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The configuration of the 17-hydroxy group variably influences the glucuronidation of β-estradiol and epiestradiol by human UDP-glucuronosyltransferases*

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UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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Abstract

The glucuronidation of 17β-estradiol (β-estradiol) and 17α-estradiol (epiestradiol) was studied to elucidate how the orientation of the 17-OH group affects conjugation at the 3-OH or the 17-OH of either diastereomer. Recombinant human UDP-glucuronosyltransferases (UGTs) 1A1, 1A3, 1A7, 1A8 and 1A10 conjugated one or both diastereomers, mainly at the 3-OH. The activity of UGT1A4 was low and unique since it was directed merely towards the 17-OH of both aglycones. UGT1A10 exhibited particularly high estradiol glucuronidation activity, the rate and affinity of which were significantly higher in the case of β-estradiol than with epiestradiol. UGT1A9 did not catalyze estradiol glucuronidation but UGT1A9-catalyzed scopoletin glucuronidation was competitively inhibited by β-estradiol. UGTs 2B4, 2B7 and 2B17 exclusively conjugated the estradiols at the 17-OH position in a highly stereoselective fashion. UGT2B4 was specific for epiestradiol, UGT2B7 glucuronidated both diastereomers, with high affinity for epiestradiol, whereas UGT2B17 only glucuronidated β-estradiol. UGT2B15 glucuronidated both estradiols at the 3-OH, with a strong preference for epiestradiol. Human UGT2A1 and UGT2A2 glucuronidated both diastereoisomers at both hydroxyl groups. Microsomal studies revealed that human liver mainly yielded epiestradiol 17-O-glucuronide, human intestine primarily β-estradiol 3-O-glucuronide, while rat liver preferentially formed β-estradiol 17-O-glucuronide. Of the 3 recombinant rat UGTs that were examined in this study, rUGT2B1 was specific for the 17-OH of β-estradiol, rUGT2B2 did not catalyze estradiol glucuronidation, whereas rUGT2B3 exhibited high activity towards the 17-OH in both diastereoisomers. The results demonstrate that while many UGTs can catalyze estradiol glucuronidation, there are marked differences in their kinetics, regioselectivity and stereoselectivity.

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UDP-glucuronosyltransferases (EC 2.4.1.17; UGTs) are important conjugation enzymes that catalyze the glucuronidation of a large number of xenobiotics and physiological compounds. In the glucuronidation reaction the glucuronic acid moiety is transferred from UDP-glucuronic acid (UDPGA) to the aglycone substrate. The aglycone substrates are mainly small lipophilic molecules and conjugation with glucuronic acid increases their water solubility and accelerates their excretion through bile or urine (Tukey and Strassburg, 2000; King et al., 2000; Wells et al., 2004). The UGTs are membrane proteins of the endoplasmic reticulum that are expressed in the liver and many other tissues, including intestine, kidney, breast, placenta, testis and prostate (Tukey and Strassburg, 2000; King et al., 2000).

The UGTs are divided into three subfamilies, UGT1A, UGT2A and UGT2B (Mackenzie et al., 2005). The *UGT1* gene locus encodes the nine members of the UGT1A subfamily, UGT1A1 and UGTs 1A3-1A10 (Mackenzie et al., 2005; Gong et al., 2001). All these enzymes share exons 2-5 and therefore the amino acid sequence of their C-terminal half is identical. The first exons are specific for the individual UGT1As and they encode the variable N-terminal domains of the proteins. Nonetheless, there are high homologies also among the N-terminal domains of many UGT1As, particularly among UGT 1A3-1A5 and UGT 1A7-1A10. Within the UGT2A subfamily, there is probably exon sharing between UGT2A1 and UGT2A2 that leads to identical C-terminal halves. In UGT2A3 or among the UGT2Bs there is no exon sharing and each enzyme is encoded by a separate gene that contains all the exons (Mackenzie et al., 2005). The human UGT2B subfamily contains 7 members: UGT 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28 (Turgeon et al., 2001; Levesque et al., 2001). UGTs 2B15 and 2B17 are 90% identical, while the percent identity among the other 2B isoforms is 70-79% (Tukey and Strassburg, 2001).

UGT substrate specificity is complex as many UGTs can glucuronidate several different compounds that vary significantly in their chemical structure. There is often a partial overlap in the substrate specificity of many human UGTs, perhaps reflecting the high sequence homology among them. On the other hand, there are also sharp differences in the activity even between highly similar isoforms such as UGTs 1A3 and 1A4, 1A9 and 1A10, or 2B15 and 2B17 (Kubota et al., 2007; Sten et al., 2006; Dubois et al., 1999). One of the major challenges in current UGT research is to better understand the factors that determine the substrate specificity of the different UGTs in order to be able to predict the glucuronidation of new compounds. Few pharmacophore-based models for the prediction of substrate specificity of individual UGTs have already been developed (Smith et al., 2003a; Smith et al., 2003b; summarized by Miners et al., 2004), but there is a clear need for additional detailed information on the preference of these enzymes with respect to regio- and stereo-selective conjugation.

17β-Estradiol (β-estradiol) is an important endogenous estrogen in humans that is also used clinically for the treatment of hormonal disorders in women, particularly hormone replacement treatment. Its xenobiotic diastereoisomer, 17α-estradiol or epiestradiol (Fig. 1), is of minor significance as its affinity for estrogen receptors is low compared to β-estradiol (reviewed by Dykens et al., 2004). Nevertheless, epiestradiol was found to have protective effects against toxic stressors in cells, that are independent of estrogen receptors (Dykens et al., 2004; Morin et al., 2002). Dykens et al. (2004) have even suggested that epiestradiol could be a potential drug candidate for Alzheimer's and Parkinson's diseases. After oral administration most of the epiestradiol is rapidly conjugated (Hobe et al., 2002), and conjugated forms of β-estradiol are readily detectable in blood (Raftogianis et al., 2000). In estrogen target tissues, such as breast, β-estradiol is extensively converted by cytochrome

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P450, catechol-O-methyltransferase, sulfotransferases, and UGT enzymes before being

released into the circulation (Guillemette et al., 2004). Hydroxylated and methoxylated

derivatives are further conjugated by UGTs to glucuronides that are usually considered

inactive. However, there were also reports that β-estradiol 17-*O*-glucuronide may be a toxic

metabolite that causes cholestasis (Meyers et al., 1980, Mackenzie et al., 1996).

Estrogens can be glucuronidated by many human UGTs and the glucuronidation of

physiological estrogens, including β-estradiol, by individual UGTs was recently examined

(Gall et al., 1999, Lepine et al., 2004, Starlard-Davenport et al., 2007). None of the studies,

however, included all the human UGTs. Moreover, the glucuronidation of epiestradiol, the

xenobiotic diastereoisomer of β-estradiol has not been explored, yet. Like previous studies

with C19- and C21- hydroxysteroids (Jin et al., 1997; Bowalgaha et al., 2007),

characterization of the glucuronidation of diastereoisomeric estrogens potentially provides

important insights into structure-activity relationships of this class of compounds and the

regio- and stereo-selectivity of human UGTs.

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Methods

Materials

17α-Estradiol, 17β-Estradiol, 17β-Estradiol-β-D-glucuronides (sodium salt), Scopoletin,

UDP-glucuronic acid trisodium salt and D-Saccharic acid 1,4-lactone were purchased from

Sigma-Aldrich. Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate

were from Fluka (Buchs, Switzerland and Steinheim, Germany). Radiolabeled [^{14}C]UDPGA

was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Pooled human

liver and intestinal (from duodenum and jejunum) microsomes were purchased from BD

Gentest (Woburn, MA) and male New Zealand rabbit liver microsomes from In Vitro

Technologies (Baltimore, MD). Rat liver microsomes were prepared in our laboratory from

Aroclor 1254-induced male Sprague-Dawley rats and pig, bovine and elk liver microsomes

from untreated animals, as previously described (Luukkanen et al., 1997).

Recombinant human UGTs were expressed in baculovirus-infected insect cells as previously

described (Kurkela et al., 2003; Kurkela et al., 2007). In recent studies we have noticed that

our recombinant human UGT2B15 preparation contains a rather low level of active enzyme

(M. Finel, unpublished observation) and, therefore, recombinant human UGT2B15 was also

purchased from BD Gentest (Woburn, MA). The cDNAs for rat UGTs r2B1-r2B3 were

expressed in the same baculovirus system that was employed for the human UGTs, including

the addition of a short C-terminal fusion peptide that ends with 6 His residues (His-tag)

(Kurkela et al., 2003). The cDNAs for the rat UGTs 2B1 and 2B2 were isolated from an

expression library as previously described (Mackenzie et al., 1984; Mackenzie, 1986a;

Mackenzie, 1986b). The cDNA of rUGT2B3 was isolated by RT-PCR from total liver RNA

of Wistar rat. The sense primer upstream to the first ATG included an Xba1 site and the

antisense primer downstream from the stop codon contained an Sph1 site. The amplified full-

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length DNA was subcloned as a Xba1-Sph1 fragment into the pUC118 vector and sequenced in both directions. The cDNAs for the 3 rat UGTs were transferred to the modified shuttle vector pFBXHA following insertion of a Sal1 restriction site just upstream to the original stop codon by PCR. Thereafter virus preparation and protein production were performed as previously described (Kurkela et al., 2003). The relative expression levels of all the recombinant UGTs, with the exception of commercial UGT2B15, were determined by dot-blot analyses using anti-His-tag antibodies (Kurkela et al., 2004; Kurkela et al., 2007).

Screening of Microsomes and Recombinant UGTs

The activity of 19 human recombinant UGTs and 3 rat recombinant UGTs towards the two estradiol diastereomers, β-estradiol and epiestradiol, was initially measured using three protein concentrations (40, 100 and 200 μg/ml) and three aglycone concentrations (1, 10 and 100 μM). The samples were incubated for 30 min at 37°C as described below. Glucuronidation of the estradiol diastereomers by human intestinal microsomes and human, rat, rabbit, pig, bovine and elk liver microsomes were studied at a substrate concentration of 100 μM. The protein concentration in the incubations were between 0.4 and 3.0 mg/ml and they were carried out in duplicates at 37°C for 60 min.

UGT Activity Assays

Recombinant UGTs that exhibited activity towards either estradiol diastereoisomer in the initial screening were subjected to kinetic analyses. All enzyme assays, performed in triplicate, contained 1 mM UDPGA, 5 mM saccharolactone, 50 mM phosphate buffer (pH 7.4), 5 mM MgCl$_2$ and 5% dimethyl sulfoxide in total volume of 250 μl. The aglycone concentrations in the kinetic analyses assays were selected using approximate K_m values from the initial screening. Hence, for each UGT we have selected eight different β-estradiol and

eight different epiestradiol concentrations, all of which were in the range of 0.5 and 300 μM.

In most cases the aglycone concentration range was at least from 0.3 to 5 times the K_m value

of the tested UGT, but in few cases the range was more narrow due to solubility problem at

substrate concentrations over 300 μM and detection limitation at very low substrate

concentrations. The incubation time and protein concentration varied between enzymes and

aglycones, from 10 to 45 min and from 8 to 200 μg/ml, respectively, and they were within the

linear range for the tested UGT. The glucuronidation reactions were terminated by adding 25

μl of chilled 4 M perchloric acid and cooling the tubes in a cold block. The precipitated

proteins were removed by centrifuging the samples for 5 min at $16,100 \times g$, and a 50 μl

aliquot of the supernatant fraction was injected into the HPLC.

Inhibition of UGT1A9

Inhibition of UGT1A9 by epiestradiol and β-estradiol was studied using scopoletin as the

aglycone. The concentration of scopoletin was 25 μM, which is close to its K_m with UGT1A9

(see Results). The estradiol concentrations were 5, 25 and 150 μM and the protein

concentration was 40 μg/ml. The samples were incubated at 37°C for 15 min, the reaction was

terminated with 4 M perchloric acid, and an 80 μl aliquot of the supernatant fraction was

injected into the HPLC.

In order to determine the K_i and the mode of inhibition by β-estradiol, the kinetics of

scopoletin glucuronidation was studied in the absence and presence of three concentrations of

β-estradiol (5, 10 and 20 μM). Seven scopoletin concentrations, between 5 and 500 μM, were

used and all the samples were prepared in duplicates. The data was fitted to the Michaelis-

Menten equation and to the competitive inhibition equation (global fit) using GraphPad Prism

version 4.03 for Windows (GraphPad Software Inc., San Diego, CA).

Quantification of Estradiol Glucuronides

β-Estradiol glucuronides were quantified using authentic standards. Seven concentrations between 10 to 1000 nM were used to construct calibration curves, and the linearity was excellent for both the 3-*O*- and 17-*O*-glucuronides ($R^2 > 0.99$). For epiestradiol glucuronides, for which authentic standards were not available, radiochemical and fluorescence detection were combined in order to quantify these metabolites. Samples were incubated in the presence of 6 μM radiolabeled [^{14}C]UDPGA and 50 μM unlabeled UDPGA for 60 min. The appropriate amount of human liver microsomes or recombinant UGT1A10 was used to catalyse the glucuronidation of epiestradiol. Following centrifugation, aliquots, 2 to 50 μl, of the supernatant fraction were injected into two different HPLC systems, which were separately equipped with radioactivity and fluorescence detectors. First, the amount of glucuronides formed during the incubation was determined by HPLC coupled with a radioactivity detector and calculated using the equation $x = \dfrac{A \times B}{B + C + D}$, where x is the amount of one of the glucuronides formed (pmol), A is the amount of UDPGA (labeled + unlabeled) added to the reaction mixture before incubation (pmol), B is the peak area of the glucuronide under examination, C is the peak area of the other glucuronide formed and D is the UDPGA peak area in the radioactivity detector (Kaivosaari et al., 2001). Same amounts of the same samples were subsequently injected to the HPLC with fluorescent detector and the linearity of each glucuronide standard curve was determined. The linearity of the epiestradiol 17-*O*-glucuronide standards determined this way was good ($R^2 > 0.9$) and that of epiestradiol 3-*O*-glucuronide was acceptable ($R^2 > 0.7$). The reaction products were proven to be glucuronides because they gave signals on the radioactivity detector. The epiestradiol glucuronides were well separated by the HPLC and they were assumed to elute from the column in the same order as the respective β-estradiol glucuronides. The latter assumption

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was supported by the type of glucuronides formed by most UGTs of subfamily 1A (see

Discussion).

HPLC Analyses

The formation of estradiol glucuronides was analyzed by HPLC (Shimadzu, Kyoto, Japan)

with fluorescence detection (ex 216 nm, em 316 nm), using a Chromolith SpeedRod RP18e

column (50×4.6 mm, Merck, Darmstadt, Germany). The mobile phase consisted of 50% 25

mM phosphate buffer (pH 3.0) and 50% methanol and the flow rate was 1 ml/min. The

column temperature was 30°C. The retention times were (mean ± S.D.): 2.4 ± 0.05 min for

epiestradiol 3-*O*-glucuronide, 4.2 ± 0.17 min for epiestradiol 17-*O*-glucuronide, 2.2 ± 0.04

min for β-estradiol 3-*O*-glucuronide and 3.0 ± 0.04 min for β-estradiol 17-*O*-glucuronide. The

retention times of β-estradiol and epiestradiol were 7.6 min and 8.3 min, respectively.

Radioactive samples, used for quantification of epiestradiol glucuronides, were analyzed

using two separate HPLC systems. The Shimadzu equipment combined with fluorescence

detector was used as described above and an Agilent 1100 series HPLC (Agilent

Technologies, Waldbronn, Germany) combined with a radioactivity detector (Model 9701,

Reeve Analytical, Glasgow, U.K.) was also used. In the latter case the mobile phase consisted

of 55% 25 mM phosphate buffer (pH 3.0) and 45% methanol. Two to 50 μl aliquots of the

supernatant fraction of centrifuged samples were injected into each HPLC systems.

The formation of scopoletin glucuronide by UGT1A9 was analyzed using the Agilent 1100

series instrument fitted with a Chromolith SpeedRod column and fluorescence detector (ex

335 nm, em 455 nm). The mobile phase was 90% 50 mM phosphate buffer (pH 3.0) and 10%

methanol. The initial flow rate was 0.8 ml/min for 5 min, which was then increased to 2.5

ml/min for an additional 6 min. The retention times of scopoletin glucuronide and scopoletin

were 4.6 min and 8.9 min, respectively. The column temperature was 40°C.

Kinetic Analyses

Kinetic constants were estimated by fitting the experimental data to either the Michaelis-

Menten equation [$v = V_{max} \times S/(K_m + S)$, where v is the initial velocity of the reaction, S is the

substrate concentration, V_{max} is the maximal velocity and K_m is the substrate concentration at

0.5 V_{max}], substrate inhibition equation [$v = V_{max} \times S/(K_s + S + S^2/K_i)$, where K_i is the constant

describing the substrate inhibition interaction] or the Hill equation [$v = V_{max} \times S^n/(K_A^n + S^n)$,

where K_A is the substrate concentration at 0.5 V_{max}] using non-linear least squares regression

(GraphPad Prism version 4.03 for Windows, GraphPad Software Inc., San Diego, CA or

EnzFitter, Biosoft, Cambridge, UK). Goodness of fit was evaluated by considering the

randomness of the residuals, R^2 and standard error estimates, and the best equation to describe

the data was selected after that. In order to be able to compare the results of different

enzymes, the initial velocity data and thereby also the V_{max} values were normalized for the

relative expression of recombinant UGTs. The expression level of UGT1A1 was given the

reference value 1 and those of the other enzymes were proportioned to that.

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Results

The analyses of β-estradiol and epiestradiol glucuronidation at both the 3-OH- and 17-OH-sites, by 19 human and 3 rat recombinant UGTs, were carried out in two rounds. Firstly, all the enzymes were assayed with both aglycones, as described in the Methods section. The UGTs that did not exhibit detectable activity were the human 1A5, 1A6, 1A9, 2A3, 2B10, 2B11, and 2B28, as well as the rat 2B2. The UGTs that catalyzed the glucuronidation of at least one of the estradiol diastereoisomers were subjected to kinetic analyses. The kinetic curves for the different UGTs are presented in Fig. 2-5 and the derived kinetic constants, where applicable, are listed in Table 1. The reactions generally followed either Michaelis-Menten or substrate inhibition kinetics. There were several exceptions, however, where the initial velocities data could not be fitted to any equation e.g. glucuronidation of epiestradiol by UGT1A8, UGT2A2 and UGT2B7 and glucuronidation at the 3-OH of β-estradiol by UGT2B15 (Fig. 2-5 and Table 1). The glucuronidation of β-estradiol by UGT2A2 was the only reaction that was best fitted to the Hill equation, but also in this case substrate inhibition was obvious at substrate concentrations above 150 μM.

UGT1A1 exhibited prominent regio-selectivity towards the 3-OH of both substrates while glucuronidation at the 17-OH position was not detected. The rate of glucuronidation at the 3-OH was, however, dependent on the configuration of the 17-OH so that UGT1A1 catalyzed β-estradiol glucuronidation about 6 times faster than epiestradiol glucuronidation (Fig. 2). Nevertheless, the kinetic analyses indicate that the Cl$_{int}$ values of UGT1A1 for both diastereoisomers were similar due to higher affinity but lower V_{max} value for epiestradiol in comparison to β-estradiol.

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UGT1A3 revealed clear regio-selectivity for the 3-OH position in both substrates, but also

conjugated the two estradiols at the 17-OH position, albeit at low rates. Similar to UGT1A1,

the α orientation of the 17-OH in the substrate leads to lower K_s value in UGT1A3 when

glucuronidation at the 3-OH position is compared (Table 1). In contrast to UGT1A1,

however, the V_{max} values of UGT1A3 for epiestradiol and β-estradiol were similar, resulting

in a higher Cl_{int} value for epiestradiol (Table 1).

Unlike most UGTs of the 1A family, UGT1A4 glucuronidated the two diastereoisomers

solely at the 17-OH position, but only at low rates. Interestingly, the glucuronidation of the

two substrates at the 17-OH position by UGT1A4 was similar to the corresponding activities

in UGT1A3, except that UGT1A3 glucuronidated β-estradiol at the 17-OH position slightly

faster than it glucuronidated epiestradiol at this position, whereas in UGT1A4 this ratio was

reversed (Table 1). However, the major difference between these two homologous enzymes

was that UGT1A3 was significantly more active towards the 3-OH of the two substrates,

while UGT1A4 did not conjugate the 3-OH position in either compound.

UGT1A7 exhibited a low rate of activity, yielding only the 3-O-glucuronide of epiestradiol.

The K_s value for this reaction was similar to the respective values for UGT 1A3 and 1A10,

but there were large differences in V_{max} (Table 1). UGT1A8 exhibited considerable activity,

but data of epiestradiol glucuronidation by this isoform could not be fitted in any enzyme

kinetic equation. Thus, the K_m or K_s could not be determined and the maximum reaction rate

was estimated by visual inspection (Fig. 2).

The most active human UGT in estradiol glucuronidation was UGT1A10, particularly when

considering glucuronidation at the 3-OH position. The activity of UGT1A10 towards

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epiestradiol was very high, as was its activity towards the 3-OH group of β-estradiol (Fig. 2, Table 1). In the latter case, a combination of low K_s and, particularly, high V_{max} value led to an exceptionally high Cl_{int} value. The very high estradiol glucuronidation activity of UGT1A10, much greater than that of UGT1A8 (Fig. 2, Table 1), was in disagreement with a previous significant report on this topic (Lepine et al., 2004). Therefore, we took further steps to ensure that our findings were correct and examined the estradiol glucuronidation activity of both UGT1A8 and UGT1A10 that were expressed in HEK293 cells, without any C-terminal fusion peptide (Uchaipichat et al., 2004). The results were broadly consistent with the findings presented in Fig. 2 and Table 1. In particular, the normalized (relative to UGT1A1 expression) V_{max} value for UGT1A10 was 2 orders of magnitude higher than that of UGT1A8 (data not shown).

The human UGT2A subfamily enzymes are rarely included in glucuronidation studies. We have expressed these three enzymes as His-tagged proteins in baculovirus-infected insect cells in the same manner as the other UGTs that are included in the present study (Sneitz, Court, Zhang, Ding and Finel, manuscript in preparation). The results revealed that UGTs 2A1 and 2A2 can catalyse glucuronidation of both β-estradiol and epiestradiol at both positions. UGT2A1 revealed stereo-selectivity as it conjugated epiestradiol at the 3-OH position at much higher rate than β-estradiol. When considering the 17-OH position, the difference was smaller and with an opposite stereoselectivity (Fig. 3, Table 1).

Human UGT2B4 exhibited both regio- and stereo-selectivity. It only catalyzed epiestradiol glucuronidation and solely at the 17-OH position. UGT2B7, in turn, revealed a clear preference for epiestradiol over the physiological steroid β-estradiol, but it also glucuronidated the latter substrate. Like UGT2B4, UGT2B7 was fully regio-selective for the

17-OH site and did not catalyze the formation of 3-*O*-glucuronides. An interesting finding in

the case of the human UGT2B7 was its exceptionally high affinity for epiestradiol (Fig. 4,

Table 1). The precise K_m value could not be determined for this reaction, because when the

incubation conditions were kept within the linear range with regard to both time and protein

concentration, the amount of glucuronide formed was not detectable at aglycone

concentrations lower than 0.5 μM.

UGT2B15 was the only member of subfamily 2B that conjugated the two estradiols at the 3-

OH rather than the 17-OH position. Like UGTs 2B4 and 2B7, it exhibited a clear preference

for epiestradiol over β-estradiol. In the initial studies with our recombinant human UGT2B15

only low glucuronidation activity of epiestradiol at the 3-OH position was detected (results

not shown). Since this was in sharp contrast with the other human UGT2Bs that are largely

restricted to the 17-OH position, and because other studies gave us reasons to suspect that the

amount of active UGT2B15 in the insect cell microsomes prepared locally is significantly

lower than in our other recombinant UGT preparations (M. Finel, unpublished observation),

we purchased a commercial sample of the human UGT2B15. The results revealed that,

qualitatively, the two enzymes are the same since the commercial sample exhibited strong

preference for the 3-OH position of epiestradiol. The commercial UGT2B15 sample was

clearly more active and also produced a trace amount of 3-OH glucuronide of β-estradiol, but

17-*O*-glucuronides were still not detected.

UGT2B17 was the only human UGT with a strict regio- and stereo-selectivity for the 17-OH

of β-estradiol. Its activity was among the highest of all human isoenzymes at this position,

suggesting that it may be an important enzyme for steroids metabolism in tissues containing

it. It may be noted that with respect to UGT2B17, our results also differ sharply from the

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observations of Lepine and co-workers since they reported that UGT2B17 lacks β-estradiol

glucuronidation activity (Lepine et al., 2004).

In addition to the 19 human UGTs, we have also tested 3 rat UGTs from the 2B subfamily,

r2B1 - r2B3. rUGT2B2 was not active towards either estradiol diastereomer, rUGT2B1 only

glucuronidated β-estradiol, while rUGT2B3 glucuronidated both diastereomers, but only at

the 17-OH position. The regio- and stereo-selectivity of rUGT2B1 were similar to those of the

human UGT2B17, nearly restricted for the 17-OH of β-estradiol. Interestingly, rUGT2B3 was

highly and similarly active toward the 17-OH of both diastereomers, a clear exception among

all the tested UGTs (Fig. 5 and Table 1). The only difference between the kinetics of these

reactions was in the substrate inhibition that was slightly stronger in the case of epiestradiol

(Fig. 5).

One of the intriguing findings of this work is the very large difference in estradiol

glucuronidation activity between UGTs that are highly similar to each other at the level of

amino acid sequence. The best example for this is the contrast between the high activity of

UGT1A10 towards the 3-OH of β-estradiol (Fig. 2 and Table 1) and the lack of estradiol

glucuronidation activity by UGT1A9. In order to clarify whether or not UGT1A9 binds one or

both estradiol diastereomers, we examined the inhibition of scopoletin glucuronidation

activity of UGT1A9 by the two estradiols. The results reveal that β-estradiol can inhibit

scopoletin glucuronidation activity substantially, while the inhibitory effect of epiestradiol on

this activity was limited (Fig. 6A). To further clarify the mode of inhibition by β-estradiol, we

have determined the apparent K_m values for scopoletin glucuronidation in the absence and

presence of three concentrations of the inhibitor. The outcome of this experiment (Table 2,

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Fig. 6B and 6C) revealed that β-estradiol is a competitive inhibitor of UGT1A9 with a K_i

value of 5.8 ± 1.1 μM, a very close value to the K_s of UGT1A10 for β-estradiol (Table 1).

The results with individual human UGTs prompted us to compare human liver and intestinal

microsomes in order to verify the applicability of the findings to the native UGTs. In addition,

we have examined liver microsomes from several different animal species. The results (Fig.

7) show that both human and rat liver microsomes exhibit higher glucuronidation activity at

the 17-OH position than at the 3-OH. However, the estradiol glucuronidation activity of

microsomes from rabbit, pig, bovine and elk was high and glucuronidation occurred mainly at

the 3-OH position. Looking more closely at the relative levels of 17-OH glucuronides formed

by human and rat liver microsomes, it is interesting to note that while human liver

microsomes catalyzed mainly epiestradiol glucuronidation, rat microsomes exhibited a clear

preference for β-estradiol (Fig. 7). The results also show that in human intestinal microsomes

there is a very high activity towards the 3-OH position of β-estradiol, in good agreement with

the high activity of human UGT1A10 (Fig. 2 and Table 1) and with the previous study by

Czernik et al. (2000).

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Discussion

The current study has revealed extensive new information on the activity of individual UGTs and, hence, their potential role in estradiol metabolism in different tissues. The focus of the present work was mainly on structure-activity relationships of the human UGTs. The sex hormone β-estradiol and its xenobiotic diastereoisomer, epiestradiol, were selected for this study since each of them can be glucuronidated at two different positions, providing an interesting system to examine the substrate specificity of individual UGTs and the overlap between them in this respect. Moreover, the use of these two diastereoisomers could reveal how the configuration of the 17-OH affects its own glucuronidation by different UGTs, as well as its longer-range effects on the glucuronidation at the 3-OH of these compounds.

We have investigated the activity of all known recombinant human UGTs and 3 recombinant rat UGTs. Seven human UGTs, namely 1A5, 1A6, 1A9, 2A3, 2B10, 2B11, 2B28, and the rat 2B2, did not glucuronidate either estradiol at detectable rates. The other enzymes, human UGT 1A1, 1A3, 1A4, 1A7, 1A8, 1A10, 2A1, 2A2, 2B4, 2B7, 2B15 and 2B17 along with rat UGT r2B1 and r2B3, glucuronidated at least one of the two substrates at one position. The regio- and stereo-selectivity among these enzymes varied considerably, revealing general patterns among the different UGTs, but also interesting exceptions. The results are discussed here keeping in mind the sequence homology among individual enzymes.

The general picture that emerges from this study is a distinct regio-selectivity between the 3 subfamilies of the human UGTs. The UGT1As that glucuronidate estradiol, do it preferentially on the 3-OH. The human UGT2Bs mainly glucuronidate estradiol at the 17-OH, depending on its configuration. The human UGTs of subfamily 2A that exhibit estradiol glucuronidation activity, 2A1 and 2A2, have more promiscuous binding sites since they can

glucuronidate these steroids at both positions. These patterns of estradiol glucuronidation by members of the different UGT subfamilies are partial, however, and exceptions to these rules have been found. For example, UGT2B15 glucuronidated epiestradiol at the 3-OH rather than the 17-OH and UGT1A4 glucuronidated both steroids at the 17-OH, but not the 3-OH position (Table 1).

The rules of stereo-selectivity among the different UGTs are even more complex than those governing regio-selectivity, and may be viewed as a dimension of the substrate specificity. This is particularly striking in the case of the three human UGT2Bs that glucuronidated either substrate at the 17-OH position. Hence, UGT2B4 is specific for the 17-OH of epiestradiol, UGT2B17 is strictly specific for β-estradiol, while UGT2B7 exhibited a clear preference, but not strict specificity, for epiestradiol (Table 1). In addition, UGT2B7 exhibited an exceptionally high affinity for epiestradiol (Fig. 4). It was much higher than what we have observed in other UGTs, and such low K_m values are rarely encountered in glucuronidation studies. Another interesting example for complex stereo-selectivity is provided by the 3 rat UGTs that were examined in this study. Whereas r2B1 resembles the human UGT2B17 as far as stereo-selectivity is concerned, r2B3 stands out as an interesting exception since it was both highly active and practically insensitive to the configuration of the 17-OH, while still exhibiting a strict regio-selectivity.

It is also interesting to examine the estradiol glucuronidation activity results from the point of view of protein homology. The human UGTs of the 1A subfamily can be divided into four subgroups based on amino acid sequence identity. Two groups contain a single enzyme each, namely UGT1A1 and UGT1A6. The 3rd group is comprised of UGTs 1A3-1A5 and the 4th of UGTs 1A7-1A10 (Mackenzie et al., 2005; Gong et al., 2001). It is noteworthy, that the

highly homologous UGTs 1A3 and 1A4 differ dramatically in their glucuronidation activity

towards the 3-OH of the two steroids. On the other hand, both enzymes exhibited very similar

activity, even if low, towards the 17-OH of both estradiols. These observations may provide a

starting point for identifying the residue(s) responsible for the high activity of UGT1A3, but

not UGT1A4, towards the 3-OH of the steroids, similarly to what was done previously for the

glucuronidation of 1-naphthol (Kubota et al., 2007). Our findings on the activity of UGT1A3

are also relevant to previous observations on the role of UGT1A3 in the glucuronidation of

estrone, and therefore in regulating estradiol biosynthesis (Caillier et al., 2007). The present

work show that UGT1A3 may also play a direct role in the glucuronidation of estradiol, not

only its precursor estrone. In addition, it may be pointed out that since UGT1A3 is expressed

in the liver (Tukey and Strassburg, 2001), it may contribute to estradiol glucuronidation in

this tissue, even if its affinity towards the 3-OH of β-estradiol is lower than that of UGT1A1.

There are large differences in estradiol glucuronidation activity between the human UGTs

1A7-1A10, despite the high degree of sequence similarity among them. UGT1A10 is by far

the most active human enzyme in β-estradiol glucuronidation at the 3-OH position, and its

activity towards the 3-OH of epiestradiol is also high (Fig. 2, Table 1). The estradiol

glucuronidation activity of UGT1A10 was reportedly low in a previous study (Lepine et al.,

2004), perhaps due to the use of a low activity batch of recombinant UGT1A10. With respect

to the glucuronidation of β-estradiol by UGTs 1A7-1A9, however, our results are in

agreement with the previous study (Lepine et al., 2004). The similarities include the lack of

detectable activity of either UGT1A7 or UGT1A9, but significant activity of UGT1A8

towards the 3-OH of β-estradiol. In the present study we have also detected high activity of

UGT1A8 towards the 3-OH of epiestradiol, indicating that the α configuration of the 17-OH

significantly increases its activity towards the 3-OH (Fig. 2). The configuration of the 17-OH

also affects the activity of UGT1A10 towards the 3-OH, but in the case of UGT1A10 the

direction of the effect was opposite, namely higher activity towards β-estradiol than

epiestradiol.

The configuration of 17-OH also affected the interactions between the estradiol diastereomers

and UGT1A9 (Fig. 6), an enzyme that does not catalyze estradiol glucuronidation. This

observation, along with the inhibition kinetics of β-estradiol (Table 2, Fig. 6B and 6C),

supports the suggestion that although UGT1A9 does not glucuronidate β-estradiol, it binds

this steroid at nearly the same affinity as UGT1A10. Hence, no activity of a given UGT

towards a test compound does not necessarily mean no binding.

One of the most striking findings of the present study is that while certain UGTs exhibit strict

stereo-specificity, others essentially lack specificity. Human UGT2B4 glucuronidates the 17-

OH only in epiestradiol whereas human UGT2B17 was highly specific for the beta

configuration of this hydroxyl group. In contrast, rUGT2B3 can catalyze the glucuronidation

of the 17-OH in both steroids with efficiency. Future detailed structural information on the

UGTs may help to understand the molecular basis for these features, as well as for the regio-

selectivity differences among the UGTs.

Taken together, our results demonstrate that many UGTs are capable of estradiol

glucuronidation, but they catalyze these reactions with varying regio- and stereo-selectivities

and differ in terms of catalytic efficiency. Hence, the assumption of broadly overlapping

specificities of UGTs is not justified, particularly when dealing with compounds that may be

glucuronidated at more than one position.

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Acknowledgments

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Footnotes

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Figure legends

Fig. 1. Chemical structures of epiestradiol (A) and β-estradiol (B).

Fig. 2. Glucuronidation of estradiol diastereomers by recombinant human UGTs of subfamily 1A. The normalized initial rates were fitted to either Michaelis-Menten or substrate inhibition equation. See Table 1 for kinetic constants.

Fig. 3. Glucuronidation of estradiol diastereomers by recombinant human UGTs of subfamily 2A. The normalized initial rates were fitted to either substrate inhibition or Hill equation. See Table 1 for kinetic constants.

Fig. 4. Glucuronidation of estradiol diastereomers by recombinant human UGTs of subfamily 2B. The normalized initial rates were fitted to the substrate inhibition equation. See Table 1 for kinetic constants.

Fig. 5. Glucuronidation of estradiol diastereomers by rat recombinant UGTs 2B1 and 2B3. The normalized initial rates were fitted to either Michaelis-Menten or substrate inhibition equation. See Table 1 for kinetic constants.

Fig. 6. Inhibition of the scopoletin glucuronidation activity of UGT1A9 by estradiol diastereomers. **A**. Inhibition at a single scopoletin concentration, 25 μM, by several concentrations of epiestradiol and β-estradiol. **B**. Kinetic analysis and global fit of the data to competitive inhibition equation for the inhibition of scopoletin glucuronidation by β-estradiol. The derived K_i value was 5.8 ± 1.1 μM. **C**. Determination of the mode of inhibition of

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UGT1A9 by β-estradiol. The apparent K_m and V_{max} values were first determined by fitting the data separately to untransformed Michaelis-Menten equation. The obtained values were substituted into the reciprocal transformation of the equation ($1/v = K_m/V_{max} \times 1/[S] + 1/V_{max}$) and the straight lines in the figure represent the equations that were obtained in this way (see Table 2 for the $K_{m(app)}$ and $V_{max(app)}$ values). Actual data points were eventually added onto the calculated straight

Fig. 7. Formation of the four estradiol glucuronides by microsomes from different sources. The samples were incubated at 37˚C for 60 min in the presence of 100 μM of the aglycone substrates. The relative activities were measured as peak areas and the largest peak area of each microsomal sample was given the reference value of 100%. The subsection shows an enlargement of the smallest bars from the main chart.

<u>Table 1</u>. The enzyme kinetic parameters of glucuronidation of epiestradiol and β-estradiol.

The K_m, K_s, K_A, K_i and n values are best-fit values ± standard errors.

| UGT	K_m, K_s or K_A (μM)				Normalized V_{max} (pmol/min/mg)				K_i (μM)			
	Epi-3G	βE-3G	Epi-17G	βE-17G	Epi-3G	βE-3G	Epi-17G	βE-17G	Epi-3G	βE-3G	Epi-17G	βE-17G
1A1	11.2 ± 2.70	60.6 ± 15.7	n.d.	n.d.	115	704	n.d.	n.d.	347 ± 172	196 ± 84.9	n.d.	n.d.
1A3	34.2 ± 13.6	250 ± 107	-[a]	66.7 ± 14.2	683	653	~ 26[a]	86.1	134 ± 64.7	71.5 ± 35.0	-[a]	184 ± 53.5
1A4	n.d.	n.d.	21.7 ± 5.79	10.6 ± 1.85	n.d.	n.d.	41.0	14.0	n.d.	n.d.	408 ± 160	-
1A5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1A6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1A7	37.1 ± 27.0	n.d.	n.d.	n.d.	94.2	n.d.	n.d.	n.d.	130 ± 101	n.d.	n.d.	n.d.
1A8	-[a]	61.7 ± 11.5	n.d.	10.0 ± 1.24	~ 200[a]	217	n.d.	4.75	-[a]	352 ± 116	n.d.	-
1A9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1A10	21.8 ± 1.5	4.07 ± 0.325	n.d.	2.63 ± 1.08	2100	6340	n.d.	83.6	85.7 ± 6.4	167 ± 22.1	n.d.	571 ± 507
2A1	99 ± 44	174 ± 5	36.0 ± 8.8	73.0 ± 19.3	693	128	78.3	265	38 ± 18	48.4 ± 1.1	196 ± 71	209 ± 76.5
2A2	-[a]	45.4 ± 5.57[b]	-[a]	-[a]	~ 70[a]	62.8[b]	~ 25[a]	~ 4[a]	-[a]	n=1.6 ± 0.2[b]	-[a]	-[a]
2A3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2B4	n.d.	n.d.	4.77 ± 1.62	n.d.	n.d.	n.d.	135	n.d.	n.d.	n.d.	404 ± 317	n.d.
2B7	n.d.	n.d.	< 0.5[a]	5.96 ± 0.929	n.d.	n.d.	~ 590[a]	631	n.d.	n.d.	-[a]	303 ± 93.9
2B10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2B11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2B15	26.1 ± 5.61	-[a]	n.d.	n.d.	350[c]	~ 5[a,c]	n.d.	n.d.	204 ± 57.6	-[a]	n.d.	n.d.
2B17	n.d.	n.d.	n.d.	11.4 ± 2.46	n.d.	n.d.	n.d.	327	n.d.	n.d.	n.d.	432 ± 196
2B28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
r2B1	n.d.	-[a]	n.d.	14.3 ± 1.04	n.d.	~ 55[a]	n.d.	1630	n.d.	-[a]	n.d.	-
r2B2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
r2B3	n.d.	n.d.	30.2 ± 9.30	19.5 ± 2.56	n.d.	n.d.	8110	5890	n.d.	n.d.	89.6 ± 29.4	610 ± 171

Epi-3G, Epiestradiol-3-*O*-glucuronide; βE-3G, 17β-Estradiol-3-*O*-glucuronide; Epi-17G, Epiestradiol-17-*O*-glucuronide; βE-17G, 17β-Estradiol-17-*O*-glucuronide; n.d., No activity was detected; [a]Kinetic parameters could not be determined; [b]The data was fitted to the Hill equation; [c]No normalization was done

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Table 2. The kinetic constants for scopoletin glucuronidation by UGT1A9 in the presence and

absence of β-estradiol. The K_m values are best-fit values ± standard errors.

β-Estradiol (μM)	$K_{m(app)}$ (μM)	V_{max} (Peak area)
0	26.0 ± 5.8	130
5	53.4 ± 9.1	135
10	95.6 ± 16.2	132
20	139 ± 16.6	147

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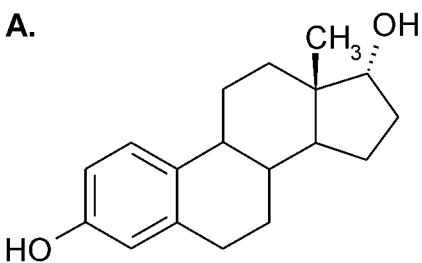
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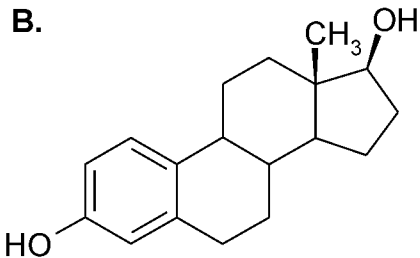
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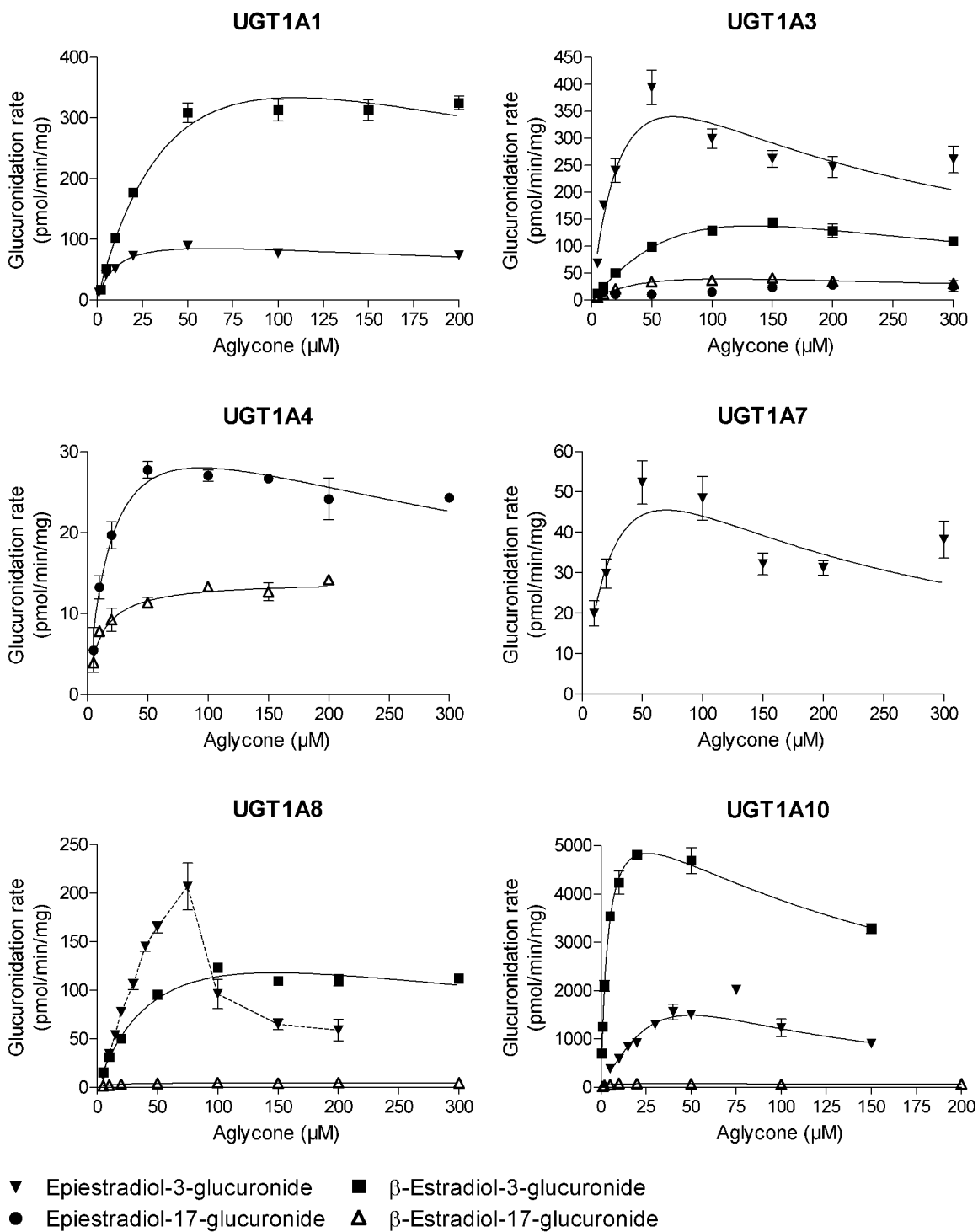
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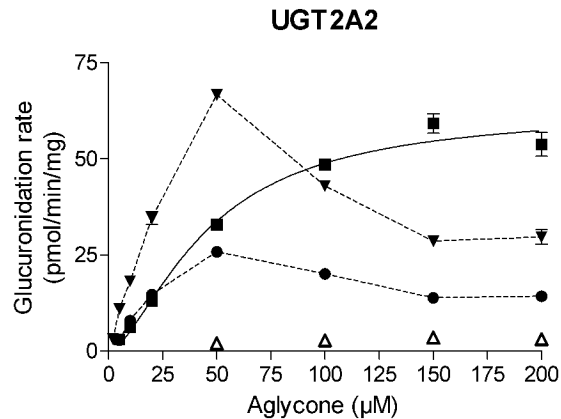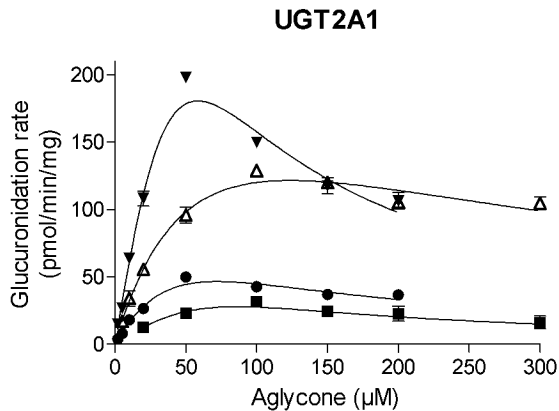
Figure 3.

UGT2B4

UGT2B7

UGT2B15

UGT2B17

▼ Epiestradiol-3-glucuronide   ■ β-Estradiol-3-glucuronide

● Epiestradiol-17-glucuronide   △ β-Estradiol-17-glucuronide

Figure 4.
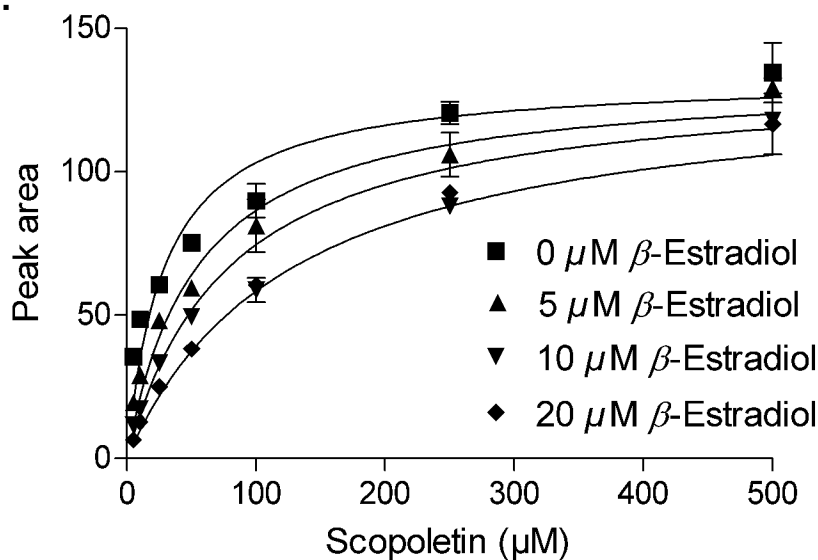
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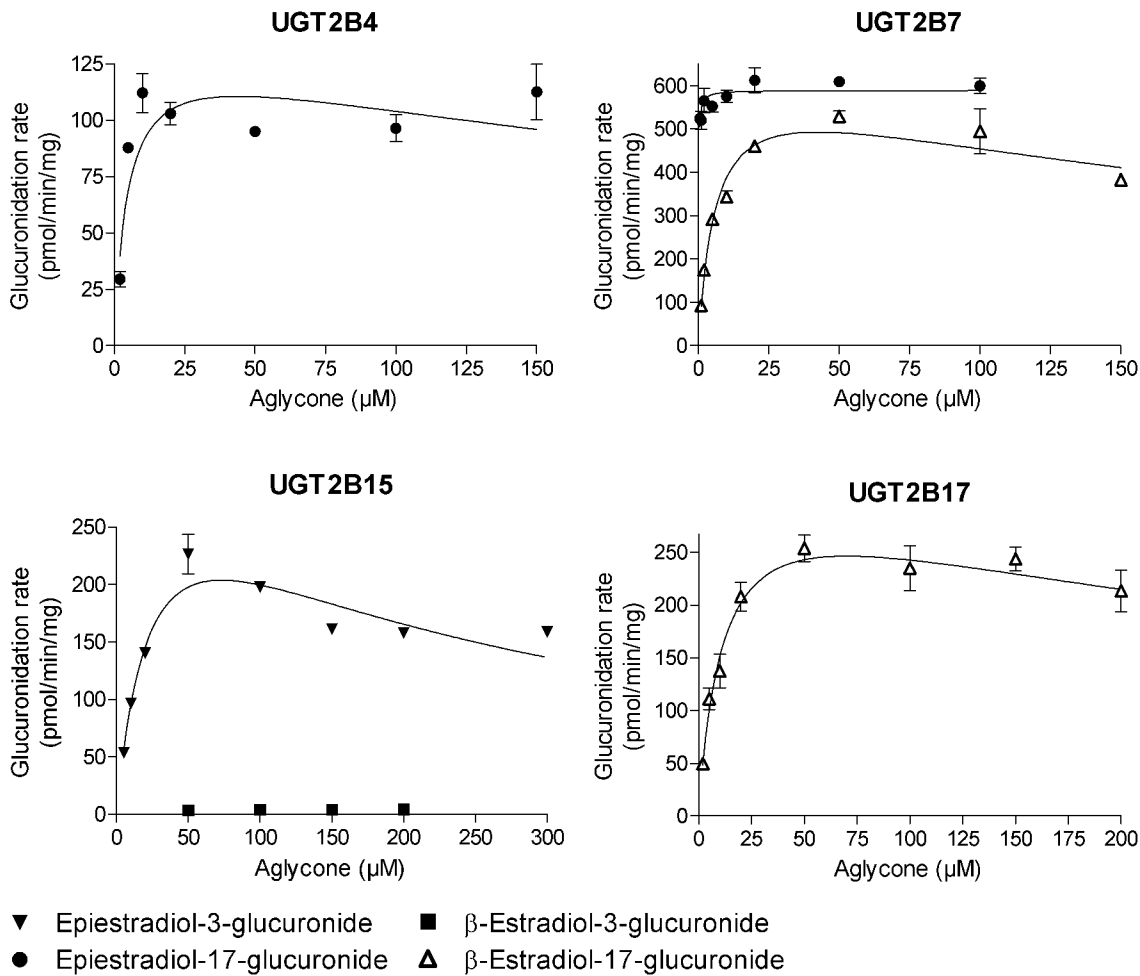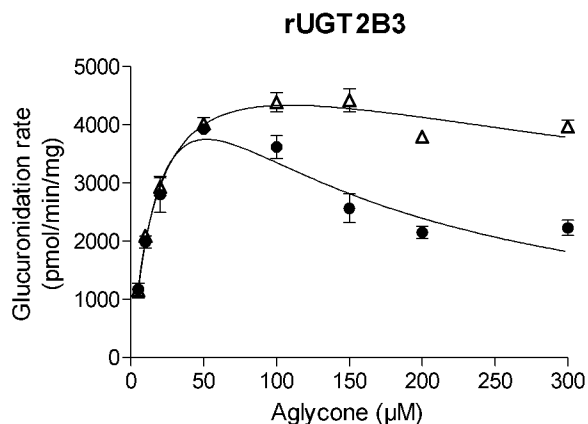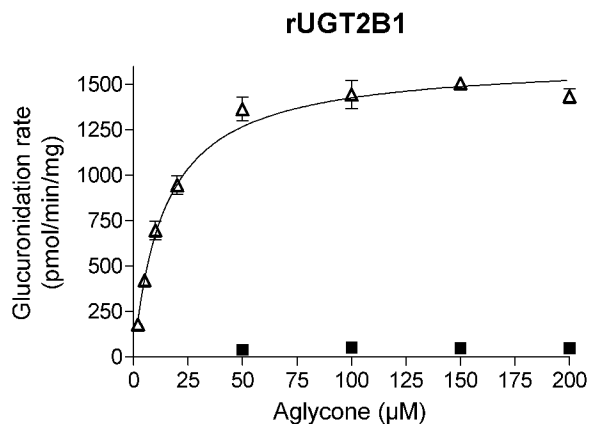
▼ Epiestradiol-3-glucuronide ■ β-Estradiol-3-glucuronide
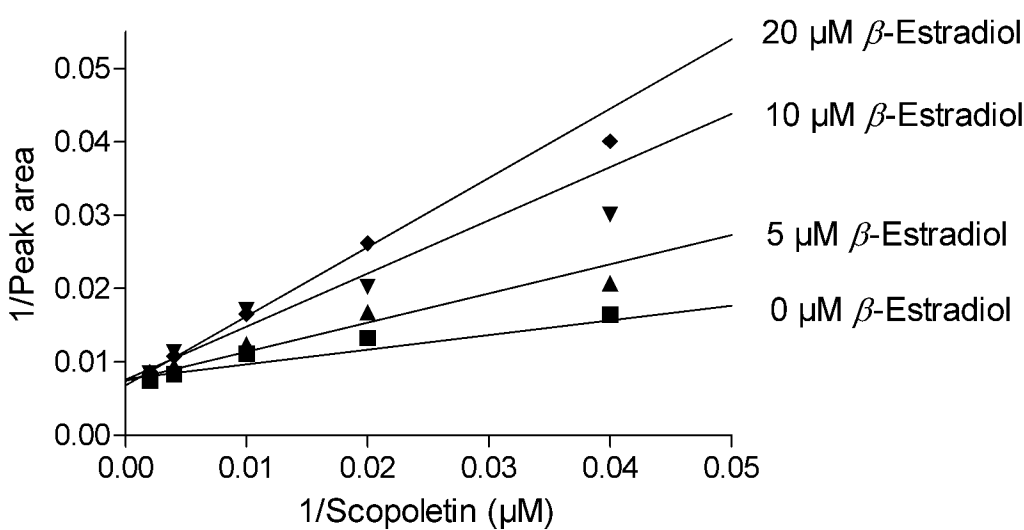● Epiestradiol-17-glucuronide △ β-Estradiol-17-glucuronide
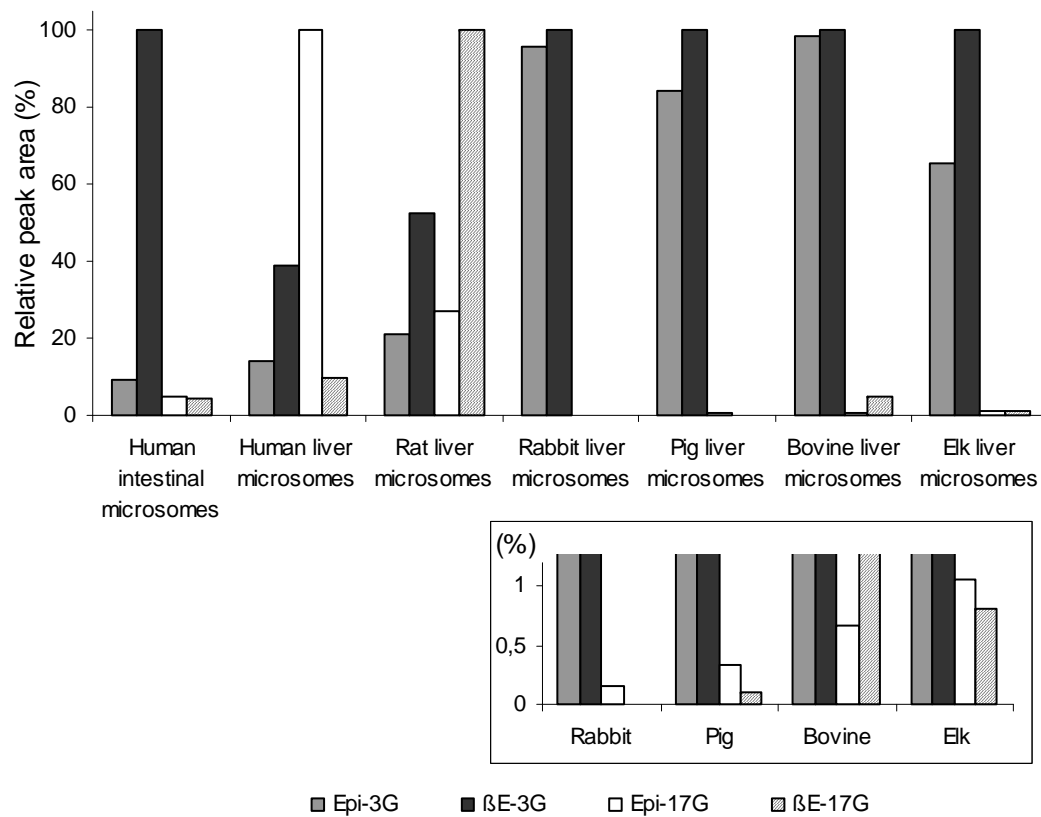
Figure 5.

A.

B.

C.

Figure 6.

Figure 7.