Stereoselectivity of the Human
UDP-glucuronosyltransferases (UGTs);

Studies on Androgens and Propranolol Glucuronidation

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ACADEMIC DISSERTATION

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The publications are referred to in the text by their roman numerals (I-IV).

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Abbreviations

CYP cytochrome P-450
DHEA dehydroepiandrosterone
DHT dihydrotestosterone
DMSO dimethyl sulfoxide
E enzyme concentration
ES enzyme-substrate complex
ESI electrospray ionization
HIM human intestine microsome
HLM human liver microsome
HPLC high pressure liquid chromatography
I inhibitor concentration
$K_i$ inhibition constant
$K_m$ Michaelis constant, substrate concentration resulting in 50% of $V_{\text{max}}$
LC liquid chromatography
MS mass spectrometry
MS/MS tandem mass spectrometry
NSAID non-steroidal anti-inflammatory drug
$n$ Hill constant
Q-TOF quadrupole time-of-flight
R-pg R-propranolol glucuronide
S substrate concentration
SL saccharic acid lactone
$S_{N2}$ bimolecular nucleophilic substitution
S-pg S-propranolol glucuronide
$S_{50}$ substrate concentration resulting in 50% of $V_{\text{max}}$ in Hill equation
T/E testosterone to epitestosterone ratio
UDP uridine 5’-diphosphate
UDPGA uridine 5’-diphospho glucuronic acid
UGT UDP-glucuronosyltransferase
$UGT$ UDP-glucuronosyltransferase gene
UPLC® Ultra performance liquid chromatograph Acquity, Waters Co.
UV ultraviolet
$V_{\text{max}}$ maximum reaction velocity
3α-HSD 3α-hydroxysteroid dehydrogenase
3β-HSD 3β-hydroxysteroid dehydrogenase
5α-diol 5α-androstane-3α-,17β-diol
5β-diol 5β-androstane-3α-,17β-diol
5α-R 5α-reductase
5β-R 5β-reductase
17β-R 17β-reductase
Abstract

Metabolic reactions are divided into functionalization and conjugation reactions. Uridine diphosphoglucuronosyltransferases (UGTs) are an important group of conjugative enzymes that catalyze the transfer of a sugar molecule to the substrate. In humans, 19 UGT enzymes are expressed in a tissue specific manner. Human UGTs are divided into subfamilies: UGT1A, UGT2A and UGT2B. Most UGTs have broad substrate selectivity and are involved in the metabolism of different endogenous and exogenous molecules. The steroids in the human circulation are mainly glucuronidated by UGT2B subfamily enzymes. Among steroids many stereoisomeric pairs are found. Stereoisomers differ from each other in the spatial orientation of the atoms. Mirror images are called enantiomers. In biological environments stereoisomers are differentiated. The current study is concerned with stereo- and regioselectivity of UGTs toward propranolol enantiomers and endogenous steroids. Stereoisomers offer a valuable tool in examination of UGT selectivity toward the substrates. Kinetic characterization of the reactions and inhibition studies can be used to further analyze the efficiency and type of the enzymatic reaction.

The studies of this thesis were conducted with human recombinant UGTs incubated with the selected androgens and propranolol. Analysis was done by liquid chromatograph equipped with either ultraviolet or fluorescence detector, or a mass spectrometer. Propranolol was glucuronidated with opposite stereoselectively by UGT1A9 and UGT1A10, two closely related enzymes. The results also suggest the role of intestinal UGT1A10 in the stereoselective first pass metabolism of propranolol. Extrahepatic UGT2A1 was also active, showing low affinity and not discriminating propranolol enantiomers. Positive cooperativity was suggested in the reaction between propranolol enantiomers and UGT2A1.

Endogenous C19-steroids were studied. Testosterone was mainly glucuronidated by UGT2B17, an enzyme that is absent in some individuals. Testosterone glucuronidation by UGT2B17 was inhibited with its diastereomeric pair epitestosterone, which is a high affinity substrate of UGT2B7, but not UGT2B17. Minor inhibition also occurred by a common non-steroidal anti-inflammatory drug (NSAID) diclofenac, but not ibuprofen. Both NSAIDs, however, inhibited UGT2B15. Other studied androgens were mostly glucuronidated by UGT2B7, 2B15, 2B17 and, often, UGT2A1. UGT2B15 was strictly regio and stereoselective toward 17β-OH bearing steroids, whereas UGT2B17 accepted both 3α- and 17β-OH. If both were available, the 17β-OH was favored over 3α-OH. UGT2B7 accepted both 3α- and 17α-OH, but not 17β-OH, which also significantly impaired the glucuronidation reaction when available in the molecule. For UGT2B7, structures with 17β-OH seem to act as a competitive inhibitor for 3-O-glucuronidation if the $K_m$ is not very low. Interestingly, 17α-OH as in epitestosterone served as a low $K_m$ substrate that was not inhibited by testosterone with 17β-OH. The results shed new light on the selectivity of human UGTs toward endogenic C19 steroids.
1 Introduction

Glucuronide conjugation is catalyzed by uridine 5’-diphosphoglucuronosyltransferases, a group of microsomal enzymes expressed in many organs (Ohno and Nakajin, 2009; Tukey and Strassburg, 2000). UGTs catalyze the conjugation of the glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to many endogenous and exogenous substrates, thus making it easier to excrete them into urine or bile. UGTs are divided into different subfamilies according to their sequence similarities (Mackenzie et al., 2005). The substrate specificity of UGTs is broad and partly overlapping.

The present work focuses on the stereoselectivity of UGTs, structural aspects of the model substrates, and structure-activity relationships between the aglycones and the proteins. When authentic standards for glucuronides are not available, enzymatic synthesis of those metabolites is possible using biological catalyst, such as rat liver microsomes (I). Enzymes are biological catalysts with three-dimensional structures, and thus the interactions of drugs with drug-metabolizing enzymes are often stereoselective. It is of interest to investigate the interactions of chiral entities, propranolol enantiomers, by the use of chiral HPLC (I). The next line of the study was directed at steroid metabolism by recombinant UGTs using Ultra Performance LC with both UV and MS detection (II, III, IV). The studies on these steroids concentrated on both structure-function aspects (II, III), and inhibition of different UGTs and substrates (II, IV).

1.1 Conjugation reactions in metabolism; UDP-glucuronosyltransferases

Conjugation reactions, also referred to as phase II metabolic reactions, are primarily catalyzed by transferase enzymes. A molecular group from a cosubstrate needed for these reactions is conjugated with a functional group on the molecule. Typical functional groups able to undergo conjugation are hydroxyl-, carboxyl-, and amine groups that are often introduced or exposed during functionalization biotransformation reactions, also referred to as phase I metabolism. Conjugation biotransformation reactions include glucuronidation, sulfonation (more commonly called sulfation), acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis), and conjugation with amino acids such as glycine, taurine, and glutamic acid. With the exception of methylation and acetylation, conjugation reactions usually result in a large increase in substrate hydrophilicity. The conjugation facilitates excretion of the compound into bile or urine through efflux transporters. UDP-glycosyltransferases (UGTs, EC 2.4.1.17) catalyze glucuronidation reactions, and are involved in about 35% of all drugs metabolized by conjugation (Evans and Relling, 1999). Glucuronic acid conjugation is one of the main metabolic pathways in detoxification of about 10% of the 200 most prescribed drugs (Williams et al., 2004).
1.1.1 Structural aspects of the UGTs

Human UGTs belong to the GT1 family of the glycosyltransferase (GT) superfamily. Mammalian UDP-glycosyltransferases are divided into four families: UGT1, UGT2, UGT3, and UGT8 (Mackenzie et al., 2005; Meech and Mackenzie, 2009). UGT1 and UGT2 family members catalyse conjugation of glucuronic acid and are further discussed below. The abbreviation UGT in the text thus refers to UDP-glucuronosyltransferases. To date, 28 human UGT genes have been identified, including nine pseudogenes containing truncation mutations (Mackenzie et al., 1997; Mackenzie et al., 2005). UGTs are divided into subfamilies, UGT1A, UGT2A, and UGT2B, primarily according to gene sequence similarity (Mackenzie et al., 2005) (Fig. 1). Between the families of UGT1 and UGT2, there is at least 50% homology, and within each subfamily at least 60% homology in their DNA sequence (Guillemette, 2003). In humans, 19 UGTs are expressed in a tissue specific manner (Finel et al., 2005; Nakamura et al., 2008; Ohno and Nakajin, 2009; Tukey and Strassburg, 2000). Full-length crystal structure of any UGT is yet to be resolved. A crystal structure of the UDP-glucuronic acid binding domain of human UGT2B7 was recently published (Miley et al., 2007).

The UGT1A family consists of nine functional proteins that are encoded by a single gene complex, located on chromosome 2q37 (Mackenzie et al., 1997; Owens and Ritter, 1995). The mRNA transcripts all share exons 2-5, but have a distinct exon 1. Thirteen exon 1 sequences in the gene locus encode nine functional enzymes. The four common exons are believed to yield the C-terminal part of the enzyme that includes the UDPGA binding domain (Mackenzie et al., 2003). The variable first exon yields the N-terminal domain of the protein, the substrate binding domain, explaining the different substrate selectivity between the enzymes despite the common C-terminal part. (King et al., 2000). The UGT enzymes exhibit distinct, although sometimes overlapping, substrate selectivities. It is not common that UGT-mediated metabolism would cause drug interaction, or a dysfunction in UGT metabolism would lead to a certain disease state. An important exception is UGT1A1, which is essential for bilirubin glucuronidation (Petit et al., 2008). The total lack of UGT1A1 activity in type I Crigler-Najjars syndrome leads to severe jaundice and kernicterus.

The UGT2 enzymes are products of separate genes located on chromosome 4q13, and are further divided into two subfamilies, UGT2A and UGT2B (Fig. 1) (Mackenzie et al., 2005). Unlike the UGT1 family, the mRNA transcript in the UGT2B family is spliced from six exons. Even with the unique genes encoding the UGT2B family enzymes, they share very conserved protein sequences at the C-terminal portion, which both makes up the UDPGA binding domain and anchors the protein to the membrane (King et al., 2000; Tephly and Burchell, 1990). Analogously with the UGT1A family, the N-terminal part contributes to substrate selectivity (Lewis et al., 2007). UGT2A family enzymes are much less studied, and presumably of less importance in systemic metabolism. UGT2A1 is found mainly in olfactory epithelium, and with very low expression also in the brain and lungs (Jedlitschky et al., 1999; Somers et al., 2007). UGT2A2 was suggested to have a unique exon 1, and to share exons 2-6 that encode the C-terminal half of the enzyme, with UGT2A1 (Mackenzie et al., 2005). To date, UGT2A2 has been found in the liver and
small intestine (Tukey and Strassburg, 2001). A recent research paper, however reported UGT2A2 expression in nasal tissue but not in adult liver (Sneitz et al., 2009). UGT2A2 activity toward different substrates is broad but quite low, following substrate selectivity of UGT2A1 (Sneitz et al., 2009). UGT2A3 expression was recently published, but its activity was limited to bile acids (Court et al., 2008). UGT2A3 is mainly found in the small intestine, liver, and adipose tissue.

Of the UGT2 family, mainly UGT2Bs play significant roles in drug and endogenic substances metabolism. They are extensively expressed in both liver and extrahepatic tissues, including reproductive organs (Nakamura et al., 2008; Ohno and Nakajin, 2009; Radomsinska-Pandya et al., 1998; Turgeon et al., 2001).

![Figure 1. A phylogenetic tree of the 19 human UDP-glucuronosyltransferases (UGTs) (Patana, 2009).](image-url)
1.1.2 Function and localization of the UGTs

Glucuronidation is a conjugation reaction, during which α-D-glucuronic acid moiety is transferred from the co-substrate UDP-glucuronic acid (UDPGA) to the substrate, which in glycosylation processes is referred to as an aglycone, literally meaning the nonsugar component of a glycoside molecule (Fig. 2). UGTs are membrane bound enzymes, located in the endoplasmic reticulum and facing the luminal side of the organelle (Tephly and Burchell, 1990; Tukey and Strassburg, 2000). The reaction is deduced to be similar to the bimolecular nucleophilic substitution reaction (SN2) producing only β-D-glucuronides (Miley et al., 2007; Yin et al., 1994). The high energy bond between UDP and glucuronic acid holds the energy to form the new bond in the product. Glucuronic acid is very hydrophilic, thus increasing the substrates water solubility (Fisher et al., 2001; Wells et al., 2004).

A study on UGT1A enzymes substrate binding order suggests that those enzymes exhibit a compulsory order mechanism, and that UDPGA is most likely the first binding substrate (Luukkanen et al., 2005). Recent studies support those findings, suggesting an ordered bi bi -mechanism, in which the aglycone may act as an inhibitor by binding to the enzyme-UDP complex (Patana et al., 2008).

Metabolic reactions occur mainly in the liver, but also in other tissues exposed to xenobiotics. Endogenous compounds, such as steroid hormones, are also metabolized by UGT enzymes. Ubiquitous UGTs are found in tissues such as the gastrointestinal tract (Finel et al., 2005; Radominska-Pandya et al., 1998; Tukey and Strassburg, 2000), lungs (Somers et al., 2007; Turgeon et al., 2001), and kidneys (Nakamura et al., 2008; Ohno and Nakajin, 2009), in a diverse expression pattern considering different UGTs. The UGT1A subfamily members UGT1A7-8 and UGT1A10 have a unique expression pattern as they are notably found only in extrahepatic tissues, particularly the gastrointestinal tract (Ohno and Nakajin, 2009; Radominska-Pandya et al., 1998; Tukey and Strassburg, 2000).
Glucuronidation is generally an end point reaction, and once formed, the glucuronic acid conjugates are not usually broken down. The chemical instability of conjugates must still be taken into consideration. Preanalytical stability of drugs and metabolites can be controlled with e.g. temperature, pH and light conditions (Shipkova et al., 2003; Skopp and Potsch, 2004). A reversible reaction to glucuronidation, namely dissociation of the glycosidic bond, is possible by β-glucuronidase. It is a protein of either human or bacterial origin, present in e.g. serum and urine (Ho, 1995; Lampe et al., 2002). Bacterial beta-glucuronidase is produced by certain intestinal bacterial strains, and thus mainly present in the gastrointestinal tract, but also in bile (Reddy et al., 1974; Skar et al., 1988). The activity of beta-glucuronidase compared to that of UGT enzymes is low, and only a limited amount of enzymatic degradation takes place (Bock and Kohle, 2009). Hence, the glucuronidated compound usually remains to be excreted. The organisms benefit from the glucuronidation reaction might also be due to neutralization of the pharmacological effect of substances, at least in most cases (Chouinard et al., 2008; Jedlitschky et al., 1999). Rare exceptions to this trend arise, such as morphine 6-glucuronide, which is a more potent analgetic than the parent compound (Osborne et al., 1990). In recent years, further metabolism of glucuronide conjugates has been detected especially for steroids. A repeated glucuronidation of steroid sex hormones, forming diglucuronides where two glucuronosyl groups are bound to a single hydroxyl group in tandem, was depicted in vitro (Murai et al., 2005). Dihydrotestosterone, for one, was shown to be metabolized in vitro via a previously unknown pathway involving sequential reactions of UGT2B17 and aldo-keto reductase (Jin et al., 2009). The clinical studies strongly suggest that this pathway would also be relevant in vivo.

Different approaches have been developed to predict glucuronidation in vivo with help from in vitro data (IVIVC; in vitro in vivo –correlation), but even the simplest attempts, using substrates that do not undergo functionalization prior to glucuronidation, did not usually lead to good predictability (Miners et al., 2004; Miners et al., 2006). Typically, the in vitro resolved kinetic characteristics lead to underestimation of the in vivo clearance of UGT metabolized drugs. The reasons for these differences might lay in the incubation study conditions (Boase and Miners, 2002; Kilford et al., 2009; Soars et al., 2003). A noteworthy matter is the presence of long-chain fatty acids present in or released during incubations. Those fatty acids are inhibitory to certain UGT enzymes, at least UGT1A9 and UGT2B7, a reaction that can be overcome by the addition of bovine serum albumin to the incubation mixture as a free fatty acid sequestering agent (Rowland et al., 2007; Rowland et al., 2008). Addition of albumin to incubations provides physiologically relevant \( K_m \) values. Recombinant UGTs are generally produced as membrane enzymes, which are not subject to other purification steps than differential centrifugation to concentrate the microsomal membrane fractions. The presence of a lipid bilayer membrane is thought to maintain the recombinant enzyme in its native membrane-bound conformation (Crespi and Miller, 1999). The luminal orientation of UGTs requires the action of different transporter proteins (Bock and Kohle, 2009). The systems in which the recombinant UGTs are produced, anyhow, do not generally express the transporter proteins that are present in human endoplasmic reticulum membranes. This can hinder both the availability of the substrates at the active site, and the release of the glucuronide
conjugates. The access through the membranes is especially critical in microsomal preparations, which can also be activated by using different mediators. The most common is probably the pore forming agent alamethicin (Al-Zoughool and Talaska, 2006; Cubitt et al., 2009). Saccharolactone (SL) is a β-glucuronidase inhibitor that has often been used in glucuronidation studies with microsomes and recombinant UGTs. A recent study, however, suggests exclusion of SL on a routinely basis in the incubation mixtures, as it might cause inhibition of glucuronide formation in some cases (Oleson and Court, 2008).

### 1.1.2.1 Oligomerization and expression regulation

At least some, if not all, UGT enzymes are oligomeric, forming different quaternary structures, such as homo- and heterodimers (Finel and Kurkela, 2008; Kurkela et al., 2003; Kurkela et al., 2007; Radominska-Pandya et al., 2005). Although not always requisite, oligomerization might be necessary for enzymatic activity, and may alter the rates or even partially restore the glucuronidation capability in some cases (Fujiwara et al., 2007a; Kurkela et al., 2007; Olson et al., 2009; Ouzzine et al., 2003). Homo- and heterodimerization of certain UGTs was also recently shown by other researchers (Fujiwara et al., 2007a; Fujiwara et al., 2007b; Fujiwara et al., 2010; Nakajima et al., 2007; Operana and Tukey, 2007). Moreover, it was suggested that UGTs are acting together with other drug metabolizing enzymes such as cytochrome P-450 (CYP) via xenobiotic nuclear receptor coordinated regulation (Bock and Kohle, 2004). UGTs are inducible enzymes, and the induction is mediated by different receptors and transcription factors. Important nuclear receptors identified in UGTs regulation in human or animal models include the aryl hydrocarbon receptor, constitutive androstane receptor, and pregnane X receptor (Buckley and Klaassen, 2009; Court et al., 2008; Kohle and Bock, 2009). They can affect the expression of different UGT enzymes in a diverse manner.

### 1.1.2.2 Polymorphism and metabolic interactions

Some of the UGTs are polymorphic enzymes (Argikar et al., 2008; Evans and Relling, 1999; Lampe et al., 2000). Polymorphism has been identified for UGT1A1, UGT1A3-4, UGT1A6-9, UGT2A1, UGT2A3, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17 and UGT2B28 (Chen et al., 2007a; Court et al., 2008; Girard et al., 2006; Guillemette, 2003; Villeneuve et al., 2003; Wilson et al., 2004). Polymorphisms often show distinctive population differences (Bhasker et al., 2000; Jakobsson et al., 2006; Lampe et al., 1999; Thomas et al., 2006; Villeneuve et al., 2003). In the UGT1A subfamily the best known and most important polymorphism affects the UGT1A1 enzyme. Depending on the severity of the state, it leads to impairment or total lack of glucuronidation of bilirubin, a degradation product of heme bearing proteins such as CYPs, hemoglobin and myoglobin. The condition is known as either Crigler-Najjar or Gilberts syndrome (Petit et al., 2008; Strassburg, 2008). This is one of the rare cases when UGT-dependent metabolism leads to disease or severe impairment. The UGT-mediated clinical drug-drug interactions are not
typical either (Kiang et al., 2005). The high $K_m$ and general involvement of multiple UGTs in a definite pathway cause glucuronidation to be less susceptible to drug-drug interactions in comparison to CYP-dependent pathways (Williams et al., 2004). Situations in which UGT genotype is taken into consideration when using medication are also atypical. The only occasion so far is the UGT1A1*28 polymorphism (Gilbert’s syndrome), which affects the metabolism of a chemotherapeutic agent irinotecan. The UGT1A1 genotype is often tested in the case of irinotecan treatment, although its relevance has received criticism (Akiyama et al., 2008; Deeken et al., 2008; Perera et al., 2008).

Recent evidence, however, associates an increased risk of certain diseases with polymorphism in UGT1A subfamily enzymes. The allelic variant UGT1A6*2 is associated with more rapid glucuronidation of salicylic acid, a major metabolite of a common NSAID acetylsalicylic acid (Chen et al., 2007b). The extrahepatic enzyme UGT1A7 is a key enzyme in catalyzing the glucuronidation of various carcinogens. For example, UGT1A7 haplotype is related to raised susceptibility of hepatocellular carcinoma in hepatitis B carriers (Kong et al., 2008). A UGT1A7 high activity genotype was more common in patients with laryngeal cancer, whereas low-activity genotype indicated risk for liver cirrhosis (Lacko et al., 2009; Tang et al., 2008). The expression level of UGT1A10, an estrogen conjugating UGT enzyme recently found in breast tissue, may be associated with breast cancer (Starlard-Davenport et al., 2008).

Among the UGT2B family there are many polymorphic enzymes. Polymorphs UGT2B4(D$_{385}$E), UGT2B7(H$_{268}$Y), and UGT2B15(D$_{85}$Y) were found by Lampe et al. (Lampe et al., 2000). Also, UGT2B17 and UGT2B28 polymorphisms exist, but they are copy number variations (McCarroll et al., 2006; Wilson et al., 2004).

UGT2B7H$_{268}$Y polymorphism is common, showing distinctive interethnic difference between Caucasian and Japanese populations (Bhasker et al., 2000; Coffman et al., 1998). It may be of clinical importance e.g. with interaction of lorazepam and valproic acid (Chung et al., 2008) but not with epitestosterone glucuronide formation and androgen disposition, nor in the metabolism of menthol and morphine (Bhasker et al., 2000; Schulze et al., 2008a). Despite a minor difference in polymorphs of UGT2B7 in androsterone glucuronidation was shown in vitro, it was not evident in vivo (Bhasker et al., 2000; Swanson et al., 2007a). The genetic susceptibility to diclofenac-induced hepatotoxicity in correlation to UGT2B7 genotype was recently reported (Daly et al., 2007).

UGT2B15 is encoded by a polymorphic gene, yielding polymorphs UGT2B15(D$_{85}$Y), (T$_{352}$I), and (K$_{523}$T) (Court et al., 2004). The isolation and characterization of UGT2B15(D$_{85}$Y) was first reported by (Levesque et al., 1997) and is of some clinical importance in S-oxazepam glucuronidation (Court et al., 2004). (Swanson et al., 2007b) showed that UGT2B15(D$_{85}$Y) and 2B17 deletion polymorphisms also predict the glucuronidation pattern of androgens and fat mass in men.

Deletion polymorphism of the UGT2B17 gene was first reported by Wilson et al. (2004), and it was shown to be genetically linked to UGT2B15. Later studies suggest that lack of the UGT2B17 enzyme may be compensated for by increased UGT2B15 transcription (Schulze et al., 2008a). Prominent interracial differences in UGT2B17 copy-number occur, the influence of which is also observed in certain endogenous compound glucuronidation, such as testosterone (Jakobsson et al., 2006; Schulze et al., 2008c; Xue et
Deletion of UGT2B17 leads to decreased level of urinary testosterone, which may lead to misinterpretation of doping control assays. UGT2B17 gene is associated with an increased risk for prostate cancer and correlates to gene expression in the prostate (Karypidis et al., 2008). Moreover, the gene is a strong determinant of androgen excretion in healthy pubertal boys, and associated with the pathogenesis of osteoporosis (Juul et al., 2009; Yang et al., 2008).

Studies on the impact of single polymorphic UGTs on a disease state or as a risk factor, however, are probably insufficient. The coexistence of the polymorphisms may have more or less profound effects on hormonal metabolism (Menard et al., 2009). Thus, the haplotype analyses are needed for further evaluation of the importance of the UGTs polymorphism.

### 1.2 Stereoisomers

Molecules that have identical atomic constitution and bonding, but different three-dimensional arrangement of the atoms are called stereoisomers, a general term for all isomers that differ only in the spatial orientation of the atoms. Within the group of stereoisomers, different subcategories can be distinguished. Mirror images that differ only by one chiral center are called enantiomers. Isomers of compounds with more than one chiral center, that are not mirror images of one another, are referred to as diastereomers. Also geometric (cis/trans) isomers are included under the definition of stereoisomers. Diastereoisomers and geometric isomers are chemically diverse molecules, and possess different pharmacological effects (unless they are interconverted 
\textit{in vivo}). In the pharmaceutical industry and pharmacotherapy, enantiomers are probably the most interesting stereoisomers. They essentially have identical physical (except for optical rotatory) and chemical properties. In a chiral environment, however, enantiomers are distinguished. This is of interest clinically, as living organisms represent chiral environment and, hence, in biological systems enantiomers are discriminated. They may have different pharmacokinetic properties (absorption, distribution, biotransformation and excretion) and quantitatively or qualitatively different pharmacologic or toxicologic effects. Another relevant point is the complication in analyses of the enantiomers, as they are only separated in chiral environment and thus require specific chiral separation techniques.

#### 1.2.1 Chiral compounds as drugs

As discussed above, chiral substances are discriminated in a homochiral environment (Fig. 3). Thus, it is of considerable interest to separately examine and evaluate the pharmacokinetic and pharmacodynamic characteristics of each individual stereoisomer. This is partly because of the U.S. Food and Drug Administrations (FDA) guidelines for
the development of new stereoisomeric drugs in 1992 (www.fda.gov). It was recently estimated, that of marketed drugs today, approximately one half are optically active and of those approximately 50 % were used as a racemate (Campo et al., 2009). Among the new drugs approved by FDA, in 2005-2008, however, one half are still optically active, but only 8% were racemates. Many of those racemic drugs’ therapeutic action is due to the eutomer, and the distomer can be less active or inactive as in the cases of citalopram and ibuprofen (Hao et al., 2005; Hyttel et al., 1992), or cause a totally different pharmacological effect such as propranolol (Zipper et al., 1983). A safer pharmacological profile, as in the case of levobupivacain, can also be a reason to choose either of the two active enantiomers (Burlacu and Buggy, 2008). Some drug molecules, such as the anti-inflammatory agent ibuprofen, undergo chiral inversion to the other enantiomer in vivo (Hao et al., 2005). The study of the enantiomers’ metabolism in the human body is of considerable interest. Examples of differences in biological action and metabolism of drug enantiomers are numerous. Racemization of the once pure enantiomer can happen, however, during storage and in vitro studies, as well as in the body (Ali et al., 2007). This is particularly important if the opposite enantiomer was toxic.

![Figure 3](image-url)  

Figure 3. The molecule on the left with contact points A, B and C matches the corresponding receptor sites A', B' and C'. The mirror-imaged enantiomeric form of the molecule (right) does not match this receptor, thereby enabling chiral discrimination. According to (Kuhnle et al., 2002).

Conjugation of chiral drugs is a research field, which has recently gained increasing attention. The vast amount of optically active substances on the market, together with the prevalence of UGT contribution to drug metabolism, makes this study field an ever growing theme. Substances are not always directly conjugated, and this metabolic reaction may take place via a functionalization reaction. Thus, it is also possible that initially a non-optically active substance, bearing a prochiral group such as ketone, is metabolized in vivo into two stereomers capable of undergoing conjugation reactions (Chiarotto and Wainer, 1995). Chiral inversion from one enantiomer to another in vivo is also possible (Wsol et al., 2004), the metabolism in a living organism is a sum of multiple processes and is hard to evaluate in vitro.
Propranolol, [1-isopropylamino-3-(1-naphtoxy)-2-propanol] is an example of a widely used chiral drug. It is a non-selective β-adrenergic blocking agent that is used as a racemic mixture in the treatment of hypertension, cardiac arrhythmias and angina pectoris. Propranolol is metabolized through different pathways such as conjugation, side-chain oxidation and ring oxidation. It is glucuronidated to two diastereomeric O-glucuronides in human, as well as in dog and rat (Bai and Walle, 1984; Thompson et al., 1981). The role of presystemic glucuronidation has been evaluated, but the results have been somewhat contradictory. Extensive presystemic glucuronidation of propranolol in human was suggested following oral propranolol administration (Midha et al., 1983). The major site of propranolol glucuronidation, anyhow, is thought to be the liver rather than intestine (Buch and Barr, 1998). Approximately 17% of the dose is metabolized through stereoselective glucuronidation in human (Silber et al., 1982; Walle et al., 1985). When racemic propranolol was administered to man, the concentration of S-propranolol glucuronide (S-pg) was higher than R-propranolol glucuronide (R-pg) in both plasma and urine (Luan et al., 2005; Silber et al., 1982). It was previously reported that at least UGT1A9, UGT2B4 and UGT2B7 are involved in propranolol glucuronidation in human (Coffman et al., 1998; Hanioka et al., 2008; Li et al., 2001). UGT2B7 did not exhibit a clear preference to either R- or S-propranolol, whereas UGT1A9 prefers catalyzing S-enantiomer to R-enantiomer (Coffman et al., 1998; Yu et al., 2009).

1.2.3 Analysis of stereoisomers

In analytical perspective, enantiomers can be directly separated by chiral chromatography, or analyzed after derivatization with a homochiral derivative either online or offline, and separating the resulting diastereomers by conventional, achiral chromatographic methods (Bobbitt and Linder, 2001; Haginaka, 2002). Analytically, conjugates of drug enantiomers are not usually too challenging, since they are chemically diastereomers and can be considered the same as derivatized chiral compounds, as with glucuronides (Laethem et al., 1996). On the other hand, if there is a need for separation of unconjugated substrates or other possible chiral metabolites, as often in clinical studies, chiral separation is usually needed. Metabolite standards are also needed, not only for clinical studies, but also therapeutic and legal drug monitoring. Different metabolites of a drug or an endogenous compound can also mirror certain disease states or biopharmacologically important genotypes. The drugs are often readily metabolized, and the parent drug can be detected in blood or urine only for a limited time period. The conjugated metabolites can usually be detected for a longer period of time, which is not only used in therapeutic drug monitoring but also doping analysis (Peng et al., 2000; Pozo et al., 2008). The need to use non-invasive methods, such as urine collection, also fortifies the importance of the hydrophilic metabolites as a research focus. The metabolite standards are limitedly available, thus decreasing the possibility to use these as biological markers. Several attempts to synthesize glucuronides have been taken place. Chemical synthesis (Alonen et al., 2009; Lucas et al., 2009; Oatis et al., 1983) and enzymatic
approaches (Alonen et al., 2005; Jäntti et al., 2007; Luukkanen et al., 1999; Soars et al., 2002; Stevenson and Hubl, 1999) have been generated. An alternative way of acquiring metabolites standards is to extract them from the urine of either humans or test animals treated with the parent drug (Bai and Walle, 1984). The advantage of different enzymatic or in vivo approaches is that only the biologically relevant β-anomers are formed, but the purification can be difficult and the yields are generally low. This methodology can also raise discussion about study ethics. The chemical syntheses often give better yields, but several impurities, including the non-biological α-glucuronide anomers can be formed, thus leading to additional purification steps.

1.3 Enzyme kinetic studies

Enzyme kinetics is a study of enzyme catalyzed chemical reactions, which aim at resolving the catalytic mechanism of the enzyme, its role in the metabolism, and controlling the activity. Enzyme kinetic studies focus on how compounds are metabolized, on the reaction rates, substrate affinity, and how do the substances affect a certain enzyme. The interaction between enzyme and substrate is often described by the Michaelis Menten equation (Equation 1).

\[ E + S \overset{k_1}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} E + P \]

The Michaelis-Menten model is somewhat simple for many purposes, but it is the most used enzyme kinetic model. The Michaelis constant, \( K_m \), is defined as \((k_2 + k_{-1})/k_1\). It should be noted that \( K_m \) is not a binding constant that measures the strength of binding between the enzyme and substrate. Often \( K_m \) is called affinity, but actually its value includes the affinity of substrate for enzyme, and also the rate at which the substrate bound to the enzyme is converted into product. Only if \( k_2 \) is much smaller than \( k_{-1} \) will \( K_m \) equal a binding affinity. \( V_{\text{max}} \) is the maximum reaction velocity, and the ratio of \( V_{\text{max}}/K_m \) can be used as an indication of the catalytic efficiency.

Enzyme reaction is commonly visualized as a plot of rate of product formation (velocity) versus substrate concentration (Fig. 4). Metabolism of a compound is classically described kinetically with the Michaelis-Menten equation, which yields a hyperbolic rate profile (Fig 4A). Not all enzyme and substrate reactions are best described by this equation, and different models and equations are also used, two examples of which are presented in the Fig. 4. The equations concerning these models are introduced in chapter 4.2.4.
Formerly, linear transformations of Michaelis-Menten equation were widely used, since they are easily exploited with simple conventional tools (Table 1). These linear plots were used for rapid identification of kinetic parameters like $K_m$ and $V_{\text{max}}$.

**Table 1.** *Linear transformations of Michelis-Menten equation.*

<table>
<thead>
<tr>
<th>Transformation</th>
<th>On horizontal (x) axis</th>
<th>On vertical (y) axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eadie-Hofstee</td>
<td>Y/X</td>
<td>No change</td>
</tr>
<tr>
<td>Hanes-Woolf</td>
<td>No change</td>
<td>X/Y</td>
</tr>
<tr>
<td>Lineweaver-Burk</td>
<td>1/X</td>
<td>1/Y</td>
</tr>
<tr>
<td>Scatchard</td>
<td>Y</td>
<td>Y/X</td>
</tr>
</tbody>
</table>

As nonlinear regression methods, however, are easy to use with modern computer software, and significantly more accurate, the linearization in parameter calculation is not longer generally used. Nevertheless, these methods offer a valuable way to easily visualize the properties of enzyme kinetics. For example in the case of atypical kinetics, the Eadie-Hofstee plot is characteristically changed from a straight line to a certain parabola (Fig 4. insets).

### 1.4 Enzyme inhibition

Many drugs used today exert their pharmacological effect via enzyme inhibition. They either decrease the enzymes ability to bind substrates, or lower the enzymes catalytic activity, or both. Two main classes exist, irreversible and reversible inhibitors, differing by their affinity to the enzyme. Reversible inhibitors bind to and dissociate from the protein in an equilibrium process. Irreversible enzymes bind tightly to the enzyme to form
an essentially permanent complex. Drugs that irreversibly inhibit proteins, and are not
dissociated once bound, can lead to toxicity that could not be reversed in poisoning cases.
The time course of the irreversible inhibition is determined by the kinetics of enzyme
synthesis, activation, and elimination. Thus, this type of enzyme inhibitor can only be used
for certain target proteins. Finasteride is used for treatment of prostate hyperplasia and
hair loss. It irreversibly inhibits 5α-reductase type II enzyme in the prostate and hair
follicles (Span et al., 1999). Another example contains the gastric acid lowering agent
omeprazole whose target protein is H⁺,K⁺-ATP-ase, and acetylsalicylic acid that inhibits
cyclo-oxygenase I -enzyme. Irreversible inhibitors bind to and inactivate enzymes, thus
lowering the available enzyme concentration [E], leading to decreased \( V_{\text{max}} \). But then, \( K_m \)
would be unchanged because it is an intrinsic constant of the enzyme, not dependent on
the [E].

Reversible inhibitors can be classified as competitive, noncompetitive, and
uncompetitive (Copeland, 2005). If the detailed mechanism of inhibition is known,
classification into these groups can be done by relation to type of interference. This means
the place on the enzyme that the inhibitor binds to, and the order in which the inhibitor
binds relative to substrate. One mechanism is blocking the substrate binding, namely a
competition for the binding site. As the inhibitor competes with a substrate for an enzymes
active site, it lowers the enzymes likelihood of binding the substrate and reduces the
reaction velocity, but leaves the maximal activity at saturating substrate concentration
unchanged.

Determination of simple kinetic parameters can often be used to identify the type of
inhibition. Typical steady-state kinetic experiments can be performed, in which the
reaction velocity is measured in the presence of varying substrate concentrations. If the
inhibitor is then added, and the data shows an increase in \( K_m \), yet the \( V_{\text{max}} \) is unaffected, it
is the sign of a competitive inhibitor (Fig. 5). Linearizations, such as double reciprocal
Lineweaver-Burk plot, have widely been used before the development of efficient
computer programs. The linearized plots still serve an easily visualized plot, which also
can clarify the inhibition type (Cortes et al., 2001). Uncompetitive inhibitors bind
exclusively to the enzyme-substrate (ES) complex. In contrast to the competitive type, the
inhibition is more pronounced when the substrate concentration is higher, as more ES
complexes are available. A noncompetitive inhibitor can bind to both the unoccupied
enzyme and ES complex. All inhibition types show distinctive patterns in direct
Michaelis-Menten plot, but the inhibition type is easier visualized using Lineweaver-Burk
plot. (Fig. 5). Dixon plot is a graphical method for determination of the inhibition
constant (\( K_i \)) and the type of enzyme inhibition (Dixon, 1953). The effect on the reaction
velocity (v) is determined at two or more substrate concentrations [S], and over a range of
inhibitor concentrations [I]. Keeping [S] constant, 1/v is plotted against [I] and a straight
line will be obtained. Data for each [S] will cut one another at \( I=-K_i \) and 1/v=1/V_{max}
(competitive inhibition), or at the abscissa (1/v=0) and \( I=- K_i \) (non-competitive inhibition.
For uncompetitive inhibition the lines are parallel. If the binding affinity to E and ES is
equal, the inhibitor is a true noncompetitive type.
A mixed inhibitor binds to a site on the enzyme, and interferes with both the parent substrates affinity and catalytic turnover (Cornish-Bowden et al., 1983). Mixed inhibitors do not bind directly to the active site. Therefore, they do not block substrate binding, but instead bind to another site that can be proximal or distal to the active site. Mixed inhibitors can bind to free enzyme prior to substrate, causing the active sites conformation to change to an unoptimal form for the substrate binding and for catalysis. The enzymatic turnover rate is slowed. Mixed inhibitors can also bind after substrate to the ES complex, decreasing the apparent substrate binding. Mixed inhibitors can either increase or decrease $K_m$ and decrease $V_{max}$.

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**Figure 5.** A: Competitive inhibition, leading to increased $K_m$ but unaffected $V_{max}$. B: Non-competitive inhibition; $V_{max}$ is decreased, $K_m$ is unaffected. C: Uncompetitive inhibition, both $V_{max}$ and $K_m$ are decreased. The kinetic parameters changed due to inhibition are written in italics.
1.5 Anabolic steroids

Human male sex steroids consist of a 19 carbon structure (Fig. 6). Male sex steroids that possess androgenic potential are also called androgens. To include all structurally related compounds regardless the androgenic activity, steroids discussed here are referred to as C19-steroids, according to their structure.

![5α-androstane and 5β-androstane](image)

**Figure 6.** Androstane structure of the C19-steroids. The numbering of the carbons as well as nomenclature of the ring structure is depicted. Note the major conformational difference in the steroid backbone between 5α- and 5β-androstane.

1.5.1 Anabolic steroids metabolism

1.5.1.1 Metabolism of testosterone

Testosterone is the main male hormone, secreted by the testes and adrenal gland. It is metabolized to various metabolites, and both testosterone and many of the metabolites are available for conjugation reactions, such as sulfonation and glucuronidation (Fig. 7).

1.5.1.2 Glucuronidation of steroids

Glucuronidation of endogenous steroids and their metabolites is an important metabolic pathway, also of interest in the fight against illegal steroid abuse in sports. The most important UGTs with respect to androgen steroid metabolism are UGT2B family members, 2B4, 2B7, 2B15 and 2B17 (Belanger et al., 2003; Turgeon et al., 2001). Testosterone and its active metabolite dihydrotestosterone (DHT) are glucuronidated mainly by UGT2B17 (Turgeon et al., 2001). Androsterone glucuronide is the major androgen metabolite found in the circulation (Belanger et al., 1991). Androsterone is glucuronidated by UGTs 2B4, 2B7 and 2B17 (Turgeon et al., 2001).
Figure 7. Metabolism of testosterone. Functionalization metabolism of androgen hormones and enzyme that are involved are presented. DHEA, dihydroepiandrosterone; DHT, dihydrotetosterone. 3α- and 3β-HSD, 3α- and 3β-hydroxysteroid dehydrogenase, respectively; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 5α- and 5β-R, 5α- and 5β-reductase, respectively. The numbering of the carbons 1-19 is explained in the DHEA structure. (Dufort et al., 2001; Labrie et al., 2003; Schänzer, 1996).

The $K_m$ values in androsterone glucuronidation by UGT2B7 and UGT2B17 in previous studies were 9.7 µM and 0.4 µM, respectively (Beaulieu et al., 1996; Coffman et al., 1998). Interestingly, even though UGT2B17 seems to be of great importance in vitro, its deletion did not effect the androsterone glucuronide serum concentration (Swanson et al., 2007b).

Most, if not all, steroids are conjugated to some degree by UGT2B7, which is abundantly expressed in the liver, intestine, kidney, and bladder (Nakamura et al., 2008; Turgeon et al., 2001). Etiocholanolone is glucuronidated by UGTs 2B7 and 2B17 (Turgeon et al., 2001). Although it seems to be preferentially glucuronidated by UGT2B17, when $V_{max}$ is considered, (relative $V_{max}$ 46 pmol/mg protein/min compared to that of 19.5 pmol/mg protein/min for UGT2B7), the total absence of UGT2B17 does not
have a significant effect on etiocholanolone glucuronide excretion in urine (Jakobsson et al., 2006; Turgeon et al., 2001). The etiocholanolone $K_m$ for UGT2B17 is lower than for UGT2B7, 1.3 and 4.8 µM, respectively and thus its glucuronidation efficiency is about 20 fold compared to UGT2B7. As endogenous concentrations of etiocholanolone in serum are at the sub-nanomolar range, (Kancheva et al., 2007), the high affinity to the enzyme is important, and the kinetic parameters can not directly explain why the conjugation in vivo is not disturbed by the deficiency of UGT2B17. For the urinary excretion of etiocholanolone glucuronides, like other steroid glucuronides, the conjugation in the kidneys and bladder can be important. It may be significant that in both organs UGT2B7, but not 2B17, is extensively expressed (Nakamura et al., 2008). The construction of the in vitro study may also offer an explanation. As discussed above, UGT2B7 is inhibited by long chain fatty acids present or released during incubation (Rowland et al., 2007). Including fatty acid sequestering agent, such as albumin, in the incubation mixture would most likely lead to lower estimated $K_m$ value for UGT2B7.

Another androgen metabolite, $5\alpha$-diol (Fig. 7), is glucuronidated by UGT2B7, 2B15 and 2B17 with high affinity ($K_m$ 0.5, 2.2 and 1.0 µM, respectively) (Beaulieu et al., 1996; Levesque et al., 1997; Turgeon et al., 2001). There is, however, a limitation in comparing literature kinetic values on the glucuronidation of $5\alpha$-diol and $5\beta$-diol. These diols are glucuronidated by different UGTs at variable regioselectivity, while the published results often contain both glucuronide regioisomers. Formerly it has been assumed that of $5\alpha$-diol, only 17-O-glucuronide exists in the circulation (Rao et al., 1987). Both glucuronides were, however, detected in serum (Chouinard et al., 2008; Swanson et al., 2007b).

### 1.5.2 Implications for doping control

Doping in sports is a problem. The list of prohibited substances in sports is published yearly by the World Antidoping Agency (WADA) (www.wada-ama.org). For over 50 years some athletes have used exogenous testosterone supplements (Bowers et al., 2009). Testosterone induces growth of muscle size and strength (Bhasin et al., 1996). Doping control became possible in the late 60s, along with the development of suitable methods, such as mass spectroscopy, capillary gas chromatography, and radioimmunoassay (Shackleton, 2009). The use of anabolic steroids was first prohibited by the International Olympic Committee at the Montreal Olympic Games in 1976. Since 1983 the method of choice for detecting exogenous testosterone misuse has been based on the urinary testosterone to epitestosterone ratio (T/E) (Donike et al., 1983). Testosterone and epitestosterone exist in approximately equal amounts in urine. Presently, T/E >4.0 is considered suspicious and requires further examination. The parent steroids are extensively metabolized, and can only be detected in the urine for a short time after use. Generally the analytes are excreted as glucuronides and sulphates, and steroids are analyzed after hydrolysis of the conjugates (Schänzer and Donike, 1993; Schänzer, 1996). Glucuronidation of endogenous steroids and their metabolites is thus also of interest in illegal steroid abuse. Both synthetic and naturally occurring steroids (e.g. testosterone,
DHT, and 4-androstenedione) are abused in sports (Cawley et al., 2008; Dehennin and Matsumoto, 1993; Donike et al., 1995). Endogenous steroids are metabolized to several androgens such as testosterone, DHT, DHEA, androstenedione, androsterone and etiocholanolone (Fig. 7). The problem with endogenous steroid determination is that since they are naturally present in the samples, a simple qualitative approach is not adequate. Screening for the misuse of naturally occurring anabolic steroids is based on not only monitoring the absolute amounts in urine, but also the ratios of certain endogenous steroids, the most important of which has been the testosterone to epitestosterone ratio (T/E) (Donike et al., 1983). Establishment of proper threshold levels is a consistent issue. The levels are based on population statistics, and many studies have shown significant population differences in the average basal levels of the studied metabolites (Bowers, 2008; Jakobsson et al., 2006; Schulze et al., 2008c; Sottas et al., 2008; Strahm et al., 2009). Current research is mostly concentrated on a UGT2B17 deletion polymorphism, as it reduces testosterone glucuronidation to negligible levels. This has led to alternative suggestions for steroid abuse detection, such as population or genotype based T/E cut-off levels, or individual longitudinal steroid profile follow up (Bowers, 2008; Schulze et al., 2008b; Schulze et al., 2008c; Sottas et al., 2008; Strahm et al., 2009). Samples with adverse analytical findings are submitted to isotope ratio mass spectroscopy analysis to verify the origin of the steroid, as the percentage of C13 in endogenous molecules is different from that of synthetic origin. Other suspicious findings include higher than threshold concentration of epitestosterone, testosterone, androsterone, etiocholanolone, and DHEA (all equivalent to the glucuronide) (WADA technical report 2004).
2. Aims of the study

This work was done to study the reactions of UDP-glucuronosyltransferases with stereoisomeric molecules. The more detailed aims of the included publications were:

- Biosynthesis of R- and S-propranolol glucuronides for reference compounds (I)
- Examination of the enantioselective glucuronidation of R- and S-propranolol by human UGTs along with microsomes from human liver and intestine (I).
- To study the glucuronidation of several structurally related androgens by recombinant UGTs, for determining the regio- and stereoselectivity in such reactions (II, III).
- To study the inhibition on testosterone glucuronidation by UGT2B15 (IV) and UGT2B17 (II, IV).
3. Materials and Methods

3.1 Chemicals

Figure 8. Structures of the studied compounds: A - R-propranolol; B - S-propranolol; C - testosterone; D - epitestosterone; E - 5α-diol; F - 5β-diol; G - androsterone; H - etiocholanolone; I - diclofenac; J - ibuprofen.
Stereoisomeric pairs of compounds were studied, the structures of which are shown in figure 8. In propranolol [1-isopropylamino-3-(1-naphtoxy)-2-propanol] enantiomers, the conjugative OH-group is at the chiral center (I). A similar difference occurred between the diastereomers testosterone (4-androsten-17β-ol-3-one) and epitestosterone (4-androsten-17α-ol-3-one) (II). Androsterone (5α-androstan-3α-ol-17-one), etiocholanolone (5β-androstan-3α-ol-17-one), 5α-diol (5α-androstan-3α,17β-diol), and 5β-diol (5β-androstan-3α-17β-diol) have more extensive differences at C5 that do not result in simple configurative changes right at the stereocenter, but also in the steroid backbone (III). Additionally, the four latter compounds let us study the regioselectivity of the UGTs with respect to the 3-OH and 17-OH groups of those androgens. The inhibition of testosterone glucuronidation by common NSAIDs, ibuprofen and diclofenac was studied using human liver microsomes and recombinant UGTs (IV). Detailed descriptions of the chemicals used can be found in the original publications I-IV. All reagents were of analytical or chromatographic grade.

3.2. General experimental conditions

Experimental methods used in the studies included in this thesis are described in detail in the original publications I-IV or references therein. The general methods are outlined briefly below.

3.2.1 Recombinant enzymes and other protein sources

Human recombinant UGTs were cloned and expressed in baculovirus-infected insect cells in our laboratory (Finel et al., 2005; Kurkela et al., 2003; Kuuranne et al., 2003; Sneitz et al., 2009). The recombinant UGTs from our laboratory carried a short C-terminal fusion peptide ending with 6 His residues, a His-tag. The relative expression level of each recombinant UGT was determined by dot-blot analyses using tetra-His antibodies (Qiagen, Hilden, Germany), as detailed elsewhere (Kurkela et al., 2004; Kurkela et al., 2007). Protein concentrations were determined by the BCA method (Pierce Biotechnology Inc., Rockford, IL, USA). Due to observed poor activity of the UGT2B15 produced in our laboratory, recombinant human UGT2B15 of commercial origin (BD Supersomes™ Gentest, BD Biosciences, MA, USA) was included in certain studies (II, III, IV). This enzyme, like our recombinant UGTs, is expressed in baculovirus-infected insect cells, but does not carry a His-tag. Hence, its expression level could not be compared to the other UGTs. Human liver and intestinal microsomes (HLM and HIM, respectively) were purchased from Gentest (BD Biosciences, MA, USA) (I).

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3.2.2. Reaction conditions

The incubation reactions are described in detail in the original publications I-IV. The studies with recombinant UGTs and liver microsomes were carried out with 2.5-5 mM co-substrate UDPGA in 50 mM phosphate buffer (pH 7.4) containing 5 mM MgCl₂. The substrates were dissolved in DMSO (I-IV) or ethanol (IV) and the organic solvent concentration in the reactions was 1-5 %. When saccharic acid lactone (a β-glucuronidase inhibitor) was used, its concentration was 5 mM (I-III). The reactions were started by the addition of UDPGA, incubated in a heat bath at +37°C and terminated by the addition of cooled methanol (I) or 4 M perchloric acid (II-IV), chosen according to the analytes and chromatographic conditions. The control incubations were carried out in the presence of UDPGA, but in the absence of substrate, and vice versa. The glucuronides were identified by HPLC/MS/MS analyses. For kinetic analyses, 2-3 protein concentrations were first incubated with 2-3 different substrate concentrations for varying time spans, to specify the conditions when product formation is linear with respect to protein concentration and time. The determination of linear conditions was done individually for each pair of recombinant protein and substrate.

3.2.3 Analytical methods

The chromatographic devices used for the studies are listed below (Tables 2 and 3). When the analyses were done in another laboratory, namely GC/MS analyses (III) and HPLC-UV analysis of HLM samples (IV), the equipment is not listed here.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromatographs and columns</strong></td>
<td><strong>Detection</strong></td>
<td><strong>Note</strong></td>
</tr>
<tr>
<td>Acquity UPLC®</td>
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<td>Empower software</td>
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<tr>
<td>Acquity BEH Shield RP18 column (100x 1mm i.d. with 1.7-µm particle size)</td>
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<tr>
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<td>Mass spectrometry</td>
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<tr>
<td>Agilent HPLC 1100</td>
<td>Fluorescence 227 (ex) 336 (em) nm</td>
<td>ChemStation software</td>
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<tr>
<td>Chirobiotic T (250 x 4.6 mm i.d. with 5-µm particle size)</td>
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</table>
Table 3. Instrumentation used in the studies I-IV.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
<th>Study</th>
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<tr>
<td>Mass Spectrometers</td>
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<td>Analysis</td>
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<td>ESI</td>
<td>LC-MS/MS</td>
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<tr>
<td>Waters Micromass Q-TOF</td>
<td>ESI</td>
<td>LC-MS and LC-MS/MS</td>
</tr>
</tbody>
</table>

3.2.4. Data analysis

Computer analyses of the enzyme kinetic data were done using GraphPad Prism software (version 4.02 for Windows; GraphPad Software, San Diego, CA, USA). In addition to computer analyses, data transformation and visualization in Eadie-Hofstee (II) and Dixon plots (IV) was done. The equations used are as follow, with symbol explanations below.

1. Michaelis-Menten equation  
   \[ v = \frac{V_{\text{max}} \times S}{K_m + S} \]  
   I-III

2. Substrate inhibition:  
   \[ v = \frac{V_{\text{max}}}{1 + (K_m/S) + (S/K_{si})} \]  
   I, III

3. Hill equation:  
   \[ v = \frac{V_{\text{max}} \times S^n}{S_{50}^n + S^n} \]  
   I-III

4. Competitive inhibition model equation:  
   \[ v = \frac{V_{\text{max}} \times S}{K_m \left(1 + \frac{I}{K_i}\right) + S} \]  
   II

5. Modified sigmoidal dose response equation  
   \[ Y = \frac{Top + (Bottom - Top)}{1 + 10^{\left((X - \text{LogIC50}) \times \text{HillSlope}\right)}} \]  
   IV
\( \nu = \text{velocity} \)
\( V_{\text{max}} = \text{maximum velocity} \)
\( S = \text{substrate concentration} \)
\( K_m = \text{Michaelis-Menten constant (substrate concentration at 0.5 } V_{\text{max}}) \)
\( K_i = \text{inhibition constant} \)
\( K_{si} = \text{the constant describing the substrate inhibition interaction} \)
\( S_{50} = \text{the substrate concentration resulting in 50\% of } V_{\text{max}} \)
\( n = \text{Hill coefficient} \)
\( I = \text{inhibitor concentration} \)
\( IC_{50} = \text{half maximal inhibitory concentration} \)
\( X = \text{logarithm of the inhibitor concentration} \)
\( Y = \text{degree of inhibition, expressed as } \% \text{ of remaining activity while the uninhibited activity is taken as 100\%. } Y \text{ starts at the } \text{Top} \text{ and goes to the } \text{Bottom} \text{ with a sigmoid shape.} \)
4. Results and Discussion

The results of this study are mainly presented in the attached articles (I-IV). Additional data about propranolol glucuronidation by UGT2A-family enzymes, and alternative data processing of the inhibitory study (IV) are presented here. The parts of the studies (II, IV) that were carried out by the researchers other than the writer of the present study are excluded from this dissection.

4.1. Propranolol glucuronidation (I)

4.1.1 Stereoselective glucuronidation of propranolol

Glucuronidation of propranolol enantiomers by HLM and HIM as well as human recombinant UGTs was studied and analyzed by HPLC with UV-detection. Racemization of the propranolol enantiomers did not happen during the incubation. Glucuronidation of propranolol enantiomers was stereoselective in HLM and HIM (Table 4). The contribution of certain UGT enzymes to this selectivity was established during incubation of propranolol enantiomers with these enzymes, both in the presence and absence of the opposite enantiomer. The concentration of an individual enantiomer was kept constant.

Table 4. Glucuronidation rates of propranolol enantiomers and their dependence on the presence or absence of the opposite enantiomer.

<table>
<thead>
<tr>
<th>UGT1A1</th>
<th>S-pg ± SD (pmol/mg/min)</th>
<th>R-pg ± SD (pmol/mg/min)</th>
<th>S-pg ± SD (pmol/mg/min)</th>
<th>R-pg ± SD (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGT1A9</td>
<td>0.7 ± 0.0</td>
<td>detectable</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>8.3 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>21.6 ± 0.6</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>UGT2B4</td>
<td>5.5 ± 0.8</td>
<td>26.8 ± 4.2</td>
<td>8.7 ± 1.3</td>
<td>36.7 ± 1.7</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>4.9 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>19.5 ± 0.5</td>
<td>25.2 ± 0.6</td>
<td>32.9 ± 3.3</td>
<td>41.3 ± 2.0</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>1.1 ± 0.2</td>
<td>detectable</td>
<td>1.4 ± 0.3</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>UGT2A1</td>
<td>1.9 ± 0.0</td>
<td>1.7 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>UGT2A2</td>
<td>0.7 ± 0.0</td>
<td>detectable</td>
<td>0.6 ± 0.0</td>
<td>detectable</td>
</tr>
<tr>
<td>HLM</td>
<td>70.7 ± 4.9</td>
<td>50.4 ± 3.2</td>
<td>74.2 ± 8.9</td>
<td>47 ± 1.8</td>
</tr>
<tr>
<td>HIM</td>
<td>7.3 ± 0.7</td>
<td>13.1 ± 1.2</td>
<td>10.0 ± 2.8</td>
<td>21.8 ± 1.8</td>
</tr>
</tbody>
</table>
The most active enzymes were the UGTs 1A9, 1A10, 2B4, and 2B7. The most prominent stereoselectivity was observed for UGT1A9 and UGT1A10, the first favoring S-propranolol, and the latter R-enantiomer (Table 4). These results might suggest the enantioselective first pass glucuronidation of propranolol, as seen with HIM. Generally, the presence of the other enantiomer diminished the glucuronide formation, as in the case of UGT1A9. An exception to this was found with UGT2A1, since the glucuronide formation was somewhat stimulated by the presence of both enantiomers.

According to the kinetic data, even the most active UGTs were low to medium capacity enzymes in both propranolol enantiomers glucuronidation (Table 5). Sub-millimolar \( K_m \) values of UGTs 1A9-10 and UGT2B7 indicated rather high affinity, whereas UGT2B4 and UGT2A1 were low affinity enzymes. According to the present knowledge, however, some of these results could be questioned. At least UGT1A9 and UGT2B7 are shown to be inhibited by long chain fatty acids present in the incubation media, a phenomenon that can be overcome with the addition of albumin in the reaction mixture (Rowland et al., 2007; Rowland et al., 2008). Another matter worth to consider in the studies is the use of SL in the reaction (Oleson and Court, 2008). It changes the pH of the incubation mixture, and the 50 mM phosphate buffer may not have had sufficient buffering capacity. Organic solvents also variably affect UGT enzyme activity, which may have obscured contributions of some UGT enzymes (Uchaipichat et al., 2004). Considering the data processing, application of multi-site kinetic models might have offered further insights into the kinetic studies.

### Table 5. Kinetic constants (± SE) for S- and R-propranolol aglucuronidation by recombinant UGTs 1A9-10, 2B4, 2B7, and 2A1.

<table>
<thead>
<tr>
<th></th>
<th>S-propranolol</th>
<th>R-propranolol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{max} )</td>
<td>( K_m ) or ( S_{50} )</td>
</tr>
<tr>
<td>( (\text{pmol/mg/min}) )</td>
<td>( (\text{mM}) )</td>
<td>( (\text{pmol/mg/min}) )</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>35.3 ± 3.7</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>11.4 ± 0.7</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>UGT2B4a</td>
<td>25.6 ± 2.4</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>73.4 ± 4.4</td>
<td>0.60 ± 0.1</td>
</tr>
<tr>
<td>UGT 2A1</td>
<td>74.1 ± 3.1</td>
<td>5.0 ± 0.3</td>
</tr>
</tbody>
</table>

\( a) \) Substrate inhibition. \( Ki = 1.2 ± 0.2 \)

\( b) \) Saturating concentration not reached; experimental data up to 5 mM fitted to the equations.

\( c) \) Hill equation. \( n = 1.9 ± 0.2 \)

\( d) \) Hill equation. \( n (S-p)=1.8 ± 0.1; n (R-p)=2.5 ± 0.3 \)

N.D., not determined

The most interesting result in catalytic activity point of view was the prominent, but opposite, stereoselectivity of the closely related enzymes UGT1A9 and UGT1A10. This
inverse preference for the propranolol enantiomer was a significant finding, especially considering the highly similar primary structures of UGT1A9 and UGT1A10. In the total of 505 residues in these mature proteins, 32 are nonidentical, denoting 93% similarity in the amino acid sequence. It is also worth mentioning that UGT1A7 and UGT1A8 were practically inactive in propranolol glucuronidation despite the considerable homology that they share with UGTs 1A9 and 1A10. In UGT1A9, 14 amino acids are unique compared to UGT1A7-8 and 1A10, and in a recent study Arg42 and Asn152 were suggested to contribute largely to the dissimilarity in the substrate specificity between UGT1A8 and UGT1A9 (Fujiwara et al., 2009). The critical amino acids affecting the substrate selectivity of UGT1A9 and UGT1A10 still remain to be studied.

4.1.2 Atypical kinetics in propranolol glucuronidation

Atypical kinetics refers to kinetics diverged from the Michaelis-Menten type. In the case of propranolol glucuronidation, both substrate inhibition and cooperativity were evidenced (Fig. 9). Those types were also clearly illustrated by the Eadie-Hofstee plots for S-propranolol glucuronidation by UGT1A9 and UGT2A1 (Fig. 9).

![Figure 9](image)

**Figure 9.** Atypical kinetics of S-propranolol glucuronidation by UGT1A9 (A) and UGT2A1 (B). Substrate inhibition with UGT1A9 and autoactivation kinetics by UGT2A1 are illustrated with Eadie-Hofstee plots in the insets.

The mechanism of substrate inhibition is not fully known. Substrate inhibition may arise from multiple mechanisms, although all arise from the binding of two or more substrate molecules. One possible explanation of observed inhibition with certain UGTs might be the product inhibition caused by the released UDP (Fujiwara et al., 2008). When one of the enantiomers has higher glucuronidation turnover with the enzyme, the other might hinder the conjugation via non-productive binding as a competitive inhibitor. In both enantiomers conjugation by UGT1A9, the reaction rate was diminished when the opposite enantiomer was also present (Table 4). The effect that the opposite enantiomers presence induces on the kinetic values of propranolol glucuronidation by UGT1A9 has
recently been elucidated (Yu et al., 2009). The $V_{max}$ of either enantiomer was significantly lower when the substrate was used as a racemic compound. The assay done with the racemic substrate most likely represents the situation *in vivo* better, if the compound is used as a racemate. The determination of the true kinetic values and possible inhibition constant of the opposite enantiomer, however, is unreliable if neither of the enantiomers concentration can be held steady.

When an enzyme does not discriminate between the two enantiomers, the activity toward both is similar. Such was the case with the propranolol enantiomers glucuronidation by UGT2A1 (Table 4). This finding was further studied by conducting the kinetic experiments of propranolol glucuronidation by UGT2A1. Both propranolol enantiomers were glucuronidated by UGT2A1 with very high $S_{50}$ values, suggesting low affinity to the enzyme (Table 5). Furthermore, the data illustrated positive autoactivation kinetics and suggested multiple binding sites. The sigmoidal kinetics is depicted in figure 9. Positive cooperativity explains the positive influence in the glucuronidation rate of either of the propranolol enantiomers by the opposite enantiomer, particularly at a concentration range significantly below the $S_{50}$ (Table 4). The study could continue with full kinetic characterization of propranolol enantiomers in the presence of the opposite enantiomer to further ascertain the positive cooperativity in action.

The study of propranolol glucuronides has yielded new information on the stereoselectivity of UGTs, particularly for the closely related enzymes UGT1A9 and UGT1A10. The studies have been repeated and extended by other authors with a kinetic study of racemic propranolol, and stereoselectivity of propranolol glucuronidation in cynomolgous monkey liver microsomes (Hanioka et al., 2008; Yu et al., 2009). The results also suggest that the use of chiral drugs as probe substrates in studies of structurally related enzymes, such as UGT1A9 and UGT1A10, may be very valuable for elucidating substrate selectivity differences. For example, stereoselective glucuronidation of oxazepam was previously used as a probe of induced forms of UGTs in rabbits (Yost and Finley, 1985). In humans, S-oxazepam is conjugated mainly by UGT2B15, that is inactive in R-oxazepam glucuronidation, whereas R-oxazepam is glucuronidated by UGT1A9 and UGT2B7 (Court et al., 2002).

### 4.2. Steroids glucuronidation (II, III)

The glucuronidation of six structurally related endogenic C19-steroids was screened with 19 recombinant human UGTs (Table 6).
Table 6.  Glucuronidation rates of androsterone, etiocholanolone, and 5α- and 5β-diol glucuronidation by recombinant UGTs. Det., glucuronide was detected but below quantification level. n.d., no glucuronide was detected. UGT2B15* was of commercial origin (BD Biosciences, USA).

Glucuronide formation, pmol/mg protein/min

<table>
<thead>
<tr>
<th>UGT</th>
<th>Testosterone 17-glucuronide</th>
<th>Epitestosterone 17-glucuronide</th>
<th>Androsterone 3-glucuronide</th>
<th>Androsterone 3-glucuronide</th>
<th>Etiocholanolone 3-glucuronide</th>
<th>Etiocholanolone 3-glucuronide</th>
<th>5α-diol* 3-glucuronide</th>
<th>5β-diol* 3-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A4</td>
<td>5.5 ± 0.3</td>
<td>4.9 ± 0.8</td>
<td>11.7 ± 0.0</td>
<td>Det.</td>
<td>5.2</td>
<td>77.4</td>
<td>Det</td>
<td>Det</td>
</tr>
<tr>
<td>1A8</td>
<td>2.7 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1A9</td>
<td>10.4 ± 0.5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1A10</td>
<td>7.9 ± 0.8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>11.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>2A1</td>
<td>253.0 ± 5.4</td>
<td>172.0 ± 0.9</td>
<td>25.0 ± 1.3</td>
<td>565.4 ± 18.5</td>
<td>n.d.</td>
<td>97.6</td>
<td>52.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>2A2</td>
<td>13.5 ± 0.5</td>
<td>69.7 ± 5.4</td>
<td>15.0 ± 1.7</td>
<td>26.1 ± 1.0</td>
<td>12.0</td>
<td>38.4</td>
<td>60.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>2B4</td>
<td>n.d.</td>
<td>8.1 ± 0.6</td>
<td>Det.</td>
<td>Det.</td>
<td>10.5</td>
<td>n.d.</td>
<td>8.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>2B7</td>
<td>4.6 ± 0.1</td>
<td>312.5 ± 20</td>
<td>687.0 ± 47.2</td>
<td>192.0 ± 39.8</td>
<td>264.6</td>
<td>n.d.</td>
<td>63.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>2B15</td>
<td>7.9 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>70.7</td>
<td>4.1</td>
</tr>
<tr>
<td>2B15*</td>
<td>63.0 ± 2.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>570.3</td>
<td>n.d.</td>
<td>30.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>2B17</td>
<td>508.9 ± 19.6</td>
<td>n.d.</td>
<td>365.0 ± 8.3</td>
<td>786.0 ± 5.4</td>
<td>12.8</td>
<td>745.9</td>
<td>27.9</td>
<td>361.2</td>
</tr>
</tbody>
</table>

The protein concentration was 0.5-1.25 mg/ml, substrate concentration 50 µM and incubation time 120 min, n=3. On protein concentration of 0.5 mg/ml, LOQ corresponds to reaction velocity of 4.6 pmol/mg/min (testosterone and epitestosterone) and 2.8 pmol/mg/min (androsterone and etiocholanolone).

* The diol glucuronides are quantified based on androsterone glucuronide standard curve.
Both stereo- and regioselectivity was evident in steroid glucuronidation (Table 6). Formation of steroid diglucuronides was not evident. Kinetic characterization of the most active UGTs in androgens glucuronidation revealed generally high affinity (low $K_m$) of 2B7 and 2B17 (Table 7). Recent study (Bowalgaha et al., 2007) determined a somewhat lower $K_m$ in testosterone glucuronidation, 3.8 µM versus our 10 µM (II). The substrate concentrations we used for studying kinetic constants should have been lower for better $K_m$ value estimation.

UGT2A1 was capable of glucuronidation of all studied steroids, but with variable activity and regioselectivity (Table 6). In a bent steroid skeleton, such as etiocholanolone and 5β-diol, 3-O-glucuronide was formed abundantly (etiocholanolone), but 17-O-glucuronide formation was negligible (5β-diol) (Fig. 7). Apparently, non-productive binding of 5β-diol in direction, when the D-ring comes close to the active site, reduced otherwise abundant 17-O-glucuronidation. In contrast, planar structure of androsterone and 5α-diol was only insignificantly conjugated at the 3-position, but was a rather good substrate for 17-O-glucuronidation, which was also evident in the case of testosterone and epitestosterone (Table 6). The glucuronidation efficiency of UGT2A1 toward the substrates, however, was rather poor (Table 7). The $K_m$ in etiocholanolone glucuronidation, 250 µM, was exceptionally high. Regardless of the high capacity, UGT2A1 is not presumed to play a major role in etiocholanolone glucuronidation, as a consequence of both low affinity and the local expression pattern of UGT2A1. The results, nonetheless, might be misleading due to the fact that UGT2B7 is shown to be inhibited by long chain fatty acids (Rowland et al., 2007). Thus the actual $K_m$s are supposed to be even lower than the reported ones. The effect of the fatty acids in UGT2A1 mediated glucuronidation is to be investigated.

| Table 7. Kinetic parameters of testosterone, epitestosterone, androsterone, and etiocholanolone glucuronidation by UGTs 2A1, 2B7 and 2B17. Values are mean ± S.E. |
|-----------------|------------------|------------------|------------------|------------------|
|                 | UGT2A1 $V_{max}$ | UGT2B7 $V_{max}$ | UGT2B17 $V_{max}$ |                 |
|                 | pmol/mg/min µM   | pmol/mg/min µM   | pmol/mg/min µM   |                 |
| Testosterone    | 427 ± 9.5 38.7 ± 6.7 | n.d. 337 ± 4.8 | 1002 ± 16.7 10.0 ± 0.8 |                 |
| Epitestosterone | 271 ± 6.7 11.6 ± 1.2 | 713 ± 41 | 713 ± 41 |                 |
| Androsterone    | n.d. n.d. | 713 ± 41 | 713 ± 41 | 713 ± 41 |
| Etiocholanolone | 4900±700 250 ± 50 | 189 ± 4.7 | 814 ± 4.3 |                 |
|                 |                 |                 |                 | n.d. stands for “not detected” |

$^a$ Data fitted to substrate inhibition equation Ksi 658 ± 333 µM
$^b$ Data fitted to substrate inhibition equation, Ksi 494 ± 211 µM
4.2.1 Substrate selectivity of UGT2B7, UGT2B15 and UGT2B17

Prominent difference appeared between UGT2B7 and UGT2B17 stereoselectivity in glucuronide conjugation at the C17 position of the steroids (Table 6). UGT2B17 was strictly stereoselective in 17β-OH glucuronidation (testosterone), whereas UGT2B7 strongly favored 17α-configuration (epitestosterone) (II). This was consistent with other findings on steroids glucuronidation (Itäaho et al., 2008). UGT2B17 is also active in 3-O-glucuronidation of androsterone, etiocholanolone and dehydroepiandrosterone (III) (Turgeon et al., 2001). The aglycones have two possible alignments in the conjugating enzymes, either the A- or D-ring toward the catalytic site of the enzyme. For conjugation with glucuronic acid by UGT2B17, the configuration of the carbon that is bearing the hydroxyl undergoing the conjugation has to be in either the 17β- or 3α-orientation (Fig. 10). Although no structure bearing the hydroxyl in a 3β-orientation was included in this study, it has been shown by others (Beaulieu et al., 1996) that epiandrosterone, an androsterone stereoisomer with 3β-OH instead of 3α-OH, was not a substrate for UGT2B17. Additional information on the substrate selectivity of UGT2B17 still has to be acquired, but our study adds both new insights and a proof of concept to the current knowledge on UGT2B17 substrate selectivity in steroids glucuronidation.

A possible explanation for UGT2B7 stereoselectivity could be that methyl groups cause hindrance for glucuronidation if they are positioned in cis-configuration with respect to the hydroxyl group, considering the plane of steroid skeleton. This would explain why androsterone, etiocholanolone, epitestosterone, and 5α-diol-3-O-glucuronide, but not testosterone glucuronide, are formed by UGT2B7. If it is assumed that a steroid structure can occupy the enzyme active site in two possible directions, then both 17α-OH and 3α-OH bearing steroids would be conjugated by this enzyme. In fact, the earlier results on androgens glucuronidation by UGT2B enzymes also indicate that 3β- as well as 17β-hydroxylated androgens are rather poor substrates to UGT2B7, while 3α-hydroxylated serve as good aglycones (Turgeon et al., 2001).

The metabolite formation is dependent on both binding affinity and catalytic turnover. Epitestosterone serves as a particularly good substrate for UGT2B7, as it has not only a high V_{max} but also a low K_{m}, yielding high efficiency (Table 7). The notable difference in affinity became evident since testosterone was not inhibiting epitestosterone glucuronidation by UGT2B7, although the opposite was true in UGT2B17-catalyzed testosterone glucuronidation (II). The diols glucuronidation at 3-OH was diminished when an additional OH-group was available at C17 (5α-diol) (Table 6). The diol may exhibit non-productive binding on the C17-direction, decreasing the production of 3-O-glucuronides.

UGT2B15 is strictly selective for 17β-O-glucuronidation, according to both our studies (II, III) and previous results (Turgeon et al., 2001). Possibly due to the direction of the hydrophobic methyl groups, which in the case of UGT2B15 would prevent reaction when directing the opposite (trans) side of the steroid plane.
Figure 10. Selectivity of UGT2B7 and UGT2B17 toward 3α- and 17α/β-OH groups in C19 steroids.

4.2.2 Correlation of in vitro results with in vivo findings

The results on steroid glucuronidation also have clinical implications. Most, if not all, steroids are conjugated by UGT2B7, which is abundantly expressed in liver, intestines, and kidneys (Nakamura et al., 2008; Ohno and Nakajin, 2009; Turgeon et al., 2001). The
UGT2B subfamily enzymes are all present in liver and it has recently been reported that UGT2B4 expression is particularly high in this organ (Izukawa et al., 2009; Ohno and Nakajin, 2009). Hence, UGT2B4 might have a prominent contribution to metabolism in vivo, despite its relatively low activity in vitro. Although not only testosterone, but also androsterone and etiocholanolone are preferentially glucuronidated in vitro by UGT2B17 (Table 6), the deletion of UGT2B17 does not seem to have a significant effect on these steroid glucuronide excretion in urine (Jakobsson et al., 2006; Juul et al., 2009). Since endogenous concentrations of steroids are low (unconjugated etiocholanolone in plasma 1.4 nM) (Sato and Ohsawa, 1977) the high affinity to the enzyme is important, and therefore the kinetic parameters can not directly explain why the conjugation in vivo is not disturbed by the deficiency of UGT2B17. The explanation might lay in the relatively low expression level of UGT2B17 in the liver, and its near absence from the kidney (Nakamura et al., 2008; Ohno and Nakajin, 2009).

Since 5β-diol-17-O-glucuronide is only produced by UGTs 2B15 and 2B17, its concentration could be expected to be related to UGT2B17 deletion polymorphism, as seen with testosterone (Jakobsson et al., 2006). The total 5β-diol glucuronide amount in urine is reduced among samples with a UGT2B17 deletion polymorphism (Jakobsson et al., 2006), suggesting that in vivo a considerable amount is glucuronidated at the 17-OH by UGT2B17. On the other hand, the effect of UGT2B17 deletion was not seen or was minor in 5α-diol glucuronidation (Jakobsson et al., 2006; Juul et al., 2009). Our results can explain these findings, as especially 5β-diol-17-glucuronide formation is almost exclusively due to UGT2B17 activity, whereas UGT2B15 can largely compensate the lack of UGT2B17 in the case of 5α-diol (Table 6). Even though the study by Juul et al. discussed all conjugated metabolites of the studied androgens, and the diol glucuronides concentrations consisted of both 3-O- and 17-O-glucuronide, the difference was highly significant, suggesting a central role of UGT2B17 in 5β-diol glucuronidation in vivo.

UGT2A1 was active in all androgens glucuronidation, at least to some extent, but generally the $K_m$ in every substrates glucuronidation was quite high (Table 6-7). The contribution of UGT2A1 to steroids systemic metabolism is presumed negligible, as it is only abundantly expressed in the nasal cavity, although reports about low level expression of UGT2A1 mRNA in the lung and trachea have been published (Jedlitschky et al., 1999; Nishimura and Naito, 2006; Somers et al., 2007). The UGT2A1, however, should not be ignored in prospective studies. Nasal delivery in brain targeting has gained increasing interest (Costantino et al., 2008; Hanson and Frey, 2008), and even testosterone administration to the brain of a mouse, using the intranasal route, was recently described (Banks et al., 2009).

4.3 Inhibition of testosterone glucuronidation (II, IV)

We studied inhibition of testosterone glucuronidation by different compounds using recombinant UGT2B17 (II, IV) and UGT2B15 (IV). Inhibitory effects of diclofenac and ibuprofen on testosterone glucuronidation by HLM, UGT2B15, and UGT2B17 were investigated at fixed substrate concentrations and variable inhibitor concentrations (IV).
Competitive inhibition was evident in most cases with the exception of ibuprofen inhibition of UGT2B17-catalyzed testosterone glucuronidation. Weak inhibition was seen in this case, but no clear conclusion of the inhibition type could be drawn based on the Dixon plots (IV). The study of inhibition potency of epitestosterone on testosterone glucuronidation by UGT2B17 (II) was conducted by full kinetic characterization in presence of varying inhibitor concentrations. As computer programs for calculating global values for kinetic and inhibition parameters offer better estimation of true values, such a study design is advisable (Kakkar et al., 1999). This study design also allows the use of Lineweaver-Burk plots in visualizing the inhibition type. Fairly good estimations with only two substrate concentrations could be acquired when our data (IV) was analyzed with the above mentioned techniques (Fig. 11). Lineweaver-Burk plots strongly suggest competitive type inhibition by diclofenac and epitestosterone, but uncompetitive type by ibuprofen (Fig. 11).

Figure 11. Testosterone glucuronidation by UGT2B17 and inhibition by diclofenac (A), ibuprofen (B) and epitestosterone (C) represented in direct plot (A-C) and semireciprocal Lineweaver-Burk plot (D-F, respectively). Data was fit in competitive inhibition (A,C) or uncompetitive inhibition equation (B) with GraphPad Prism 5.0.

UGT2B17 is the most active enzyme in testosterone glucuronidation, both in vitro and in vivo (II, IV) (Jakobsson et al., 2006; Turgeon et al., 2001). If UGT2B17 is inhibited in vivo, it could have a minor contribution to homeostasis of certain substances, such as testosterone. The inhibitors, still, should be far more effective than the tested ones. The $K_i$ of diclofenac inhibition, calculated from the above mentioned data, was 56 µM (± S.E. 4.8 µM), and the $K_i$ for ibuprofen was 605 µM (± S.E. 78 µM). The plasma concentration observed for diclofenac varies between 3.6 and 6.1 µM (Cryer and Feldman, 1998; Vietri et al., 2000). The therapeutic plasma concentrations of ibuprofen have been reported to be between 39 and 110 µM (Cryer and Feldman, 1998) depending on the dose. This suggests that, considering UGT2B17 metabolism in vivo, these NSAIDS are not expected to significantly affect its conjugative activity. UGT2B15 was inhibited by both diclofenac
and ibuprofen with \( K_I \)s of 13 (±0.8) µM and 40 (±3.3) µM, respectively (IV). This indicates that ibuprofen, despite having a lower potential inhibitory effect, might modulate the testosterone glucuronidation more in vivo, especially in the case of \( UGT2B17 \) del/del genotypes, in which \( UGT2B15 \) is probably the main enzyme responsible in testosterone glucuronidation (II). However, both diclofenac and ibuprofen are very highly protein bound in blood, and on that basis, inhibition of testosterone glucuronidation may not be expected.
5. Summary and Conclusions

Stereoisomers offer useful probes for investigating UGT substrate specificity and structural aspects influencing substrate selectivity and affinity. Propranolol was glucuronidated by UGT1A9 and UGT1A10, two very similar enzymes, with opposite stereoselectivity. The results suggest a role of intestinal UGT1A10 in the first pass metabolism of propranolol. Extrahepatic UGT2A1 was also active, even though both propranolol enantiomers were poor low affinity substrates. Allosteric activation was suggested in the reaction between propranolol enantiomers and UGT2A1.

Studies on androgens glucuronidation by human recombinant UGTs mostly offered a proof of concept to the previous studies, but also some novel findings. Testosterone glucuronidation by UGT2B17 was inhibited by its diastereomeric pair epitestosterone, a high affinity substrate of UGT2B7. NSAIDs, diclofenac and ibuprofen, were not considerably inhibiting in testosterone glucuronidation by UGT2B17, whereas UGT2B15 was inhibited by both. The androgens were mostly glucuronidated by UGT2B7, UGT2B15, UGT2B17, and often UGT2A1. In both regio- and stereoselectivity, UGT2B15 was strictly selective. It only conjugated the androgens at the 17-OH position in the $\beta$-configuration, whereas UGT2B17 accepted both 3$\alpha$- and 17$\beta$-OH as the acceptor group. If both of those were available, the 17$\beta$-OH was favored over the 3$\alpha$-OH. The third active UGT in steroid glucuronidation, UGT2B7, accepted both 3$\alpha$- and 17$\alpha$-OH, but not 17$\beta$-OH, which also significantly impaired the glucuronidation reaction when available in the molecule. For UGT2B7, a structure with a 17$\beta$-OH, seems to act as a competitive inhibitor for 3-O-glucuronidation unless the $K_m$ of 3-O-glucuronidation is very low. Interestingly, epitestosterone with a 17-OH in $\beta$-configuration served as a low $K_m$ substrate that was not inhibited by testosterone with 17$\beta$-OH. The results shed new light on the selectivity of human UGTs toward endogenic C19 steroids.
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Tuusula

_Taina Sten_
7. References


