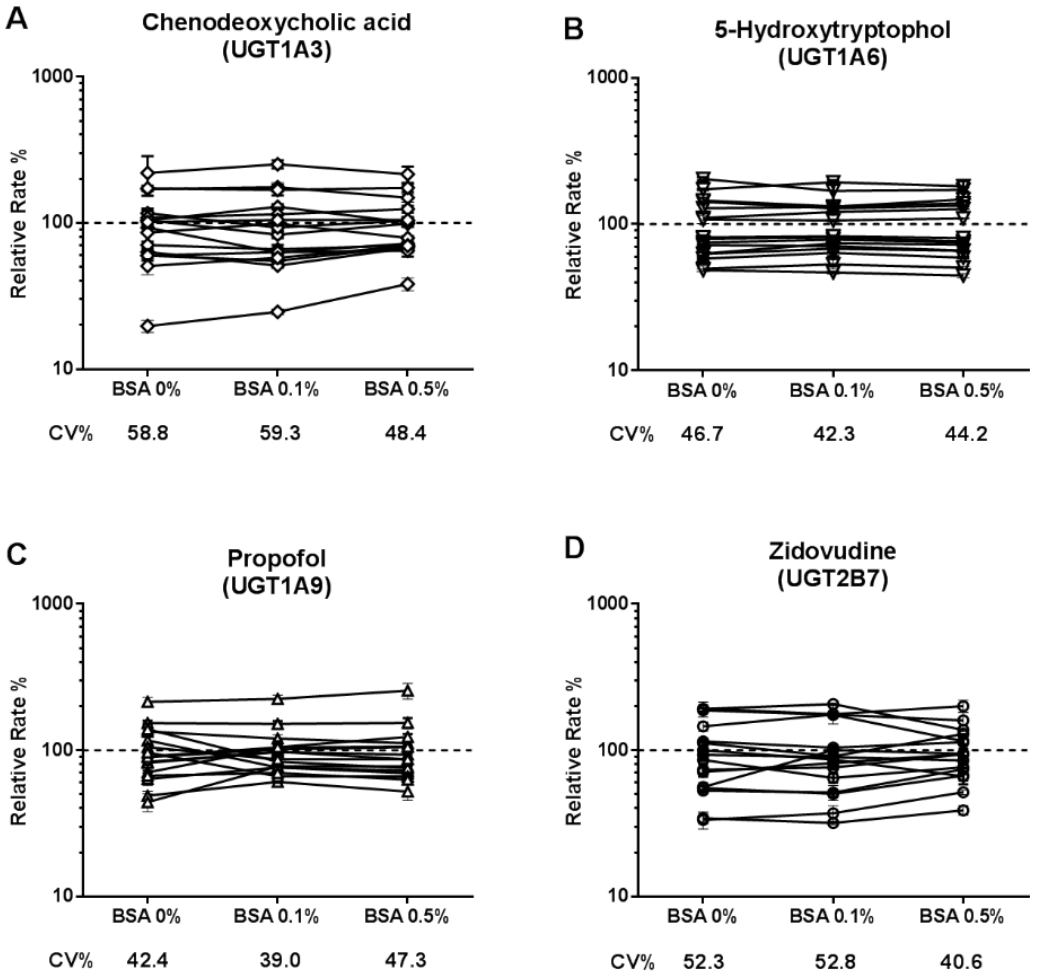

Figure 4.

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